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Isolation and identification of oxidation products of guaiacol from brines and heated meat matrix

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ABSTRACT

In this study we investigated the formation of the oxidation products of guaiacol in brines and heated meat matrix: 6-nitrosoguaiacol, 4-nitroguaiacol and 6-nitroguaiacol. For this purpose we applied a newly developed HPLC-UV and LC–MS method. For the first time, 6-nitrosoguaiacol was determined in brine and meat (containing guaiacol and sodium nitrite), which had been heated to 80 °C and subsequently subjected to simulated digestion. Application of 500 mg/L ascorbic acid to the brines reduced guaiacol degradation at pH 3 and simultaneously inhibited the formation of 6-nitrosoguaiacol compared to brines containing only 100 mg/L of ASC. The oxidation products were isolated with a new extraction method from meat samples containing 400 mg/kg sodium nitrite at pH 3.6 following simulated digestion. When oxygen was added, 6-nitrosoguaiacol was determined even at legally allowed levels (150 mg/kg) of the curing agent. Finally, we developed a new LC–MS method for the separation and qualitative determination of the four main smoke methoxyphenols.

1. Introduction

The methoxyphenols guaiacol (2-methoxyphenol), 4-methylguaiacol, syringol (2,6-dimethoxyphenol), and 4-methylsyringol have been shown to be the four main phenolic constituents of wood smoke, smoke flavoring, and smoked food products, with varying concentrations that depend on extraction method, type of wood, smoking conditions, and type of food product (Baltes & Söchtig, 1979; Knowles, Gilbert, & McWeeny, 1975a; Wittkowski, Toth, & Baltes, 1981; Hitzel, Pöhlmann, Schwägele, Speer, & Jira, 2013). Many studies have dealt with the extraction and detection of these compounds in consideration of their effects on food properties such as the enhancement of preservability and typical smoke flavoring (Baltes & Söchtig, 1979; Kjällstrand & Petersson, 2001). Due to the volatility of the analytes their analysis has mainly been achieved by means of gas chromatography mass spectrometry (GC–MS) using derivatization to their trimethylsilyl ethers in order to increase sensitivity (Kornreich & Issenberg, 1972). Analysis by liquid chromatography mass spectrometry (LC–MS) has been carried out sporadically for the detection of glycosylated derivatives (Hayasaka et al., 2010) or fluoride transfer products (Yee et al., 2013). However, these four major phenolic smoke components had not yet been separated or analyzed qualitatively under underivatized conditions. We developed a new LC–MS method for this purpose. Furthermore, we selected guaiacol as the most important phenol in food products smoked with softwood (Hitzel et al., 2013) to apply LC–MS, GC–MS, HPLC-UV; diode array detection (HPLC-DAD), and electrochemical detection (HPLC-ECD) in order to separate, detect and analyze this monomethoxyphenol and its oxidation products formed in brine and meat containing sodium nitrite as curing agent. The reaction of guaiacol in aqueous medium with nitrosating agents at low pH values and high temperatures has been well studied in regard to its importance as an air pollutant (Kung, 1968; Dimmel, Karim, Savidakis, & Bozell, 1996; Tang & Thompson, 2012; Kitanovski, Cusak, Grgic, & Claeys, 2014). Oxidation products were determined to be 4-nitroguaiacol, 6-nitroguaiacol, or 4,6-dinitroguaiacol. In one case investigators found 2-methoxy-1,4-benzoquinone-4-oxime, which is isomeric to nitrosoguaiacol, but the latter has not yet been identified either in aqueous media or in food matrix. The importance of guaiacol and possible nitrosation products arose from its concentration in smoked food products and smoke flavoring used in food processing (Baltes & Söchtig, 1979). The concentration of this phenol was determined in smoked pork belly to be as high as 35.6 mg/kg (Lustre & Issenberg, 1970). Furthermore, it must be taken into account that the concentration of smoke phenols is much higher on the surface of smoked food products than in homogenized matrix or the inner layers (Bratzler, Spooner, Weathers, & Maxey, 1969; Knowles et al., 1975a). Not only does the surface of meat products receive the main impact of heat treatment during meat processing (temperatures up to 80 °C for hot smoking (Maga, 1988)), the surface is also subjected to the most intense contact with the nitrite in curing brine, so that the phenols in the outer layers are particularly at risk of oxidative degradation

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under the formation of their nitrosated and nitrated derivatives. Nitrosated guaiacol has been shown to be genotoxic in *in vitro* experiments (Ohshima et al., 1989) and to have structural characteristics indicating probable mutagenicity (Rosenkranz, Klopman, Ohshima, & Bartsch, 1990); for these reasons its reaction behavior in food products containing nitrite is of particular interest. We therefore investigated the depletion of guaiacol and the generation of nitrosation and nitration products in curing brines and meat products under different oxidation conditions.

2. Materials and methods

2.1. Chemicals

Syringol, 5-nitroguaiacol, ammonium bicarbonate, and TEAOH (tetraethylammonium hydroxide) were obtained from Sigma–Aldrich (Steinheim, Germany). The 2-nitroso-5-methoxyphenol was purchased from Molekula (Newcastle upon Tyne, UK); guaiacol, 4-methylsyringol, and 4-methylguaiacol were obtained from TCI Chemicals (Tokyo, Japan). Methanol, ethanol, and acetonitrile of HPLC and LC–MS grade were obtained from Fisher Scientific (Loughborough, UK). Nitrite curing salt containing 0.9% sodium nitrite was purchased from the Institute of Food Quality and Food Safety, University of Veterinary Medicine Hannover, Germany. The nitrite curing solution contained 200 mg/mL sodium chloride and 8 mg/mL sodium nitrite. Sodium chloride, sodium nitrite, acetic acid, hydrochloric acid, ammonia solution, pepsin, and ascorbic acid were obtained from Carl Roth (Karlsruhe, Germany). Helium 5.0, oxygen, argon, and nitrogen gas were obtained from Linde AG (Pullach, Germany). Distilled water (18 MΩ × cm) was prepared with a purification system (Millipore, San Jose, CA, USA).

2.2. Brine preparation

Brines consisted of 1.6% nitrite curing salt (NaCl containing 0.9% sodium nitrite) in aqueous solution, resulting in a sodium nitrite concentration of 150 mg/L. Samples were prepared containing 200 mg/L of guaiacol to which either 100 mg/L or 500 mg/L ascorbic acid was added. Brines were first heated in a water bath at 80 °C for 40 min. The samples were cooled in an ice bath to 18 °C, whereupon 4 mL of the brine were removed and filtered through a syringe filter (nylon, pore diameter 0.45 μm) in order to remove the brown precipitate formed during the heating procedure. Finally, 2.5 mL of the filtered yellow brine and 250 μL of 4-methylguaiacol (1 g/L) as internal standard were transferred to a 5-mL volumetric flask and filled to volume with water. In all the dilution of brines was 1:2. The sample was analyzed with HPLC-UV detection.

The remaining brine was subjected to simulated digestion. For this the pH of the brine was adjusted to 3 with 0.5 M hydrochloric acid, and the sample was heated for 1 h in a water bath at 40 °C. Afterwards the brine was cooled in an ice bath to 18 °C and the pH was adjusted to 5 with 2 M ammonia solution. For analysis, 4 mL of brine were removed and prepared as described above. Six replications were made for each brine experiment.

2.3. Manufacturing of meat samples

Meat samples were made from minced meat consisting of 55% pork and 45% beef obtained from a local supermarket. All meat samples were prepared with 60 mg/kg guaiacol and 100 mg/kg ascorbic acid. Then, either 1.66% nitrite curing salt (containing 0.9% $NaNO_2$) or 5% nitrite curing solution (8 mg/mL $NaNO_2$) was added to the meat samples, resulting in a concentration of sodium nitrite of either 150 mg/kg or 400 mg/kg. The meat mixtures were homogenized in a blender. For experiments using egg whites, these were added to the mixture in unfoamed or foamed form to comprise 20% *w*/*w* of the samples. The foam was prepared under air or oxygen atmosphere. Six replications were made of each formulation. Meat mousses were filled in screw-lid jars and heated in a water bath for 45 min at 80 °C. The batches were cooled in an ice bath to a core temperature of 18 °C. Finally, 3 g of each sample were removed for extraction.

For experiments with simulated digestion, centrifuge tubes were filled with 10 g of the heated meat mass, 5 mL 0.5 M hydrochloric acid, and 1.5 mL pepsin solution (5 mg pepsin in 1 mL water adjusted to pH 4.5 with 0.5 M hydrochloric acid). The samples were homogenized with an Ultra-Turrax® (IKA, Staufen, Germany) and heated for 1 h in a water bath at 40 °C. Afterwards, the sample batches were cooled in an ice bath to a core temperature of 18 °C. Finally, 1 mL of 2 M ammonia solution was added and 5 g of the sample were used for extraction.

2.4. Extraction and purification of guaiacol and oxidation products from meat matrix

The samples were extracted three times with 5 mL methanol (heated to 50 °C) and once with 5 mL water using an Ultra-Turrax for homogenization. The emulsion was cooled to 0 °C, centrifuged at 0 °C for 4 min at 20.000 rpm, filtered through a folded filter, and the filtrate brought to a volume of 150 mL with water. Finally, the extract was purified by means of solid phase extraction (SPE) using LiChrolut® EN cartridges (40–120 μm, 200 mg, Merck, Darmstadt, Germany). The cartridge was rinsed with methanol and conditioned with water. After the meat extract was applied, the cartridge was rinsed with deionized water, and analytes were eluted with 4 mL methanol (at 40 °C). The extract was dried to approximately 300 μL under nitrogen gas at 37 °C. We ensured that no further product formation occurred during the concentration procedure. Concentrated meat extract and 50 μL of 4-methylguaiacol as internal standard (200 mg/L) were transferred to a 1-mL volumetric flask, which was filled to volume with water.

2.5. Recovery of guaiacol and oxidation products from meat matrix

Recovery was determined in six replications for guaiacol and oxidation products from meat matrix as follows: Either standard solution of guaiacol (60 mg/kg) or a mixture of 6-nitrosoguaiacol, 6-nitroguaiacol, and 4-nitroguaiacol (previously isolated from the heated curing brine) was added to raw minced meat containing neither ascorbic acid nor nitrite curing salt. The mixtures were homogenized in a blender and samples were heated to 80 °C and subjected to simulated digestion (see above). Extraction and purification were carried out as described above.

2.6. Analytical methods

2.6.1. HPLC-UV analysis, HPLC-DAD spectrum analysis and HPLC-ECD analysis

Preparative and analytical HPLC-UV and HPLC-EC detection were carried out on a Knauer Maxistar K-1000 HPLC pump (Knauer, Berlin, Germany), connected to a Jasco 875 Intelligent UV Detector (Jasco Corporation, Tokyo, Japan) followed by a 641 VA-Detector and a 650 Electrochemical Detector (Metrohm AG, Herisau, Switzerland) controlled by Eurochrom 2.05 software on a Knauer Interface Box (Knauer, Berlin, Germany).

Different columns, injection volumes, and wavelengths were used for the analysis of brines and meat extract because of the need for greater sensitivity in the analysis of the meat extract due to the effects of the meat matrix and the lower yields of oxidation products. Brines were analyzed with a 250 mm Nucleodur C-18 Isis® column with an inner diameter (i. d.) of 4.6 mm and a particle size of 5 μm; the meat extract was analyzed with a 150 mm Nucleodur C-18 Isis® column (particle size 3 μm, i. d. 3 mm) using a corresponding pre-column (all obtained from Macherey-Nagel, Düren, Germany). For detection in brine, 20 μL were injected and measured at a wavelength of 272 nm, whereas for meat extract, 100 μL were injected and measured at