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Formation and mitigation of *N*-nitrosamines in nitrite preserved cooked sausages

S.S. Herrmann *, K. Granby, L. Duedahl-Olesen

National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark

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Chemical compounds studied in this article:
N-nitrosothiazolidine-4-carboxylic acid (NTCA) (PubChem CID: 104890)
N-nitroso-2-methyl-thiazolidine-4-carboxylic acid (NMTCA) (PubChem CID: 115101)
N-nitrosoproline (NPRO) (PubChem CID: 24141)
N-nitrosohydroxyproline (NHPRO) (PubChem CID: 61873)
N-nitrosopiperidine (NPIP) (PubChem CID: 7526)
N-nitrososarcosine (NSAR) (PubChem CID: 25811)
N-nitrosodimethylamine (NDMA) (PubChem CID: 6124)
N-nitrosopyrrolidine (NPYR) (PubChem CID: 13591)
Erythorbic acid (PubChem CID: 54675810)
6-O-palmitoyl-L-ascorbic acid (Ascorbyl palmitate) (PubChem CID: 54680660)

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A B S T R A C T

Literature on formation and mitigation of *N*-nitrosamine (NA) and especially non-volatile NA (NVNA) in meat products is scarce and the present study is therefore a relevant contribution to the field. We found positive correlation between the levels of *N*-nitrosopiperidine (NPIP), *N*-nitrosohydroxyproline (NHPRO), *N*-nitrosoproline (NPRO), *N*-nitrosothiazolidine-4-carboxylic acid (NTCA) and *N*-nitroso-2-methyl-thiazolidine-4-carboxylic acid (NMTCA) and the amount of nitrite added to cooked pork sausages. The levels studied were 0, 60, 100, 150, 250 and 350 mg kg^{-1}. The levels of *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR) remained at or below limit of quantification. Erythorbic acid inhibited the formation of NHPRO, NPRO, NPIP and NTCA. This inhibition was for NTCA and NMTCA counteracted by addition of free iron. Ascorbyl palmitate had less inhibitory effect than erythorbic acid and a combination of the two provided no further protection. Increasing the black pepper content increased the levels of NPIP and NMTCA. Only slight effects of increased fat content and addition of tripolyphosphate were observed.

1. Introduction

Sodium nitrite (nitrite) has for decades been widely used for preservation of meat products and is an efficient inhibitor of the growth of *Clostridium botulinum* and thereby decreases the risk of this organism producing toxins and heat-resistant spores. Nitrite also provides the processed meat with its characteristic red colour, flavours and aromas, known from products such as bacon, and it inhibits lipid oxidation processes (Skibsted, 2011). However, *N*-nitrosamines (NA) may be formed during production and storage of nitrite preserved meat products. The group of NA include both the so called volatile NA (VNA) and the non-volatile NA (NVNA). The levels of these compounds in nitrite preserved meat products varies greatly, from below detectability (<1 μg kg^{-1}) to levels in the order of thousands μg kg^{-1}, depending on the type of NA. In particular the NVNA are found in high amounts (Hill et al., 1988). The NA is a large group of compounds of which the

* Corresponding author.
E-mail address: sher@food.dtu.dk (S.S. Herrmann).

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majority is carcinogenic (IARC, 1978). The VNA are generally potent carcinogens (e.g. *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR)) whereas the NVNA are weak carcinogens (*N*-nitrososarcosine (NSAR)), or assumed to be non-carcinogenic (e.g. *N*-nitrosoproline (NPRO), *N*-nitrosohydroxyproline (NHPRO), *N*-nitroso-thiazolidine-4-carboxylic acid (NTCA) and *N*-nitroso-2-methyl-thiazolidine 4-carboxylic acid (NMTCA)). However, the assumption that NVNA as NPRO, NHPRO, NTCA and NMTCA are non-carcinogenic, needs to be verified by actual toxicological *in vivo* studies. Theoretically there is a risk of these compounds being decarboxylated into their carcinogenic counterparts (NPYR, NHPYR, NThZ and NMThZ) either during heat treatment or by microbial activity in the large intestine. As long as it cannot be verified whether these presumable non-carcinogenic NA, contribute to the adverse health effects observed by consumption of processed meat or not, their formation should be prevented as much as possible.

Studies have indicated that there is a positive though not necessarily linear correlation, between the amount of nitrite added and the amount of NA formed (Drabik-Markiewicz et al., 2009; Drabik-Markiewicz et al., 2011; Yurchenko & Mölder, 2007). These studies also indicate that the effects observed on the NA levels by changes in the amount of nitrite added during preparation, i.e. the ingoing amount of nitrite, may be different for the different NA and/or for the different test systems/meat products. Furthermore, the majority of the available publications only deal with the VNA, i.e. typically NDMA, *N*-nitrosodiethylamine (NDEA), NPYR and *N*-nitrosopiperidine (NPIP). Thus, data on the possible relationship between ingoing amount of nitrite and the extent of NA formation in a meat product for both VNA and NVNA are scarce or non-existing.

Besides the ingoing amount of nitrite a wide range of factors may potentially affect the formation of NA. These factors are related to meat quality, fat content, processing, maturation and handling at home. Factors related to processing include additives, heat applied during drying or smoking, precursors (added via wood smoke, spices or other ingredients), storage/maturation conditions and packaging. Processing factors can easily be controlled and their role in NA formation have been widely studied (Hill et al., 1988; Li, Wang, Xu, & Zhou, 2012; Li, Shao, Zhu, Zhou, & Xu, 2013; Sebranek & Fox, 1985). These studies only deal with the VNA (NDMA, NPYR and in a few cases NDEA), whereas studies including the NVNA are scarce (Janzowski, Eisenbrand, & Preussmann, 1978).

Antioxidants are widely used as additives in meat processing because they increase the storage stability. There is a large amount of literature on the effects of antioxidants on lipid oxidation processes, whereas literature on the effect on the NA formation in meat products is limited (Li et al., 2012; Li et al., 2013; Mottram, Patterson, Rhodes, & Gough, 1975; Rywotycki & Ryszard, 2002; Sen, Donaldson, Seaman, Iyengar, & Miles, 1976). These studies on the effect of adding antioxidants to meat also only deal with NDMA, NPYR and NDEA and to our knowledge only one study tests the effect of adding different levels of antioxidant (Mottram et al., 1975). Thus data on the effect of adding different levels of ascorbate/ascorbic acid/erythorbic acid (i.e. varies forms of vitamin C) on the NA formation is needed in order to provide advice on the levels to be added during production and preferably regarding both VNA and NVNA.

The different forms of vitamin C are polar antioxidants and because both oxygen and nitrogen oxide produced by reduction of nitrite are more soluble in lipid (Combet et al., 2007) it has been suggested that the levels of nitrosating species produced in the lipid phase can be higher than in the aqueous lean phase of the meat. Nitrosating species liberated from the lipid phase have been suggested as the reason for the increase in NPYR during frying of bacon (Sen et al., 1976). Thus a combination of a polar (e.g. erythorbic acid) and a non-polar antioxidant (e.g. ascorbyl palmitate) might inhibit the formation of NA more efficiently than one antioxidant. Polyphosphates are often used additives in meat processing because they increase the water holding capacity of the meat as well as stabilise the emulsion created in e.g. sausages (Bianchi, 1971). When the lipid and the lean phase are emulsified it might facilitate exchange of molecules between the two phases and thereby also exchange of nitrosating species and antioxidants.

Black pepper is used in the preparation of many types of sausages, including cooked sausages and salamis, and NPIP is often detected in these types of products (De Mey et al., 2014). Black pepper contains piperidine and *N*-nitrosopiperidine (Mey et al., 2014; Tricker, Pfundstein, Theobald, Preussmann, & Spiegelhalder, 1991); however, it has to our knowledge not been confirmed by controlled studies that the addition of black pepper to meat products results in occurrence of NPIP. NDMA, NPIP and NPYR may be produced when spices are mixed with nitrite, thus spices may be a source of NA precursors (Sen, Donaldson, Charbonneau, & Miles, 1974).

Haem has been suggested to play an essential role in the endogenous formation of NA following consumption of red and processed meat (Lunn et al., 2007; Pierre et al., 2013; Santarelli et al., 2010) and haem (Cross, Pollock, & Bingham, 2003) are associated with increased endogenous formation of nitroso compounds in both rodents and humans. Thus haem may play a role in the endogenous formation of nitroso compounds in general and therefore of NA as well. The haem iron may be released from myoglobin or haem as it passes through the digestive tract and it can therefore not be ruled out whether free iron plays a role in the nitroso compound formation. Free iron induces lipid oxidation processes in meat which is inhibited by the presence of antioxidants as is the NA formation, thus there may be some link between lipid oxidation processes and NA formation. The role of haem/myoglobin and free iron in NA formation in meat has to our knowledge not been studied.

To elucidate some of the areas mentioned above for which sufficient data is lacking we set up the following aims for the present study: (1) to examine the effect of the ingoing amount of nitrite on the amount of NA formed during the production of a cooked sausages; (2) to examine whether the formation of NA in the nitrite cured cooked sausages is affected by the presence of a water soluble (erythorbic acid) and a fat soluble antioxidant (ascorbyl palmitate), fat content, tripolyphosphate or black pepper; (3) to examine which concentrations of the two mentioned antioxidants that resulted in the most efficient inhibition of the formation of NA and if a combination of the two provides a higher degree of protection against NA formation than just one of them; (4) to examine whether haem or free iron play a role in the formation of NA in cooked sausages as has been suggested for the endogenous NA formation. In order to examine the effect of each individual factor, possible interactions between them and to work with at realistic number of experimental setups, factorial designs were employed.

2. Material and methods

2.1. Chemicals

Acetonitrile (extraction solvent) was of HPLC grade (Rathburn Chemicals Ltd., Walkerburn, Scotland). Formic acid (purity 98–100% for analysis), pipecolic acid (purity 98%), and the methanol for LC eluent (purity $\geqslant$ 99.9%, Fluka-Analytical) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Erythorbic acid, ascorbyl palmitate, tripolyphosphate, iron(III)sulphate hydrate and myoglobin from equine heart were also purchased from Sigma–Aldrich Co. The pure standards of *N*-nitrososarcosine