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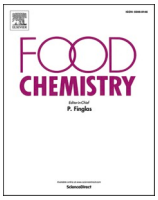

Evaluation of the potential of annatto seed powder to reduce the formation of heterocyclic amines in charcoal-grilled and pan-fried beef patties

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ABSTRACT

Various strategies are being explored to reduce the formation of undesirable compounds during the thermal processing of foods. This study investigates the impact of incorporating annatto seed powder (*Bixa orellana* L.) into beef patties to reduce the formation of heterocyclic amines (HAs) during charcoal-grilling and pan-frying. A three-level full factorial design was used to assess the effect of both annatto seed powder concentration and cooking times on HAs formation. The results showed that HA formation increased with longer cooking times and decreased with higher concentrations of annatto seed powder. A significant reduction in HA content was observed in both charcoal-grilled and pan-fried beef patties when annatto seed powder was added, with a particularly notable 91 % reduction at the 1 % addition level. These findings demonstrate that the addition of annatto seed powder is a highly effective strategy for reducing HA formation in beef patties.

Chemical compounds studied in this article: 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) (PubChem CID: 62275); 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) (PubChem CID: 104739); 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx) (PubChem CID: 104855); 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (PubChem CID: 1530); 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) (PubChem CID: 5284474); 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) (PubChem CID: 5284476); 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC) (PubChem CID: 62805); 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC) (PubChem CID: 62244); Bixin (PubChem CID: 5281226).

1. Introduction

Thermal processing used in meat preparation enhances sensory and nutritional quality and ensures microbiological safety. However, high temperature and low moisture conditions, typically used in charcoal-grilling and pan-frying, can produce thermal processing contaminants,

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such as carcinogenic heterocyclic aromatic amines (HAs) (Bouvard et al., 2015; Jägerstad & Skog, 2005; Khan et al., 2022).

HAs are nitrogenous compounds associated with heterocyclic aromatic rings, formed during the cooking of protein-rich foods. They are categorized into two groups based on their characteristics and formation mechanisms: (1) thermic HAs, generated at temperatures above 100 °C through interactions between creatine/creatinine, free amino acids, and hexoses during *Maillard reactions*; and (2) pyrolytic HAs, formed at temperatures above 300 °C due to the pyrolysis of amino acids, peptides, and/or proteins (Alaejos & Afonso, 2011).

Regarding toxicity, the 2-amino-3-metil-3H-imidazo[4,5-*f*]quinoline (IQ) is classified as probably carcinogenic (group 2 A), while 2-amino-3,4-dimetilimidazo[4,5-*f*]quinoline (MeIQ), 2-Amino-3,8-dimetilimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-1-metil-6-fenilimidazo[4,5-*b*] piridine (PhIP), 1,4-dimetil-5H-pirido[4,3-*b*]indol-3-amina (Trp-P-1), 3-amino-1-metil-5H-pirido[4,3-*b*]indol (Trp-P-2), 2-amino-9H-pirido[2,3-*b*]indol (AαC), 2-amino-3-metil-9H-pirido[2,3-*b*]indol (MeAαC), 2-amino-6-metildipirido[1,2-a:3′,2′-d]imidazol (Glu-P-1) and 2-amino-dipirido[1,2-a:3′,2′-d]imidazol (Glu-P-2) are all classified as possibly carcinogenic (group 2B). The carcinogenic potential of these compounds is mainly due to their metabolic by-products, which can bind to proteins or DNA, forming stable adducts that cause replication errors, mutations, oxidative stress, inflammation, and ultimately cancer (IARC - International Agency for Research on Cancer, 2018).

Diet is the main route of human exposure to HAs, thus it is necessary to develop effective strategies to mitigate or minimize human exposure to these compounds. The literature identifies three key strategies to reduce HA formation: selecting foods with fewer precursors, adjusting cooking methods and conditions, and incorporating exogenous inhibitors, such as natural products (Dong et al., 2020; Wang et al., 2023). Various ingredients, including condiments and spices such as garlic, onion, paprika, ginger, pepper (Lu et al., 2018), rosemary, turmeric, bay leaf (Yu et al., 2024), initially used to enhance the sensory attributes of food, have been investigated for their bioactive compounds with antioxidant properties. These ingredients, particularly rich in phenolic compounds, can inhibit the *Maillard* reaction by sequestering reactive species, eliminating intermediates, and degrading amino acids necessary for HA formation (Murkovic, 2004; Neves et al., 2021).

However, no study has yet evaluated the potential of annatto (*Bixa orellana* L.) seeds to reduce the formation of thermal processing contaminants in cooked meat. Annatto seeds have been traditionally used as coloring and flavoring ingredients in Brazil and other Latin American countries since pre-Columbian times (Rivera-Madrid et al., 2016; Ulbricht et al., 2012; Zarza-García et al., 2021), particularly in beef, pork, and fish dishes (Cuspinera et al., 2002; EMBRAPA - Empresa Brasileira de Pesquisa Agropecuária, 2009). Globally, the food industry uses annatto seeds as coloring agents in meat products due to their low allergenicity, minimal toxicity, and ability to replace artificial coloring and antioxidants (Islam et al., 2016; Raddatz-Mota et al., 2017; Ulbricht et al., 2012). The reddish color and antioxidant properties of these seeds are primarily due to the presence of the apocarotenoid bixin (Oliveira et al., 2022; Rivera-Madrid et al., 2016). While phenolic compounds are also present in annatto seeds, their concentrations are significantly lower than those of bixin (Chisté, Benassi, & Mercadante, 2011).

This study aims to evaluate the potential of seasoning beef patties with annatto seed powder to reduce HAs formation during charcoal-grilling and pan-frying. Using multivariate analysis, the study simultaneously evaluates annatto seed powder concentration and cooking time regarding their influence on HAs formation. Considering the study's objective, the following hypotheses were defined: (1) Does the addition of annatto seed powder prevent HAs formation in cooked beef patties? (2) Can annatto seed powder reduce the typically high HAs content in meat cooked under extreme conditions? (3) Is there an interaction between the variables, specifically the percentage of annatto seed powder and cooking time that contributes most to HAs formation during meat processing? (4) Is there a relationship between the concentration of bioactive compounds in annatto seed powder and the percentage of HAs inhibition in cooked beef patties?

2. Material and methods

2.1. Reagents and standards

Chromatographic grade reagents (acetic acid, acetonitrile, dichloromethane, ethanol, ethyl acetate, methanol, and petroleum ether), analytical grade reagents (acetone, methanol, sodium hydroxide [NaOH], hydrochloric acid [HCl], ammonium hydroxide, ammonium acetate, ammonia solution 25 % (*v/v*) and triethylamine), Extrelut® NT 20 columns and diatomaceous earth Extrelut® were obtained from Merck (Darmstadt, Hessen, Germany).

Bond Elut propyl sulfonic acid (PRS, 500 mg) and Bond Elut C_{18} (100 and 500 mg) cartridges were purchased from Agilent Technologies (Santa Clara, California, United States). The Folin-Ciocalteu reagent (F—C) was from Fluka (Buchs, Werdenberg, Switzerland), potassium from Panreac (Barcelona, Spain), and glacial acetic acid from VWR Chemicals Prolab (Leuven, Belgium). The reagents 2.2′-Azinobis 3-ethylbenzothiazoline-6- sulfonic acid ($ABTS^{\bullet+}$) and 2,2-diphenyl-1-picrylhydrazine ($DPPH^{\bullet}$), 2,4,6-tripyridyl-*s*-triazine (TPTZ) solution, ferric chloride, ferrous sulfate, sodium carbonate, anhydrous sodium acetate and the standards gallic acid (≥98 % purity) and 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) (97 % purity) were acquired from Sigma-Aldrich Corp (St. Louis, MO, USA).

Heterocyclic amines standards (≥97 % purity), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*] indole (Trp-P-2), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), 2-amino-6-methyldipyrido [1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), and 2-aminodipyrido[1,2-*a*:3′,2′-*d*] imidazole (Glu-P-2), were obtained from Toronto Research Chemicals (North York, Ontario, Canada). Stock standard solutions of 100 μg mL^{-1} in methanol were prepared and used.

Ultrapure water of 0.055 μS.cm^{-1} was obtained through a Seralpur Pro 90CN system (Seral, Ransbach-Baumbach, Germany).

Millipore membranes (Billerica, MA, USA) were used to filter extracts (0.22 μm) and solvents (0.45 μm) before chromatographic bixin analysis. All solutions were measured using a glass electrode connected to a pH meter (MicropH 2001, Crison, Barcelona, Spain) and filtered (0.22 μm) before HAs analysis.

2.2. Annatto seed powder preparation

Annatto seeds of the Piave variety were donated by a producer in Monte Castelo, São Paulo, Brazil, in vacuum packs protected from light and stored at −80 °C until grinding. The seeds (200 g) were ground in a knife mill (Grindomix GM 200, Retsch GmbH, Mettmann, Germany) and sieved through a 125 μm mesh (Retsch GmbH, Mettmann, Germany). The annatto seed powder was stored protected from light, under vacuum, and frozen at −20 °C until use.

2.3. Experimental design by multivariate analysis

The effect of adding annatto seed powder and varying cooking time on the formation of HAs in charcoal-grilled and pan-fried beef patties was investigated using a factorial design. A three-level full factorial design was employed for two variables - annatto seed powder concentration and cooking time - with one replicate at the central point, (supplementary material, Table S1).

The lowest level of cooking time was determined in a preliminary assay and defined as the minimum time required to reach an internal temperature of 71 °C at the geometric center of the beef patties. This temperature is recommended by the United States Department of Agriculture (USDA) to ensure the microbiological safety of ground meat (USDA - United States Department of Agriculture, 2020). The highest cooking time was set based on visual indicators of very well-done meat (Iwasaki et al., 2010).

The maximum concentration of annatto seed powder to add (1 %, *w/w*) was established based on the acceptable daily intakes of 12 mg bixin kg^{-1} bw defined by the Food and Agriculture Organization (FAO - Food and Agriculture Organization/WHO - World Health Organization, 2007) and 6 mg bixin kg^{-1} bw defined by the European Food Safety Authority (EFSA - European Food Safety Authority, 2019).

2.4. Beef patties preparation and cooking

Minced meat from beef thighs (*Gluteus biceps*) was purchased on the day of preparation from a local butcher in Porto, Portugal. Annatto seed powder was incorporated into the minced beef according to the levels specified in the experimental design, and the formulations were manually homogenized without adding other ingredients. The samples were then packed in oxygen-permeable PVC film and refrigerated overnight (4 °C). The following day, 80 g portions of beef were shaped into patties using a burger press mold (1 cm thickness, 9 cm diameter), and then either charcoal-grilled or pan-fried as described below.

Charcoal-grilled beef patties were cooked directly on disposable charcoal grills (31 cm long x 25 cm wide x 10 cm high; Algon, Alberic, Valencia, Spain). The fire was ignited according to the manufacturer's instructions, achieving a temperature of approximately 200 °C. Each sample was cooked in duplicate, placed in opposite quadrants of the grill to minimize temperature variations, and turned once at half the cooking time. Only four patties were grilled at a time, and the grills were discarded after each use.

Pan-fried beef patties were cooked without oil in a preheated frying pan equipped with a thermo-spot to determine the optimal cooking start time on a glass ceramic hob. The pan surface was maintained between 180 °C and 200 °C. To ensure consistent cooking conditions, three beef patties (one for each seasoning concentration) were placed at equidistant points in the preheated pan). They were pan-fried for the specified cooking times, turning once at half the cooking time. This procedure was repeated three times for each cooking time, rotating the positions of the patties clockwise to ensure uniform cooking. The pan was cleaned after each round. An additional round was conducted to pan-fry the central point samples in all three positions.

Representative photographs and moisture content of both pan-fried and charcoal-grilled beef patties, according to experimental design levels, are available in the supplementary material (Fig. S1 and Table S2, respectively).

In total, 50 beef patties were prepared: 20 were charcoal-grilled (n = 2 per condition), and 30 were pan-fried (n = 3 per condition). After cooking, composites of patties from the same experimental conditions were prepared using a knife mill (Grindomix GM 200, Retsch GmbH, Mettmann, Germany), resulting in 10 samples for each cooking method. These samples were then frozen at −20 °C until HAs analysis.

2.5. HAs extraction and analysis

The extraction and purification of HAs were carried out in duplicate based on the solid-phase extraction (SPE) method developed by Gross (1990), a reference in interlaboratory exercises (Santos et al., 2004), which has been widely used in our laboratory (Melo et al., 2008; Viegas, Novo, Pinto, Pinho and Ferreira, 2012; Trujillo-Mayol et al., 2021; Viegas et al., 2015).

The separation and quantification of HAs were conducted using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). A Thermo Scientific Accela quaternary pump, a thermostatted autosampler, a column oven, and a TSQ Quantum Ultra™ triple quadrupole mass spectrometer equipped with a Heated Electrospray Ionization (HESI-II) operating in positive mode were employed. Chromatographic separation was achieved on a Core-Shell C_{18} EVO column (Kinetex, 100 × 2.1 mm i.d.; 2.6 μm; Phenomenex, Torrance, USA) fitted with a SecurityGuard™ ULTRA UHPLC pre-column (C_{18}, 3 × 4 mm). The mobile phase consisted of 0.1 % ammonia in water (mobile phase A) and 0.1 % ammonia in methanol (mobile phase B), flowing at a rate of 300 μL min^{-1}, with a column temperature of 35 °C. The injection volume was 10 μL. The spray voltage was set at 3000 V, and the vaporization and capillary temperatures were maintained at 350 and 320 °C, respectively. Pressure sheath, sweep, and auxiliary gas were kept at 35, 1, and 10 arbitrary units (au), respectively. The precursor and product ions, as well as the tube lens voltage and collision energy for each HA, were determined through flow injection analysis and are detailed in supplementary material (Table S3).

For HA quantification, the standard addition method was employed using two fortified levels (5 and 10 ng g^{-1}) and one non-spiked sample. Quantification was based on peak characteristics and area. MRM parameters for quantification and confirmation, along with the limit of detection (LOD) and the limit of quantification (LOQ), are also provided in Table S3. The results were reported as the mean (standard error) of HAs in ng g^{-1}.

2.6. Bixin extraction and analysis of annatto seed powder

Extractions of bixin were conducted in triplicate based on the method proposed by Rios and Mercadante (2004). Briefly, 0.1 g of ground and homogenized seeds were weighed, and the pigments were extracted with 20 mL of ethyl acetate under agitation with an ultra-disperser (Ultra-turrax®, IKA, Staufen, Baden-Württemberg, Germany) for 3 min. This procedure was repeated three times, and the resulting extract was concentrated in a rotary evaporator at room temperature. The residue was then resuspended in 2 mL of petroleum ether, transferred to vials, dried under nitrogen, and stored in a freezer at −40 °C until chromatographic analysis. Bixin standard (99.5 % purity) was obtained by crystallization and recrystallization after extraction (Rios & Mercadante, 2004). Chromatogram characteristics and UV–visible spectra of the standard are provided in the supplementary material (Fig. S2).

Bixin analysis was performed by High-Performance Liquid Chromatography (HPLC), according to the method described by Tocchini and Mercadante (2001). The HPLC system comprised a quaternary solvent pumping set (Shimadzu®, LC-20 CE), a diode array detector (Shimadzu®, model SPD-M20A), a degassing unit (DGU-20A5). Separations were carried out on a C_{18} column (Atlantis Waters® 4 mm, 4.6 × 250 mm, 5 μm particle size), maintained at 29 °C. Acetonitrile:acetic acid 2 % (65:35; *v/v*) was used as a mobile phase in a linear gradient at a flow rate of 1 mL/min for 35 min. Spectra were acquired between 250 and 600 nm, and chromatograms were processed at a wavelength of 460 nm. The bixin peak was identified by comparing UV–visible spectra, retention time, %III/II and $\%A_B/A_{II}$, with data provided by literature (Tocchini & Mercadante, 2001).

2.7. Total phenolic content and antioxidant activity of annatto seed powder

Extractions were conducted in duplicate following the method proposed by Pérez-Jiménez et al. (2008). In a capped centrifuge tube, 0.5 *g* of annatto seed powder was weighed, and the compounds were extracted in 20 mL of acidic methanol/water (50:50, *v/v*; pH 2) under shaking at room temperature for 1 h. Then, the tube was centrifuged at 2500*g* (5810 R, Eppendorf, Hamburg, Germany) for 10 min and the supernatant was collected. Subsequently, 20 mL of acetone/water (70:30, *v/v*) was added to the residue, and the shaking and centrifugation steps were repeated. The methanolic and acetonic extracts were

combined and stored at −20 °C until spectrophotometric analysis.

The total phenolic content (TPC) was analyzed by the Folin-Ciocalteu (F—C) method, and the antioxidant activity by the radical scavenging capacity against 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation ($ABTS^{\bullet+}$) and 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$), and by the ferric reducing antioxidant power (FRAP). The procedures were carried out in 96-well microplates, and absorbance measurements were performed using spectrophotometric detection (spectrophotometer SPECTROstar Nano, BMG Labtech, Ortenberg, Germany). All assays were conducted in triplicate, according to the conditions described by Magalhães et al. (2012) for F—C, $ABTS^{\bullet+}$, and $DPPH^{\bullet}$ and according to Benzie and Strain (1996) for FRAP.

F—C assay: Gallic acid standard solution in ultrapure water (15.625 to 500 μM) or diluted extracts of annatto seed powder (1:5–1:160) were mixed with 25 μL of F—C reagent (4:10, *v/v*) in a microplate. After incubation for 10 min, sodium carbonate solution (100 μL; 6 %, *w/v*) was added, and the microplate was further incubated in the dark for 2 h. Absorbance was measured at 765 nm. For samples' intrinsic absorption, ultrapure water (25 μL) was used instead of F—C reagent, and for blank assays, water was added instead of sample extract. TPC was calculated using a gallic acid calibration curve ($r^2 = 0.9992$) and expressed as mean (standard deviation) in milligrams of gallic acid equivalents per gram of seed (mg GAE g^{-1}).

$ABTS^{\bullet+}$ assay: Microplate wells were filled with Trolox solution in methanol (15.625 to 500 μM) or diluted extracts of annatto powder (1:5–160). $ABTS^{\bullet+}$ solution (150 μL), prepared by mixing $ABTS^{\bullet+}$ and potassium persulfate in ultrapure water and acetate buffer, was added, followed by incubation in the dark for 2 h. Absorbance was read at 734 nm. For intrinsic absorption of samples, acetate buffer was used instead of $ABTS^{\bullet+}$ solution, and for blanks, ultrapure water was added instead of samples.

$DPPH^{\bullet}$ assay: Trolox standard solution in methanol (15.625 to 500 μM) or diluted extracts of annatto powder (1:5–160) were mixed with $DPPH^{\bullet}$ methanolic solution (150 μL; 50 %, *v/v*; 1 mM; 394.3 mg L^{-1}) in microplate wells. The microplate was then incubated in the dark for 120 min, and absorbance was measured at 517 nm. All procedures were conducted at room temperature. For intrinsic absorption and blanks, methanolic solution was added instead of $DPPH^{\bullet}$ solution and samples, respectively.

Standard curves for Trolox in $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays were constructed ($r^2 \geq 0.9998$), and antioxidant capacities were expressed as mean (standard deviation) in milligrams of Trolox equivalent antioxidant capacity per gram of seed (mg TEAC g^{-1}).

FRAP assay: Diluted extract (30 μL, 1:10) in methanol was mixed with FRAP reagent (270 μL) consisting of 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM ferric chloride diluted to one-third with acetate buffer. After thorough mixing, the microplate was incubated for 30 min at 37 °C, protected from light. Absorbances were subsequently read at 595 nm. For the blank assay, FRAP reagent (270 μL) was mixed with methanol (30 μL). A calibration curve was constructed using ferrous sulfate ($r^2 = 0.9991$), and ferric reducing antioxidant power was expressed as the mean (standard deviation) of micromoles of ferrous sulfate equivalents per gram of seed (μmol FSE g^{-1}).

2.8. Statistical analysis

The mean and standard deviation of the triplicate of analyses were calculated for bixin content, total phenolic content (TPC), and antioxidant activity. For individual HAs and their sum (ΣHA), the mean and standard error of the duplicate analyses were calculated. Differences between the means of ΣHA under different test conditions were determined using ANOVA followed by Tukey's multiple comparison test. Differences between the means of ΣHA for the two cooking methods – pan-frying and charcoal-grilling – were evaluated using the Mann-Whitney test. Statistical significance was considered at $p < 0.05$, and data processing was performed with GraphPad Prism 8.0.

Response surface methodology (RSM) was employed to investigate the effect of cooking time, annatto seed powder concentration, and their interactions on HAs formation. Regression analysis, ANOVA, and contour surface plots were used to evaluate and identify significant factors ($p < 0.05$) and interaction effects, as well as to compare results between different experiments. Model validity was assessed based on the coefficient of determination (R^2) values, with acceptable values being greater than 70 %. The results and contour surfaces of the experimental design were analyzed and generated using Protimiza© version 1 (Rodrigues & Costa, 2014).

3. Results

3.1. HAs content in samples

The HA content the beef patties under different test conditions (varying annatto seed powder concentrations and cooking times) is presented in Table 1. Of the 13 HAs analyzed, eight were detected and quantified. IQ, IQx, Glu-P-1, Glu-P-2, and MeIQ were consistently below the method's limit of detection (LOD) (Table S3). In charcoal-grilled samples, 7,8-DiMeIQx was never detected, and PhIP had the highest content, ranging from 0.23 to 30.96 ng g^{-1}. In pan-fried samples, Trp-P-1, Trp-P-2, AαC and MeAαC were not detected in any conditions, with MeIQx being the most abundant HA, ranging from 0.49 to 5.80 ng g^{-1}. No pyrolytic HAs were formed in the pan-fried beef patties. Additionally, pan-fried beef patties exhibited lower ΣHAs compared to charcoal-grilled patties in most tested conditions. This difference was most pronounced at the highest cooking time, with pan-fried patties having ΣHAs five times lower than charcoal-grilled patties (11.70 ng g^{-1} vs 57.45 ng g^{-1}).

Regardless of the cooking method, it was observed that the HAs content was directly proportional to the cooking time and inversely proportional to the percentage of annatto seed powder. The highest ΣHAs was observed with longer cooking time, specifically 30 min for charcoal-grilled samples and 15 min for pan-fried, without the addition of annatto seed powder [57.45 (3.96) ng g^{-1} and 11.70 (1.23) ng g^{-1}] for charcoal-grilled and pan-fried beef patties, respectively). Conversely, the lowest ΣHAs was observed with shorter cooking times, namely 14 min for charcoal-grilled samples and 8 min for pan-fried, supplemented with 1 % of annatto seed powder [0.56 (0.01) ng g^{-1} and 0.29 (0.01) ng g^{-1} for charcoal-grilled and pan-fried beef patties, respectively].

3.1.1. Charcoal-grilled samples

The addition of annatto seed powder demonstrated efficacy in reducing HAs content in charcoal-grilled samples, with the most pronounced effect observed at the maximum cooking time of 30 min. Here, the addition of 1 % annatto seed powder led to a remarkable 91 % reduction in ΣHAs (from 57.45 ng g^{-1} to 5.24 ng g^{-1}). Similarly, at the same cooking time, incorporating 0.5 % annatto seed powder resulted in a 55 % reduction in ΣHAs (from 57.45 ng g^{-1} to 25.76 ng g^{-1}). This trend of inhibition persisted across various cooking times, albeit with varying degrees of reduction – 79 % and 61 %, for 1 % and 0.5 % of annatto seed powder at 22 min, and 61 % and 14 % for 1 % and 0.5 % at 14 min (Table 1).

Moreover, the addition of annatto seed powder exhibited greater efficacy in reducing the content of thermic HAs compared to pyrolytic HAs. For instance, at a cooking time of 30 min, incorporating 1 % annatto seed powder led to a substantial 93 % reduction in ΣThermic (from 46.07 ng g^{-1} to 3.02 ng g^{-1}), and an 80 % reduction in ΣPyrolytic (from 11.38 ng g^{-1} to 2.23 ng g^{-1}). Conversely, adding 0.5 % annatto seed powder resulted in a 62 % reduction in ΣThermic (from 46.07 ng g^{-1} to 17.43 ng g^{-1}) and only a 27 % reduction in ΣPyrolytic (from 11.38 ng g^{-1} to 8.33 ng g^{-1}).

In terms of individual HAs, PhIP and MeIQx were the most abundant, with content levels of 30.96 (3.49) ng g^{-1} and 14.46 (0.06) ng g^{-1},

Table 1
Content (ng g^{-1}) of individual HAs, ΣThermic HAs, Pyrolytic HAs in charcoal-grilled and pan-fried beef patties under the experiments provided by the experimental design.

Charcoal-grilled	Cooking time (min)	Annatto concentration (%)	MeIQx	4,8-DiMeIQx	7,8-DiMeIQx	PhIP	Trp-P-1	Trp-P-2	AαC	MeAαC	ΣThermic	ΣPyrolytic	ΣHAs	Inhibition (%)
1	14	0	0.41 (0.00)	< LOD	< LOD	0.30 (0.01)	0.24 (0.00)	0.11 (0.02)	0.24 (0.00)	0.13 (0.03)	0.71 (0.01)	0.71 (0.04)	1.42 (0.04) [a,*]	–
2	14	0.5	0.27 (0.01)	< LOD	< LOD	0.30 (0.02)	0.21 (0.01)	0.10 (0.01)	0.19 (0.02)	0.15 (0.02)	0.57 (0.03)	0.65 (0.00)	1.22 (0.03) [b]	14
3	14	1.0	0.15 (0.02)	< LOD	< LOD	0.23 (0.03)	0.09 (0.02)	0.03 (0.02)	0.05 (0.00)	< LOD	0.39 (0.01)	0.17 (0.00)	0.56 (0.01) [c]	61
4	22	0	9.39 (0.71)	0.23 (0.03)	< LOD	4.24 (0.11)	1.79 (0.04)	0.66 (0.02)	1.13 (0.05)	0.60 (0.01)	13.87 (0.57)	4.18 (0.12)	18.05 (0.68) [d]	–
5	22	0.5	2.99 (0.08)	0.11 (0.00)	< LOD	1.82 (0.03)	0.78 (0.11)	0.18 (0.01)	0.84 (0.02)	0.31 (0.01)	4.91 (0.06)	2.11 (0.07)	7.01 (0.01) [e]	61
6	22	1.0	1.49 (0.12)	0.06 (0.00)	< LOD	1.11 (0.02)	0.28 (0.06)	0.10 (0.00)	0.75 (0.03)	0.04 (0.00)	2.65 (0.14)	1.16 (0.03)	3.82 (0.11) [f]	79
7	30	0	14.46 (0.06)	0.64 (0.03)	< LOD	30.96 (3.49)	4.68 (0.28)	1.34 (0.02)	3.99 (0.28)	1.37 (0.03)	46.07 (3.41)	11.38 (0.05)	57.45 (3.96) [g]	–
8	30	0.5	6.15 (0.09)	0.16 (0.04)	< LOD	11.12 (0.03)	0.98 (0.01)	1.08 (0.02)	4.47 (0.49)	1.81 (0.07)	17.43 (0.08)	8.33 (0.59)	25.76 (0.67) [h]	55
9	30	1.0	1.67 (0.05)	0.13 (0.02)	< LOD	1.22 (0.05)	0.21 (0.02)	0.42 (0.03)	1.07 (0.03)	0.52 (0.02)	3.02 (0.01)	2.23 (0.09)	5.24 (0.08) [i,**]	91
10	22	0.5	3.10 (0.28)	0.11 (0.03)	< LOD	2.03 (0.05)	0.79 (0.05)	0.16 (0.00)	1.28 (0.11)	0.33 (0.01)	5.23 (0.23)	2.55 (0.15)	7.79 (0.08) [e]	57

Pan-fried	Cooking time (min)	Annatto concentration (%)	MeIQx	4,8-DiMeIQx	7,8-DiMeIQx	PhIP	Trp-P-1	Trp-P-2	AαC	MeAαC	ΣThermic	ΣPyrolytic	ΣHAs	Inhibition (%)
1	8	0	0.84 (0.07)	< LOD	< LOD	0.39 (0.03)	< LOD	< LOD	< LOD	< LOD	1.23 (0.10)	< LOD	1.23 (0.10) [k,*]	–
2	8	0.5	0.49 (0.09)	< LOD	< LOD	0.29 (0.03)	< LOD	< LOD	< LOD	< LOD	0.78 (0.07)	< LOD	0.78 (0.07) [l]	37
3	8	1.0	< LOD	< LOD	< LOD	0.29 (0.01)	< LOD	< LOD	< LOD	< LOD	0.29 (0.01)	< LOD	0.29 (0.01) [m]	76
4	11.5	0	2.25 (0.07)	0.47 (0.09)	0.24 (0.06)	2.11 (0.11)	< LOD	< LOD	< LOD	< LOD	5.06 (0.34)	< LOD	5.06 (0.34) [n]	–
5	11.5	0.5	0.95 (0.06)	0.29 (0.01)	0.09 (0.01)	1.00 (0.02)	< LOD	< LOD	< LOD	< LOD	2.33 (0.08)	< LOD	2.33 (0.08) [o]	54
6	11.5	1.0	0.66 (0.03)	0.20 (0.02)	0.04 (0.00)	0.70 (0.02)	< LOD	< LOD	< LOD	< LOD	1.60 (0.07)	< LOD	1.60 (0.07) [o]	68
7	15	0	5.80 (0.92)	1.31 (0.10)	0.31 (0.01)	4.28 (0.21)	< LOD	< LOD	< LOD	< LOD	11.70 (1.23)	< LOD	11.70 (1.23) [p]	–
8	15	0.5	5.63 (0.15)	0.83 (0.01)	0.16 (0.02)	3.03 (0.17)	< LOD	< LOD	< LOD	< LOD	9.65 (0.01)	< LOD	9.65 (0.01) [p,**]	18
9	15	1.0	2.30 (0.02)	0.49 (0.04)	0.09 (0.02)	2.14 (0.07)	< LOD	< LOD	< LOD	< LOD	5.02 (0.03)	< LOD	5.02 (0.03) [q]	57
10	11.5	0.5	0.82 (0.08)	0.26 (0.02)	0.08 (0.01)	1.24 (0.01)	< LOD	< LOD	< LOD	< LOD	2.40 (0.04)	< LOD	2.40 (0.04) [o]	53

Results expressed as mean (standard error) of HAs and ΣHAs in ng g^{-1} of beef patties, $n = 2$. Inhibition (%) values express the percentage of inhibition of ΣHAs towards unseasoned samples at the same method and cooking time. Σ, sum. ΣThermic = MeIQx +4,8-DiMeIQx +7,8-DiMeIQx + PhIP; ΣPyrolytic = Trp-P-1+ Trp-P-2+ AαC + MeAαC. ΣHAs: Different letters and symbols in the same column indicate significantly different values (Tukey's test; $p \leq 0.05$).

respectively, f under the condition of 30 min and 0 % annatto seed powder. Conversely, at 22 min and 0 % annatto seed powder, PhIP and MeIQx content levels were 4.24 (0.11) ng g^{-1} and 9.39 (0.71) ng g^{-1}, respectively. The lowest quantified HA was Trp-P-2, with a content of 0.03 (0.02) ng g^{-1} under the condition of 14 min and 1 % annatto seed powder.

The impact of cooking time on the increase in HAs content in charcoal-grilled samples was pronounced, with significant rises observed in ΣHAs, ΣThermic, ΣPyrolytic, and individual HA content as cooking time increased. Specifically, ΣHAs increased by 13-fold (from 1.42 ng g^{-1} to 18.05 ng g^{-1}) for the 22-min cooking time and 40-fold (from 1.42 ng g^{-1} to 57.45 ng g^{-1}) for the 30-min cooking time. The increase in ΣThermic was even more substantial, rising by 20 times (from 0.71 ng g^{-1} to 13.87 ng g^{-1}) for the 22-min cooking time and 65 times (from 0.71 ng g^{-1} to 46.07 ng g^{-1}) for the 30-min cooking time. A similar trend was observed for ΣPyrolytic, albeit with a smaller magnitude – increasing by 6 times (from 0.71 ng g^{-1} to 4.18 ng g^{-1}) for the 22-min cooking time and 16 times (from 0.71 ng g^{-1} to 11.38 ng g^{-1}) for the 30-min cooking time. Regarding individual HAs, PhIP (the most abundant HA) content increased significantly with longer cooking times – rising by14 times (from 0.30 ng g^{-1} to 4.24 ng g^{-1}) and 103 times (from 0.30 ng g^{-1} to 30.96 ng g^{-1}) for the 22 and 30-min cooking times, respectively.

3.1.2. Pan-fried samples

Similar to charcoal-grilled samples, the addition of annatto seed powder proved highly effective in reducing HAs content in pan-fried samples, albeit with slightly lower inhibition percentages. For instance, at a cooking time of 30 min, the incorporation of 1 % annatto seed powder led to a 57 % reduction in ΣHAs (from 11.70 ng g^{-1} to 5.02 ng g^{-1}), while adding 0.5 % annatto seed powder resulted in an 18 % reduction (from 11.70 ng g^{-1} to 9.65 ng g^{-1}). This inhibition trend persisted across other cooking times, with higher inhibition percentages observed – 68 % and 54 %, for 1 % and 0.5 % of annatto seed powder at 22 min, and 76 % and 37 % for 1 % and 0.5 % of annatto seed powder at 14 min.

Since only thermic HAs were formed during pan-frying, the effect of annatto seed powder addition and cooking time was solely observable on ΣThermic HAs and individual HAs. Incorporating 1 % annatto seed powder resulted in a significant reduction in ΣThermic HAs across all cooking times – 76 % (from 1.23 ng g^{-1} to 0.29 ng g^{-1}) for 8 min cooking time; 68 % (from 5.06 ng g^{-1} to 1.60 ng g^{-1}) for 11.5 min cooking time; and 57 % (from 11.70 ng g^{-1} to 5.02 ng g^{-1}) for 15 min cooking time. A lower inhibition percentage was observed for all tested cooking times when 0.5 % annatto seed powder was added – 37 % (from 1.23 ng g^{-1} to 0.78 ng g^{-1}) for 8 min, 54 % (from 5.06 ng g^{-1} to 2.33 ng g^{-1}) for 11.5 min and 18 % (from 11.70 ng g^{-1} to 9.65 ng g^{-1}) for 15 min. In terms of individual HAs, no significant differences were observed between 0 % and 0.5 % annatto seed powder for MeIQx (the most abundant HA in pan-fried samples) at 15 min of cooking time. However, a significant decrease in MeIQx content was observed when 1 % annatto seed powder was used (5.80 ng g^{-1} vs. 2.30 ng g^{-1}). A similar trend was observed for PhIP, where its content decreased significantly from 4.28 ng g^{-1} (0 % annatto seed powder) to 2.14 ng g^{-1} (1 % annatto seed powder).

Although the increase in cooking time led to higher HAs content, this effect was less pronounced in pan-fried samples. For example, ΣHAs were only 4 times higher (1.23 ng g^{-1} vs. 5.06 ng g^{-1}) for 11.5 min of cooking time and 10 times higher (1.23 ng g^{-1} vs. 11.70 ng g^{-1}) for 15 min of cooking time.

3.2. Three-level full factorial design

A Pareto chart is a powerful tool used to prioritize and identify the most significant factors in a dataset. This chart presents the magnitude of effects in an ordered bar chart, with the length of each bar on the horizontal axis indicating the significance of the variables. In the case of charcoal-grilled samples, both cooking time (X_1) and annatto seed powder content (X_2), along with their interaction ($X_1 - X_2$) were statistically significant variables ($p < 0.05$) for MeIQx, 4,8-DiMeIQx, Trp-P-1, AαC, ΣThermic, ΣPyrolytic, and ΣHAs (Fig. 1). Similarly, this holds true for 7,8-DiMeIQx in pan-fried samples. As illustrated in Table 2, cooking time (X_1) consistently exhibited a positive effect on the measured response, while annatto seed powder content (X_2) and the interaction between both variables ($X_1 - X_2$) consistently exerted a negative effect on the response. Notably, very high R^2 values ($\geq$ 94 %) were obtained for MeIQx, 4,8-DiMeIQx, Trp-P-1, AαC, MeAαC, ΣThermic, ΣPyrolytic, and ΣHAs in charcoal-grilled samples and for 7,8-DiMeIQx in pan-fried samples (Table 2).

Fig. 2 illustrates contour surfaces and predictive equations for individual HAs, ΣThermic, ΣPyrolitic and ΣHAs formation in charcoal-grilled beef patties according to the experimental design variables (the same information is displayed in Fig. S3 for pan-fried beef patties). The darker regions of the contour surfaces represent the range of studied variables where the highest HAs content occurred, indicating synergistic conditions of prolonged cooking time and lower concentration of annatto seed powder seasoning.

3.3. Bixin and total phenolic content of annatto seed powder

Table 3 presents the bixin and total phenolics content in annatto seed powder. The annatto seed powder contained 2.08 (0.44) g 100 g^{-1} of bixin and 8.56 (0.26) mg g^{-1} GAE.

3.4. Antioxidant activity of annatto seed powder

The antioxidant activity of annatto seed powder, determined through the $ABTS^{\bullet+}$, $DPPH^{\bullet}$, and FRAP assays, is detailed in Table 3. Annatto seed powder exhibited an antioxidant activity of 30.40 (2.62) mg g^{-1} TEAC in the $ABTS^{\bullet+}$ assay, 18.30 (0.66) mg g^{-1} TEAC in the $DPPH^{\bullet}$ assay and 17.44 (0.40) mg FSE g^{-1} in the FRAP assay.

4. Discussion

The addition of annatto seed powder to beef patties proved to be a highly effective strategy for reducing the formation of harmful HAs during cooking, particularly for both charcoal-grilled and pan-fried preparations. Across various cooking conditions – different cooking times and methods – significant reductions (ranging from 14 % to 91 %) in HA content (both total and individual) were observed when annatto seed powder was incorporated compared to control samples. Notably, this protective effect was dose-dependent, with the most pronounced impact seen at the 1 % addition level, irrespective of cooking time or method. At the longest cooking time tested (30 min for charcoal-grilled and 15 min for pan-fried), a substantial reduction of 91 % and 57 % reduction in HAs content was observed for charcoal-grilled and pan-fried beef patties, respectively (Table 4).

Several studies have explored the efficacy of various spices and herbs in reducing HA formation during meat cooking (Table 4). For instance, Kilic et al. (2021) investigated the addition of turmeric at two levels (0.5 % and 1 %) in inhibiting the formation of HA in chicken meatballs. A maximum inhibition of 72 % was observed with the addition of 0.5 % turmeric when the chicken meatballs were cooked at 200 °C for 18 min. Similarly, Yu et al. (2024) tested the addition of various spices/seasonings to beef burgers aiming to inhibit the formation of HAs and found that the highest inhibition of HAs formation (75 %) was achieved when rosemary (0.5 %) was added. Puangsombat et al. (2011) also tested the addition of rosemary and other spices/herbs to beef patties and found that the formation of HAs could be reduced by 43 % when rosemary was added at 0.2 %. Lu et al. (2018) achieved a 78 % reduction in the HAs content in deep-fried beef balls when ginger was added at 0.5 %. In our study, the incorporation of annatto seed powder (1 %) resulted in a remarkable 91 % reduction in HA content in charcoal-grilled beef patties

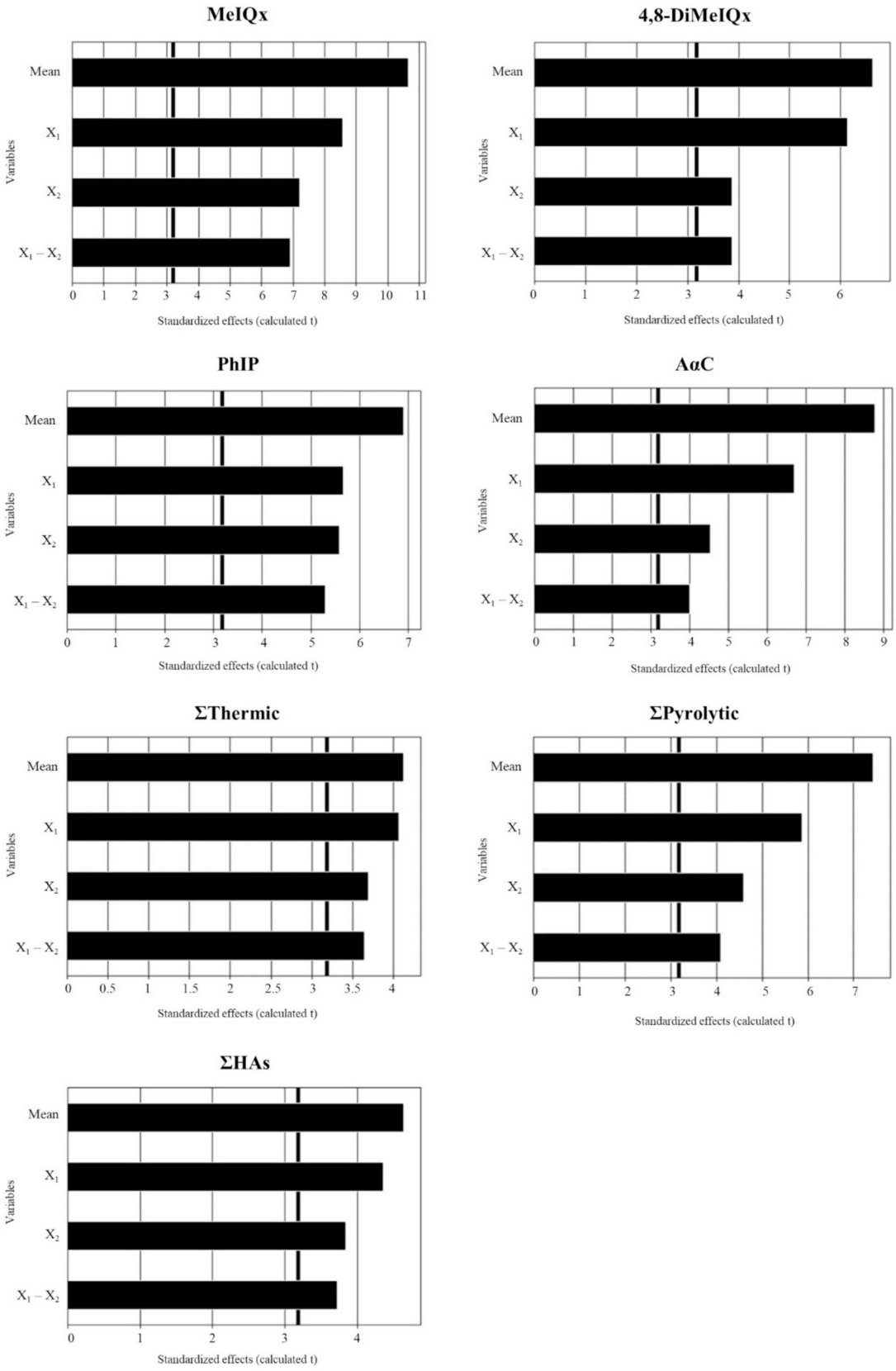

Fig. 1. Pareto charts illustrating the effects of cooking time (X_1), annatto seed powder concentration (X_2) and their interaction (X_1 - X_2) on the formation of heterocyclic amines (HAs) in charcoal-grilled beef patties.

Table 2
Statistical analysis of HAs formation on charcoal-grilled and pan-fried beef patties according to the experimental design variables X_1 - cooking time (minutes); X_2 - annatto seed powder concentration (% *w/w*) and their interaction ($X_1 - X_2$).

HAs	Charcoal-grilled samples											
	Regression table					ANOVA table						
	Variables	Effect	Standard error	Calculated t	*p*-value	Variation source	Sum of squares	Degrees of freedom	Mean square	F-calc	p-value	R^2 (%)
MeIQx	Mean	3.64	0.34	10.67	0.002	Regression	141.98	3	47.32	57.79	0.003	98
	Cooking time (x_1)	3.88	0.45	8.57	0.003	Residuals	2.46	3	0.82			
	Annatto concentration (x_2)	−3.27	0.45	−7.22	0.005	Lack of fit	2.44	1	2.44	393.54	0.002	
	Interaction x_1 - x_2	−3.13	0.45	−6.92	0.006	Pure error	0.01	2	0.006			
						Total	144.43	6				
4,8-DiMeIQx	Mean	0.16	0.02	6.62	0.007	Regression	0.26	3	0.087	22.5	0.014	96
	Cooking time (x_1)	0.19	0.03	6.14	0.008	Residuals	0.01	3	0.004			
	Annatto concentration (x_2)	−0.12	0.03	−3.87	0.030	Lack of fit	0.01	1	0.011	548.2	0.002	
	Interaction x_1 - x_2	−0.12	0.03	−3.87	0.030	Pure error	0.00	2	0.000			
						Total	0.27	6				
PhIP	Mean	5.85	1.97	2.96	0.050	Regression	808.93	3	269.64	9.87	0.046	91
	Cooking time (x_1)	8.53	2.61	3.27	0.040	Residuals	81.93	3	27.31			
	Annatto concentration (x_2)	−8.05	2.61	−3.08	0.050	Lack of fit	81.89	1	81.89	4004.86	0.000	
	Interaction x_1 - x_2	−8.03	2.61	−3.07	0.050	Pure error	0.041	2	0.020			
						Total	890.87	6				
Trp-P-1	Mean	1.11	0.16	6.89	0.006	Regression	16.64	3	5.55	30.45	0.009	97
	Cooking time (x_1)	1.19	0.21	5.58	0.011	Residuals	0.55	3	0.18			
	Annatto concentration (x_2)	−1.21	0.21	−5.67	0.010	Lack of fit	0.53	1	0.53	87.46	0.011	
	Interaction x_1 - x_2	−1.13	0.21	−5.30	0.013	Pure error	0.01	2	0.01			
						Total	17.19	6				
Trp-P-2	Mean	0.35	0.089	3.91	0.029							
	Cooking time (x_1)	0.38	0.117	3.38	0.070							
	Annatto concentration (x_2)	−0.26	0.117	−2.17	0.110							
	Interaction x_1 - x_2	−0.22	0.117	−1.86	0.160							
AαC	Mean	1.22	0.14	8.77	0.003	Regression	11.10	3	3.70	27.19	0.011	97
	Cooking time (x_1)	1.23	0.18	6.71	0.006	Residuals	0.421	3	0.14			
	Annatto concentration (x_2)	−0.83	0.18	−4.53	0.020	Lack of fit	0.23	1	0.23	2.61	0.247	
	Interaction x_1 - x_2	−0.74	0.18	−4.01	0.028	Pure error	0.18	2	0.09			
						Total	11.51	6				
MeAαC	Mean	0.42	0.05	7.89	0.004	Regression	1.10	3	0.37	18.35	0.019	95
	Cooking time (x_1)	0.43	0.07	6.08	0.008	Residuals	0.06	3	0.02			
	Annatto concentration (x_2)	−0.25	0.07	−3.47	0.040	Lack of fit	0.06	1	0.06	374.67	0.002	
	Interaction x_1 - x_2	−0.17	0.07	−2.45	0.091	Pure error	0.00	2	0.00			
						Total	1.16	6				
ΣThermic	Mean	9.64	2.33	4.12	0.030	Regression	1668.00	3	556.01	14.51	0.027	94
	Cooking time (x_1)	12.60	3.09	4.07	0.030	Residuals	114.90	3	38.31			
	Annatto concentration (x_2)	−11.44	3.09	−3.69	0.030	Lack of fit	114.90	1	114.93	10,890.81	0.000	
	Interaction x_1 - x_2	−11.28	3.09	−3.64	0.030	Pure error	0.02	2	0.01			
						Total	1783.00	6				
ΣPyrolytic	Mean	3.10	0.41	7.41	0.005	Regression	88.75	3	29.58	24.12	0.010	96
	Cooking time (x_1)	3.25	0.55	5.87	0.009	Residuals	3.67	3	1.22			
	Annatto concentration (x_2)	−2.54	0.55	−4.59	0.019	Lack of fit	3.46	1	3.46	32.92	0.030	
	Interaction x_1 - x_2	−2.26	0.55	−4.08	0.026	Pure error	0.21	2	0.10			
						Total	92.43	6				
ΣHAs	Mean	12.74	2.74	4.63	0.019	Regression	2522.06	3	840.68	15.90	0.024	94
	Cooking time (x_1)	15.85	3.63	4.36	0.022	Residuals	158.67	3	52.89			
	Annatto concentration (x_2)	−13.98	3.63	−3.84	0.031	Lack of fit	158.33	1	158.33	921.62	0.001	
	Interaction x_1 - x_2	−13.54	3.63	−3.72	0.034	Pure error	0.34	2	0.17			
						Total	2680.74	6				

(*continued on next page*)

Table 2 (*continued*)

HAs	Charcoal-grilled samples											
	Regression table					ANOVA table						
	Variables	Effect	Standard error	Calculated t	*p*-value	Variation source	Sum of squares	Degrees of freedom	Mean square	F-calc	p-value	R^2 (%)
Pan-fried samples MeIQx	Mean	1.39	0.56	2.48	0.088							
	Cooking time (x_1)	2.19	0.74	2.95	0.059							
	Annatto concentration (x_2)	−1.03	0.74	−1.39	0.256							
	Interaction x_1 - x_2	−1.03	0.74	−1.39	0.256							
4,8-DiMeIQx	Mean	0.38	0.05	6.57	0.007	Regression	1.28	3	0.42	17.55	0.02	67
	Cooking time (x_1)	0.47	0.07	6.09	0.008	Residuals	0.07	3	0.02			
	Annatto concentration (x_2)	−0.21	0.07	−2.78	0.068	Lack of fit	0.07	1	0.07	102.42	0.009	
	Interaction x_1 - x_2	−0.21	0.07	−2.78	0.068	Pure error	0.00	2	0.0			
						Total	1.35	6				
7,8-DiMeIQx	Mean	0.09	0.00	15.67	0.001	Regression	0.063	3	0.021	92.643	0.0018	99
	Cooking time (x_1)	0.10	0.01	12.73	0.001	Residuals	0.0006	3	0.000			
	Annatto concentration (x_2)	−0.06	0.01	−7.60	0.004	Lack of fit	0.0004	1	0.000	3.157	0.2175	
	Interaction x_1 - x_2	−0.06	0.01	−7.60	0.004	Pure error	0.0002	2	0.000			
						Total	0.0638	6				
PhIP	Mean	1.52	0.18	8.26	0.003	Regression	11.29	3	3.76	15.77	0.02	71
	Cooking time (x_1)	1.46	0.24	5.98	0.009	Residuals	0.71	3	0.23			
	Annatto concentration (x_2)	−0.60	0.24	−2.46	0.090	Lack of fit	0.67	1	0.68	37.49	0.02	
	Interaction x_1 - x_2	−0.56	0.24	−2.32	0.100	Pure error	0.036	2	0.018			
						Total	12.01	6				
ΣHAs	Mean	3.74	0.67	5.55	0.011	Regression	94.03	3	31.343	9.857	0.046	63
	Cooking time (x_1)	4.02	0.891	4.51	0.020	Residuals	9.53	3	3.17			
	Annatto concentration (x_2)	−2.10	0.891	−2.36	0.098	Lack of fit	9.53	1	9.53	13,318.50	0.00007	
	Interaction x_1 - x_2	−1.67	0.891	−1.884	0.156	Pure error	0.001	2	0.0007			
						Total	103.57	6				

R^2, coefficient of determination.

cooked for 30 min, underscoring the potential of this spice to curtail HA formation under severe cooking conditions.

The potent inhibitory role of annatto seed powder can be attributed, in part, to its rich array of bioactive compounds, namely the bixin and the phenolic compounds. The analysis of the total phenolic content and antioxidant activity of annatto seed powder revealed that this spice has a considerable content of phenolic compounds (8.56 mg GAE g^{-1}) as well as high antioxidant activity (18.30 and 30.40 mg of TEAC g^{-1} for DPPH and ABTS, respectively). Previous research has highlighted the role of bioactive compounds with antioxidant properties in inhibiting oxidation associated with the *Maillard* reaction by scavenging radicals, thereby mitigating reactive intermediate formation during HA synthesis (Murkovic, 2004; Neves et al., 2021). Studies comparing the inhibitory potential of spices have noted that those with higher total phenolic content and antioxidant activity tend to exhibit greater effectiveness in HA reduction. For example, in the study of Yu et al. (2024) the use of rosemary emerged as the most effective spice in reducing HAs formation (75 % reduction), and interestingly, this spice had the highest TPC (22.2 mg GAE g^{-1}) and greater antioxidant activity (42.5 mg AAE g^{-1}) of the tested spices. Similarly, in the study conducted by Lu and its colleagues, where ginger was the spice with the greatest inhibitory effect on HAs formation, a high TPC (3.41 mg GAE g^{-1}) and equally high antioxidant activity (11.2 μM Trolox 100 g^{-1}) were observed for this spice compared to the other tested. Taken together, the results of this study and those from the literature provide strong evidence that both the total phenolic content and antioxidant activity appear to be important parameters for the inhibitory capacity of spices.

Nevertheless, the antioxidative mechanism cannot be solely attributed to phenolics, as evidenced by spices with high inhibitory potential but modest antioxidant capacity. Other compounds must play a role in inhibiting the formation of HAs (Kwon et al., 2023; Zhou et al., 2023), such as the carotenoids present in paprika and red pepper (Lu et al., 2018). In this sense, some studies found that the greatest HAs inhibition occurred with tomato (72 %), compared to garlic (60 %), ginger (58 %), onion (51 %), and pepper (32 %) (Khan, 2015; Khan et al., 2017), suggesting that other compounds and mechanisms may be involved in reducing the formation of HAs in meat during cooking. One of those compounds can be, in the case of annatto seed powder, bixin, which represents 80 % of total apocarotenoids present in annatto seeds. Bixin is formed by the oxidative cleavage of lycopene, and it is present in greater quantities in annatto seeds compared to phenolic compounds (Rivera-Madrid et al., 2016; Vilar et al., 2014). The main mechanisms associated with the action of bixin would be electron transfer when exposed to reactive species, due to conjugated double bonds in its structure, and hydrogen capture by the functional molecule of apocarotenoid, which would break the structure of the radicals.

The antioxidant action of annatto extract, rich in bixin, has been previously demonstrated, showing a high capacity to remove oxygen and nitrogen reactive species (Chisté, Benassi, & Mercadante, 2011; Chisté, Mercadante, et al., 2011). Interestingly, bixin exhibits the highest measured carotenoid oxidation potential to date. This high oxidation potential enhances its radical scavenging ability exponentially, even with the use of a small amount (Tay-Agbozo et al., 2018). Recently, the inclusion of bixin in active food packaging demonstrated that, even with the loss of 85 % due to thermal processing, remained efficient for the protection of oxidation sensible goods. This highlight

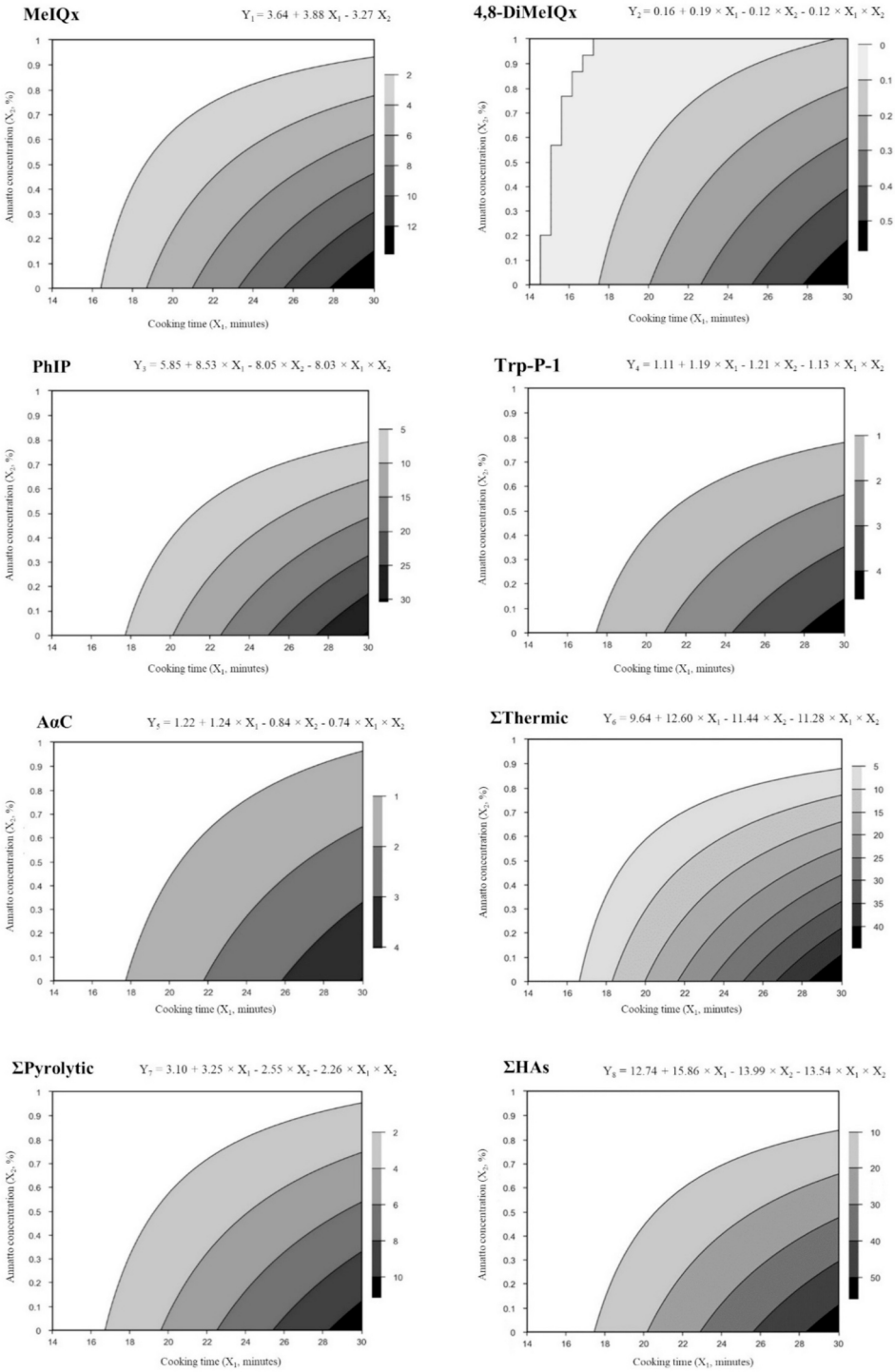

Fig. 2. Contour surfaces and predictive equations for HAs formation in charcoal-grilled beef patties according to the experimental design variables (cooking time - X_1, minutes; annatto seed powder concentration - X_2,% *w/w*).

that partially degraded bixin, particularly *trans*-bixin, continues to protect food (Stoll et al., 2023). In meat products, bixin has been shown to increase their oxidative stability (Figueirêdo et al., 2015).

In our study, we hypothesize that bixin could act by neutralizing or eliminating radicals involved in HAs formation during the thermal *Maillard* reaction, including the removal of cationic pyrazine or carbon-centered radicals observed in other reaction systems. Additionally, bixin can also form covalent adducts with electron-rich nucleophiles, thereby hindering HAs formation (Khan et al., 2022; Wang et al., 2023). The inhibition of HA formation by annatto seed powder was effective at 0.5 and 1 %, in a dose-dependent manner, corresponding to 10 and 20 mg of bixin per 100 g beef patty. This effectiveness persisted even under harsher cooking conditions with higher thermal exposure. Future studies are essential to elucidate the mechanisms responsible for the significant inhibitory effect of annatto seed seasoning on HA formation during food thermal processing. Furthermore, these studies should explore the roles of bixin and other phenolic compounds present in annatto seed in this process.

Multivariate statistical analysis, particularly employing a three-level full factorial design, enabled the identification of significant variables

Table 3
Bixin and total phenolic content as well as antioxidant activity of annatto seed powder.

Analyte	Mean (standard deviation)*
Bixin $\mu g_{100\ g}^{-1}$	02.08 (0.44)
TPC $mg_{GAE\ g}^{-1}$	08.56 (0.26)
$ABTS^{\bullet+}$ $mg_{TEAC\ g}^{-1}$	30.40 (2.62)
$DPPH^{\bullet}$ $mg_{TEAC\ g}^{-1}$	18.30 (0.66)
FRAP $mg_{FSE\ g}^{-1}$	17.44 (0.40)

*Expressed on a wet weight basis, $n = 3$. TPC, total phenolic content; GAE, gallic acid equivalents; $DPPH^{\bullet}$, 2-diphenyl-1-picrylhydrazyl radical; $ABTS^{\bullet+}$, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation; TEAC, Trolox equivalent antioxidant capacity; FRAP, ferric reducing antioxidant power; FSE, ferrous sulfate equivalents.

influencing HA formation during cooking. Specifically, the application of this type of statistical tool proved to be particularly useful in this study, as it was possible to identify that both individual variables (cooking time and annatto seed powder) and their interaction significantly influenced the response (Fig. 1 and Table 2). Cooking time exerted a positive effect on HA formation, while annatto seed powder addition showed a negative effect. The increase in HAs content with longer cooking times is a widely recognized and established relationship in the scientific literature. Several studies have already shown a very strong positive correlation between cooking time and HAs content in various cooked foods (Costa et al., 2009; Iwasaki et al., 2010; Puangsombat et al., 2011; Fan et al., 2022).

The goodness-of-fit of the proposed model can be evaluated in several ways (Pinto et al., 2018), including the R^2, diagnostic plots (e.g., normal probability of residuals, predicted versus actual values, residual versus predicted values, and standardized residuals versus run plot), ANOVA, Q^2 and others. In our study, for most individual HAs (as well as for ∑pyrolytic, ∑thermic and ∑HAs), the predicted values are highly consistent with the experimentally determined values (Fig. S4, supplementary material), which supports the validity of the model. This relationship is quite evident when observing the high R^2 values obtained in Table 2, which are a measure of the proportion of variability in the response variable explained by the model. The lack-of-fit of the model, a measure of how well the model fits the data, was significant ($p < 0.05$) for all individual HAs well as for ∑pyrolytic, ∑thermic and ∑HAs (Table 2). A significant lack of fit is not desirable, since it indicates that the variation of the design points about their predicted values is much larger than the variation of the replicates about their mean values. In practice, either the model does not predict well, or the replicates are so consistent that their variance is very small, or some combination of both (StatEase, 2022). Here, our hypothesis is that the high agreement among replicas, resulting from the tight control over the experimental conditions of meat preparation, cooking, and subsequent HAs analysis, may not reflect the true intrinsic variability of the model.

In summary, as depicted in Fig. 2, particularly in the contour plots, cooking time and annatto seed powder addition exhibit opposing effects on HA formation, with the latter mitigating the former's positive impact. This finding suggests that annatto seed powder supplementation can counteract the increase in HA formation associated with prolonged cooking times, thereby offering a practical means to reduce human exposure to these carcinogenic compounds. This is especially relevant, due to the role of HAs in cancer development through eating well-done meat. Although several classes of carcinogens and multiple mechanisms are likely to be at play, much attention has focused on HAs (Le Marchand, 2021). The current recommendations of World Health Organization (WHO - World Health Organization, 2023) suggest that red meat consumption should be between 98 g and 500 g per week for adults. Additionally, the World Cancer Research Fund International (WCRF - World Cancer Research Fund International, 2018) recommends a consumption limit of red meat to no more than about three portions per week (about 350–500 g cooked weight). Based on these recommendations, a scenario of consumption of 100 g of well-done and very well-done charcoal-grilled beef patties, entails an intake of around 1800 and 6000 ng of HAs, respectively. Considering the same scenario, but with patties seasoned with 1 % of annatto, the intake of HAs would decrease to 180 and 575 ng 100 g^{-1} for well and very-well done, respectively. Considering the same consumption scenario for pan-fry, an intake of 506 and 1170 ng are considered for the mean (11.5 min) and high (15 min) cooking times, respectively. The 1 % addition of annatto would lower these potential exposure levels for 160 and 500 ng 100 g^{-1}, respectively. The amount of daily HAs intake is different between various epidemiological surveys, one of the studies estimating that the calculated daily HAs intake is of ~420 ng/day per person (Keating & Bogen, 2004; Wang et al., 2023). However, a USA case-control study reported a daily intake of 261 ng for controls and 364 ng for cases of colorectal cancer (Nowell et al., 2002). Regarding the sum of HAs in well-done and very well-done pan-fried and charcoal-grilled beef patties, special attention must be given to the intake of these cooked meats, since extremely high amounts of HAs can be taken in a single meal/portion. Although the cooking methods used to prepare meat differ largely within countries, and the prevalence of charcoal grilling and pan-frying is quite variable in different populations (Keating et al., 2004), the use of 1 % annatto in patties, represents an extraordinary strategy to decrease the exposure to HAs without modifying the cooking method and doneness level of the meat.

5. Conclusion

This study represents the first investigation into the potential of annatto seed powder to reduce the formation of harmful HAs in meat. Incorporating annatto seed powder, rich in bixin (~2 %) and with a total phenolic of ~8.6 mg GAE g^{-1}, proved to be a highly effective strategy for curtailing HAs formation, with reductions of up to 91 % observed. Seasoning beef patties with annatto seed powder (0.5 to 1 g per 100 g of beef, with 10 to 20 mg of bixin) led to a dose-dependent decrease in the formation of heterocyclic amines (HAs) during charcoal-grilling and pan-frying. The strongest inhibitory effect was noted during longer cooking durations, which are typically associated with higher HAs levels. Multivariate analysis facilitated the identification of significant variables (annatto concentration, cooking time, and their interaction) influencing HAs formation, with predictive equations proposed to forecast the formation of these harmful compounds.

Consumption of 100 g portions of well-done and very well-done charcoal-grilled beef patties corresponds to an intake of approximately 1.8 and 6 μg of HAs, respectively. In contrast, patties seasoned with 1 % annatto exhibit a tenfold reduction in exposure (0.18 and 0.6 μg, respectively). Similarly, significant reductions were observed in pan-fried beef. These findings underscore the dual benefits of incorporating annatto seed powder in beef patties: not only does it enhance the product with value derived from Brazilian flora, but it also offers a straightforward method, that can be adopted in the practice of beef preparation at household or by meat industry, to diminish dietary exposure to HAs, thereby augmenting the overall safety profile associated with meat consumption. Future studies should investigate the mechanisms by which annatto seed seasoning inhibits the formation of HAs during food thermal processing, with a specific focus on the roles played by bixin and phenolic compounds.

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Table 4
Inhibitory effects of the different spices on ΣHAs formation in meat formulation, and TPC and antioxidant activity of the spices.

Spice	TPC (mg GAE g^{-1})	DPPH$^{\bullet}$ (*)	ABTS$^{\bullet+}$ (*)	FRAP (*)	% used	Meat	Cooking method	ΣHAs inhibition (%)	Reference
annatto seed	08.56 (0.26)	18.33 (0.66)	30.40 (2.62)	17.44 (0.40)	0.5 1 0.5 1 0.5 1	beef patties 80 g (9 cm diameter x 1 cm thickness)	charcoal-grilled, ~200 °C, 22 min, well-done charcoal-grilled, ~200 °C, 30 min, very-well-done pan-fried, 180–200 °C, 11.5 min, well-done pan-fried, 180–200 °C, 15 min, very well-done	57–61 79 **55** **91** 53–54 68 **18** **57**	Present work
rosemary	9.85 (0.91)	95.7 (0.20)	–	–	0.2	beef patties 100 g (10 cm diameter x 1 cm thickness)	pan-fried, 204 °C, 10 min well-done	43	Puangsombat et al., 2011
turmeric	13.7 (0.53)	92.5 (0.59)						39	
fingerroots	12.2 (0.14)	49.6 (1.08)						34	
galangal	2.28 (0.53)	19.9 (0.92)						18	
coriander seed	0.74 (0.06)	9.49 (0.73)						4	
cumin	1.67 (0.14)	20.1 (0.92)						3	
rosemary	22.2 (0.08)	–	–	42.5 (0.22)	0.5	beef patties 55 g (9 cm diameter x 0,7 cm thickness)	roasted, 230 °C 15 min	75	Yu et al., 2024
bay leaf	18.8 (0.03)			14.0 (0.05)				46	
turmeric	13.8 (0.05)			26.1 (0.32)				26	
ginger	3.41 (0.02)	–	11.19 (1.15)	–	0.5	beef balls 15 g (9 cm diameter)	deep-fried, 180 °C, 3 min	78	Lu et al., 2018
black pepper	2.97 (0.01)		13.57 (1.63)					65	
red pepper	3.47 (0.03)		8.45 (1.35)					54	
garlic	0.90 (0.00)		7.86 (1.29)					48	
paprika	1.18 (0.01)		7.5 (0.94)					46	
onion	0.99 (0.01)		6.55 (0.90)					43	
ginger	9.90 (0.27)	0.26	4.60	–	0.25 0.75 1.25	pork balls 20 g	deep-fried, 180 °C, 3 min	63 48 11	He et al., 2022
rosemary	61.3 (1.23)	0.03	0.93		0.25 0.75 1.25			59 32 5	
turmeric	–	–	–	–	0.5 1 0.5 1	chicken balls 60 g	grilled, 200 °C, 18 min grilled, 250 °C, 18 min	72 33 61 20	Kilic et al., 2021
turmeric	16.32 (0.36)	26.30 (0.44)	37.66 (2.80)	–	0.5	chicken wings	air fryer, 180 °C, chicken: 30 min, pork: 20 min	51	Kwon et al., 2023
						pork belly		37	
rosemary	36.47 (0.44)	46.35 (0.05)	67.25 (4.22)		0.5	chicken wings		41	
						pork belly		30	
garlic	8.93 (0.20)	14.11 (1.05)	26.90 (2.44)		15	chicken wings		33	
						pork belly		32	

TPC, total phenolic content. *Different units of measurement for antioxidant activity assays, DDPH$^{\bullet}$ (2-diphenyl-1-picrylhydrazyl radical) and ABTS$^{\bullet+}$ (2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation): % scavenging effect at 50 mg mL^{-1} (Puangsombat et al., 2011); μM de Trolox 100 g^{-1} (Lu et al., 2011); IC_{50} mg mL^{-1} (He et al., 2022); mg of Trolox equivalent antioxidant capacity (TEAC) g^{-1} of spice (Kwon et al., 2023; and the present work). FRAP (ferric reducing antioxidant power): mg of ascorbic acid equivalents (AAE) g^{-1} of spice (Yu et al., 2024); mg of gallic acid equivalents (GAE) per g of annatto seed powder g^{-1} (present work). Results expressed as mean (standard deviation) of TPC and antioxidant activity. **Values express the percentage of inhibition of ΣHAs towards unseasoned samples.

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Ethical statement

This article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

Thaís de Moura Neves-Gonçalves: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Edgar Pinto:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Olga Viegas:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Anna Rafaela Cavalcante Braga:** Formal analysis, Data curation. **Leonardo M. de Souza Mesquita:** Methodology, Investigation, Formal analysis. **Isabel Maria Pinto Leite Viegas Oliveira Ferreira:** Methodology, Funding acquisition. **Carmen García-Jares:** Methodology, Funding acquisition. **Veridiana Vera De Rosso:** Methodology, Conceptualization. **Semíramis Martins Álvares Domene:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, 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.

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.2024.141015.

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