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Assessment of the influence of some spice extracts on the formation of heterocyclic amines in meat

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ABSTRACT

Beef meat samples were heated in the presence of basil, oregano, marjoram, rosemary, sweet grass, savory, thyme and coriander extracts and the effects on the formation of heterocyclic amines (HAs) were measured by HPLC with fluorescence detection. The results obtained after heating meat in diethylene glycol showed that the addition of 0.2% and 0.5% of thyme, savory and oregano extracts slightly decreased the formation of 1-methyl-6-phenyl-1H-imidazo[4,5-*b*]pyridin-2-amine (PhIP). Other extracts did not have any influence on the concentration of PhIP, while in some cases they promoted its formation, particularly with basil extract. Oregano and basil extracts, which showed the best positive and negative effects on the formation of PhIP, were tested by heating meat in vegetable oil. Both extracts increased PhIP amount, but decreased Trp-P-2 concentration (see Section 2.1). The content of Trp-P-1 decreased in the samples with basil and increased in those with oregano. No correlation between the anti-oxidant activity of extracts and the formation of PhIP was found.

1. Introduction

Heterocyclic amines (HAs) are the most common environmental mutagens and are suspected human carcinogens (Nozawa, Nakao, Takata, Arimoto-Kobayashi, & Kondo, 2006). There are two classes of aromatic amines related to cooked food: amino carbolines and aminoimidazoazarenes (AIA). All of these compounds can be formed in products rich in proteins (amino acids) during the treatment at high temperatures. Maillard reactions are involved in the formation of HAs (Skog, 1993) and free radicals are important intermediate species in this reaction. Several studies have reported that natural and synthetic anti-oxidants decreased the mutagenic activity or inhibited the formation of HAs. In most cases, the effects of anti-oxidants on the formation of HAs were measured using mutation tests, while only a few studies have measured these effects using chromatographic methods. For example, it was observed that butylated hydroxyanisole (BHA), soy protein products and anti-oxidative green or black tea polyphenols influenced the formation of HAs (Wang, Vuolo, Spingarn, & Weisburger, 1982). *t*-Butyl hydroquinone (TBHQ) had only a slight HAs-inhibiting effect in all kinds of meat in a model system (Messner & Murkovic, 2004). In another study, vitamins C and E, and butylated hydroxytoluene (BHT), were found to be effective HAs inhibitors (Lan, Kao, & Chen, 2004). However, in fried fish, the same anti-oxidants did not show any consistent effect on the concentration of HAs (Tai, Lee, & Chen, 2001).

Spices are a promising source of natural anti-oxidants, and some of them were tested to measure their effects on the formation of HAs. For instance, rosemary oleoresin, added to the ground beef patties before frying, resulted in a reduction of PhIP (Balogh, Gray, Gomma, & Booren, 2000), while rosemary extract added to virgin olive oil, used for frying beef burgers, also decreased the concentration of HAs (Persson, Graziani, Ferracane, Fogliano, & Skog, 2003). The formation of MeIQx was effectively reduced by additions of 1.0% of pine bark extract, rosemary oleoresin or grape seed extract (Ahn & Grun, 2006). Most recently, it was reported that spice-containing marinades can be effective inhibitors of HA's formation during meat grilling (Smith, Ameri, & Gadgil, 2008). However, flavouring substances extracted from thyme, marjoram and rosemary increased PhIP in a model system, independently of their properties (pro- or anti-oxidative) (Zöchling & Murkovic, 2002).

Although a number of research studies have been performed since 1970 on HAs, they remain a challenge for scientists from various points of view (Knasmüller, Murkovic, Pfau, & Sontag, 2004). Somewhat controversial findings and still scarce information on the effects of spice extracts and anti-oxidants on the formation of HAs can be considered as an important issue for further studies in this area. Therefore, this study was aimed at further investigations of ways to reduce the formation of HAs in heated meat by using selected spice extracts.

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2. Materials and methods

2.1. Chemicals and other materials

All HA reference compounds (1-methyl-6-phenyl-1H-imidazo [4,5-*b*]pyridin-2-amine (PhIP); 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole, acetate (Trp-P-1); 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2); 2-amino-9H-pyrido[2,3-*b*]indole (AαC); 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole (MeAαC); and 2-amino-6-methyldipyrido[1,2-A:3′,2′-D]imidazole, hydrochloride hydrate (Glu-P-1)), used as standards, were purchased from Toronto Research Chemicals (Toronto, Canada).

For the first experiment, acetic acid, acetonitrile, methanol (all HPLC grade) and diethylene glycol were from Sigma–Aldrich (Germany); ethyl acetate was from Chempur (Poland); and sodium hydroxide, hydrochloric acid and ammonium hydroxide (25%) from Merck (Darmstadt, Germany). Chromoband XTR (70 ml, 14,500 mg, kieselguhr) and Chromoband PS-H^+ (strong PS/DVB cation-exchanger in H^+ form) cartridges were supplied by Macherey–Nagel (Duren, Germany).

For the second experiment, acetonitrile and methanol were purchased from Merck (Darmstadt, Germany); ammonium hydroxide (25%) was from Fluka Chemic (Switzerland), water from Carlo Erba Reagenti (Italy) and ethyl acetate from Clear Consult (Italy). Diatomaceous earth and C18 columns (500 mg Strata C18-E) were obtained from Phenomenex, USA; and PRS cartridges (500 mg Bond Elut LRC-PRS) were from Superchrom (Italy).

2.2. Preparation of spice extracts

The following plants (supplied by "Gardukas", Kaunas, Lithuania) were used to prepare the extracts: basil (*Ocimum basilicum*), oregano (*Origanum vulgare*), marjoram (*Origanum majorana*), rosemary (*Rosmarinus officinalis*), sweet grass (*Hierochloe odorata*), savory (*Satureia hortensis*), thyme (*Thymus vulgaris*), and coriander (*Coriandrum sativum*). Ten grams of dried ground leaves or seeds (coriander) were placed in a 250 ml flask and twice extracted with 100 ml of ethanol/water (70/30 v/v) by constant shaking during 2 h. The extracts were filtered and dried in a vacuum rotary evaporator (removal of ethanol) and freeze-dryer (removal of water). Dry extracts were stored in a freezer until used.

2.3. Total phenolics

The content of phenolic compounds was determined using Folin–Ciocalteu regent (Singleton & Rossi, 1965) and expressed in gallic acid equivalents (GAE). Dry extracts were diluted in methanol and 1 ml of this solution was transferred to a volumetric flask with 5 ml of Folin–Ciocalteu regent and 4 ml of 7.5% Na_2CO_3. After 30 min incubation the absorbance was measured at 765 nm, using a UV spectrophotometer and compared with a gallic acid calibration curve. All measurements were made in duplicate.

2.4. Determination of radical-scavenging in DPPH· reaction

The anti-oxidant activities of spice extracts were measured using the DPPH· radical-scavenging assay method (Brand-Williams, Cuvelier, & Berset, 1995). Dry extracts were dissolved in methanol. Two millilitres of 6×10^{-5} M methanol solution of DPPH· were used. The reaction was started by the addition of 50 μl of opportunely diluted spice extracts and the decrease in absorbance was measured on a spectrophotometer at 515 nm after 30 min. Methanol was used as a blank sample. All determinations were performed in triplicate.

The anti-oxidant activity was calculated as the inhibition (%) of the DPPH·:

$$\% \text{ Inhibition} = \left[\frac{A_{515}^{\text{control}} - A_{515}^{\text{sample}}}{A_{515}^{\text{control}}}\right] \cdot 100$$

2.5. Identification of extract composition using HPLC–MS/MS

Phenolic compounds were identified by liquid chromatography separation, coupled with tandem mass spectrometry analysis (LC/MS/MS). For this purpose, chromatographic separation was performed, using an HPLC apparatus equipped with two Series 200 micropumps (Perkin Elmer, Norwalk, CT, USA), a UV/vis series 200 (Perkin Elmer, Wellesley, MA, USA) detector set at 280 nm, and a Prodigy 5 μm ODS3 100 Å column (250 × 4.6 mm, particle size 5 μm) (Phenomenex, Torrance, CA, USA). The eluents were: (A) water solution of 0.2% formic acid; (B) acetonitrile/methanol (60:40 v/v). The gradient programme was: 0–10 min B increased from 18% to 27%; 10–20 min from 27% to 35%; 20–30 min from 35% to 50%; and 30–50 min from 50% to 100%. The flow was set at 0.8 ml/min. The LC flow was split and 0.2 ml/min was sent to the mass spectrometry. Injection volume was 20 μl. MS and MS/MS analyses of extracts were performed on an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada) equipped with a Turbolonspray source working in the negative ion mode. The following settings were used: capillary voltage (IS) −4000 V, declustering potential (DP) −60 V, and the drying (air) was heated to 400 °C.

Information-dependent acquisition (IDA) (Decaestecker et al., 2004) was used to identify the metabolites: this acquisition method generates a survey scan, single MS spectra with molecular mass information, product ion spectra (MS^2) and extracted ion fragment grams (XICs). IDA was carried out in the range m/z 50–1100 and the identified compounds were then analysed in MRM (multiple reaction monitoring).

2.6. Meat preparation and cooking

Two independent experimental procedures were applied for meat samples. For the first experiment, beef was purchased from the local market in Kaunas (Lithuania). One kilogram of raw beef meat was homogenised, freeze-dried and stored in a freezer at −18 °C until used. Freeze-dried meat samples (1 g) were heated, with or without spice extracts, with diethylene glycol (10 ml) in open crucibles at 200 °C for 20 min, using a microwave-grill oven (grill with convection programme). The crucibles with meat were immediately cooled on ice after heating.

For the second experiment, beef was purchased from the local market in Portici (Italy). Four hundred grams of minced beef were homogenised and freeze-dried. One gram of meat, with or without extracts, was heated at 200 °C for 30 min with 9 ml of sunflower oil (Olindo, Italy) in tightly closed 25 ml tubes (Pyrex, England). All tubes were immediately cooled after heating.

2.7. Extraction of HAs

The chromatographic separation of HAs for the first experiment was performed using a modified method described elsewhere (Messner & Murkovic, 2004). All samples were dissolved in 12 ml of 1 M NaOH and homogenised for 30 min at 150 rpm. The alkaline solution was poured into Chromabond XTR cartridges. Ethyl acetate (50 ml) was used as the extraction solvent and the eluate was passed into PS-H^+ cartridges. The cartridges were washed with 0.1 M HCl (2 ml) and MeOH (2 ml) and HAs were eluted with 2 ml of MeOH-concentrated ammonia (19:1 v/v). All samples were