N-nitrosamines in processed meat products
– analysis, occurrence, formation, mitigation and exposure

Susan Strange Herrmann
PhD Thesis
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Preface and acknowledgements

This PhD thesis was conducted from June 2011 to June 2014 at the Technical University of Denmark, National Food Institute, Division of Food Chemistry.

The research performed in connection with the thesis has been described in four scientific papers of which two have been published, one is submitted and one is prepared as manuscript for submission (listed under "Publications" and included in full at the end of thesis). The first part of the thesis (Chapter 2) provide introduction into the motivation for the project and for studying *N*-nitrosamines in processed meat products and general relevant background information. The second part of the thesis (Chapter 3 to 9) describes the studies performed and the results obtained, which are described in detail in the three papers and the manuscript, as well as preliminary and supplementary studies not described in the papers.

Mørkhøj, June 2014

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Summary

N-nitrosamines (NA) occur in sodium nitrite (nitrite) preserved meat products as bacon, sausages, ham and several types of luncheon meats. Several of these NA are carcinogenic and high intake of processed meat products has been associated with increased risk of cancer and other adverse health effects in some epidemiologic studies. Exposure to NA via meat products may be the underlying reason for this association. The levels of NA in processed meat products ought therefore to be as low as possible. There is a large amount of literature on the occurrence, formation and mitigation of NA in meat products already available, though several areas especially regarding non-volatile NA (NVNA) are relatively unexplored. Studies performed in actual meat products are also scarce. The more that is understood about which factors affects the formation of both volatile NA (VNA) and NVNA the more likely is it to identify strategies for the prevention of NA formation in general and not only for a few NA.

The **aim** of the present thesis was therefore to study the role of ingoing amount of nitrite, factors relevant for industrial processing of meat, fat content and the effect of heat treatment on the formation of VNA and NVNA in meat. Secondly data on the occurrence of VNA and NVNA in processed meat products on the Danish market were to be generated and used for an evaluation of the exposure level resulting from consumption of processed meat products. A method allowing for the simultaneous determination of both VNA and NVNA has not been described in the literature. In order to meet the defined aims, a method based on acetonitrile extraction and liquid chromatography tandem mass spectrometry using both atmospheric pressure chemical ionisation and electrospray ionisation was developed and validated.

Data on the occurrence of NA in processed meat products was obtained by analysing products taken from the Danish market. The mean levels of the individual VNA were generally found to be low ($\leq 0.8 \mu g\ kg^{-1}$), whereas the mean levels of the individual NVNA were considerably higher ($\leq 118\ \mu g\ kg^{-1}$). The most frequently detected VNA were *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR) and the most frequently detected NVNA were *N*-nitrosothiazolidine-4-carboxylic acid (NTCA) and *N*-nitroso-2-methyl-thiazolidine-4-carboxylic acid (NMTCA). NTCA occurred at high levels, i.e. up to $2000\ \mu g\ kg^{-1}$. Higher mean levels of both the VNA ($\leq 1.5\ \mu g\ kg^{-1}$) and NVNA ($\leq 270\ \mu g\ kg^{-1}$) were found in samples taken from the Belgian market, though the difference was not significant. Thus in spite of the National Provision that Denmark obtain allowing an ingoing amount of sodium nitrite of $60\ mg\ kg^{-1}$ instead of $150\ mg\ kg^{-1}$ according to EU regulation, no significant differences between the mean levels of NA in the Danish samples and the Belgian samples could be demonstrated.

The relationship between the ingoing amount of nitrite and the levels of VNA and NVNA was studied in both minced meat and cooked pork sausages. The levels of *N*-nitrosohydroxyproline (NHPRO), *N*-nitrosoproline (NPRO), NTCA, NMTCA, *N*-nitrososarcosine (NSAR), and *N*-nitrosopiperidine (NPIP) were found to be positively related to the ingoing amount of nitrite. The same could not be demonstrated for the commonly assayed NDMA and NPYR of which the levels remained low even when $350\ mg\ kg^{-1}$ nitrite was added. This may indicate that the relevant precursors are not present. Studies by others have indicated especially the formation of NDMA to depend more on factors as meat quality including feeding and/or breeding conditions and processing factors as temperatures and duration of drying and storage than on the ingoing amount of nitrite.

A range of studies were performed using both minced pork meat and cooked pork sausages in order to evaluate the effects of sodium chloride, antioxidants (erythorbic acid and ascorbyl palmitate), sodium tripolyphosphate, dextrose, fat content, black pepper and time on the NA formation and their interactions with nitrite and each other. Factorial experiments were employed in order to gain as much information with a reasonable number of samples. The ingoing amount of nitrite and the presence of erythorbic acid affected the levels of NA most. The levels of NHPRO, NPRO, NPIP, NTCA and NMTCA were inversely related to the amount of erythorbic acid (396-1104 mg kg^{-1}). The levels of the individual NA were reduced with up to 20 to 75%. No additional protection against NA formation was obtained by also adding ascorbyl palmitate, a fat soluble antioxidant. Sodium chloride was found to have minor effects on the NA levels compared to nitrite and erythorbic acid. The NA formation happened rapidly and was relatively unaffected by storage for up to 13 days. Black pepper significantly increased the levels of NPIP. Fe(III) increased the levels of NHPRO, NMTCA and NTCA, whereas haem had no effect on the NA levels.

A clear positive effect of heat treatment on the levels of NPIP was demonstrated in all the heat treatment experiments performed. Depending on the temperature obtained in the meat different effects were found for the other NA. If the sausages produced with different levels of nitrite were fried until a centre temperature of 100°C also the levels of NSAR, NTCA and NMTCA increased. Though when products purchased at the local supermarkets and butcher stores were heated to a higher temperature (~250°C), the levels of NTCA and NMTCA decreased. Depending on the product and heat treatment the levels of NPRO, NPYR, *N*-nitrosodiethylamine (NDEA) and *N*-nitrosomethylaniline (NMA) either increased or decreased.

From the data acquired on the occurrence of NA in meat products on the Danish market it was estimated that consumption at the 95th percentile of these products resulted in an exposure to VNA of 0.34 ng kg bw^{-1} day^{-1} and 1.1 ng kg bw^{-1} day^{-1} for Danish adults and children, respectively. The calculated Margin Of Exposure (MOE) was well above 10,000 indicating that the exposure is of low concern. Though, it cannot be ruled out that the exposure to these VNA is accountable for the stronger association between adverse health effects and consumption of processed meat than for consumption of red meat. The 95th percentile exposure to the NVNA was estimated to be considerably higher (33-90 ng kg bw^{-1} day^{-1}); though this exposure level is not possible to risk assess because data concerning the toxicological relevance of these compounds are lacking.

Overall the present thesis show that if nitrite is used for meat preservation and/or colouration the levels of NA generally increase. Because of the possible adverse health effects of NA the exposure level ought to be kept at a minimum. Based on the present knowledge it is evaluated that low levels of NA in processed meat products are best achieved by using as little nitrite as possible and use it in combination with erythorbic acid (~1000 mg kg^{-1}), ascorbic acid or ascorbate. Furthermore by storing the processed meat products protected from oxygen, depletion of the erythorbic acid is prevented. The European Food Safety Authority has concluded that microbiological safe meat products generally may be produced by the addition of 50 mg kg^{-1} of nitrite. Other means besides nitrite addition can insure the microbiological safety. However, the occurrence of the carcinogenic NDMA and perhaps NPYR seems neither to be related to the levels of nitrite or to the levels of erythorbic acid.

Resumé (in Danish)

N-nitrosaminer (NA) forekommer i kødprodukter som bacon, pølser, skinke og andre typer af kødpålæg konserveret med natrium nitrit (nitrit). Flere af disse NA er kræftfremkaldende og i nogle epidemiologiske undersøgelser er der endvidere fundet sammenhæng mellem højt indtag af forarbejdet kød og øget risiko for kræft og andre negative effekter på helbredet. Eksponering for NA via kødprodukter kan være årsag til den sammenhæng. Indholdene af NA i forarbejdet kød bør derfor være så lave som muligt. Et stort antal publikationer vedrørende forekomsten, dannelsen samt afværgelse af dannelsen af NA er allerede tilgængelige, men der er stadig flere områder, særligt vedrørende de ikke flygtige NA (NVNA), som ikke er afdækket. Antallet af undersøgelser udført på egentlige kød produkter er ligeledes begrænset. Jo mere der er kendt omkring hvilke faktorer der påvirker dannelsen af både de flygtige NA (VNA) og NVNA, jo større sandsynlighed er der for, at man kan identificere strategier, hvorved dannelsen af NA generelt kan forhindres og ikke kun for nogle få NA.

Formålet med denne afhandling var derfor, at undersøge betydningen af den tilsatte mængde af nitrit samt betydningen af faktorer af relevans for industriel forarbejdning af kød, fedt indhold og varmebehandling på dannelsen af VNA og NVNA. Ydermere skulle der genereres data vedrørende forekomsten af VNA og NVNA i forarbejdede kødprodukter på det danske marked til brug for en evaluering af eksponeringsniveauet ved konsum af forarbejdet kød. Der er ikke tidligere i litteraturen beskrevet nogen analytisk metode til samtidig bestemmelse af VNA og NVNA. For at opfylde det beskrevne formål blev der, som en del af projektet udviklet og valideret en metode baseret på acetonitril ekstraktion samt væskekromatografi tandem-masse-spektrometri med både atmosfærisk tryk kemisk ionisering og elektrospray ionisering.

Data vedrørende forekomsten af NA i forarbejdede kødprodukter blev opnået ved, at analysere produkter udtaget fra det danske marked. De gennemsnitlige indhold af de enkelte VNA var generelt lave ($\leq$0.8µg kg^{-1}), hvorimod de gennemsnitlige indhold af de enkelte NVNA var markant højere ($\leq$118 µg kg^{-1}). *N*-nitrosodimethylamin (NDMA) and *N*-nitrosopyrrolidin (NPYR) var de hyppigst detekterede VNA og de hyppigst detekterede NVNA var *N*-nitrosothiazolidine-4-carboxylsyre (NTCA) and *N*-nitroso-2-methyl-thiazolidine-4-carboxylsyre (NMTCA). Indholdene af NTCA var høje, dvs. op til 2000 µg kg^{-1}. Højere gennemsnitlige indhold af både de VNA ($\leq$1.5 µg kg^{-1}) og NVNA ($\leq$270 µg kg^{-1}) blev fundet for prøver udtaget i Belgien, denne forskel var dog ikke signifikant. Dvs. til trods for at Danmark opretholder nationale regler der tillader tilsætning af højest 60 mg kg^{-1} nitrit, hvorimod de fælles EU regler tillader 150 mg kg^{-1}, kunne der ikke demonstreres nogen klar forskel mellem de gennemsnitlige indhold i de danske og belgiske prøver.

Sammenhængen mellem den tilsatte mængde af nitrit og indholdene af VNA og NVNA blev undersøgt både i hakket kød og pølser. Der blev påvist positiv sammenhæng mellem stigende indhold af *N*-nitrosohydroxyprolin (NHPRO), *N*-nitrosoprolin (NPRO), NTCA, NMTCA, *N*-nitrososarcosin (NSAR) og *N*-nitrosopiperidin (NPIP) og stigende mængde af tilsat nitrit. Denne relation blev ikke fundet for de hyppigst undersøgte VNA, NDMA og NPYR. Indholdene af disse forblev lave selv ved tilsætning af 350 mg kg^{-1} nitrit. Studier foretaget af andre har indikeret, at særligt dannelsen af NDMA er mere relateret til faktorer som kødkvalitet herunder også fodersammensætning og/eller avlsforhold, forarbejdningsfaktorer som valg af temperatur og længde af tørring og opbevaring end til mængden af tilsat nitrit.

En række undersøgelser blev udført, for at vurdere betydningen af natriumklorid, antioxidanter (erythorbinsyre og ascorbyl palmitat), natriumtripolyfosfat, dextrose, fedtindhold, sort peber og tid for dannelsen af NA. Faktorforsøg blev anvendt for, at opnå så mange informationer som muligt med et håndterbart antal prøver. Den tilsatte mængde af nitrit samt tilsætning af erythorbinsyre viste sig, at have størst betydning for hvor indholdene af NA. Indholdene af NHPRO, NPRO, NPIP, NTCA og NMTCA var omvendt relateret til mængden af erythorbinsyre (396-1104 mg kg^{-1}) og indholdene for de enkelte NA blev reduceret med op til 20 til 75%. At også tilsætte ascorbylpalmitat, en fedtopløselig antioxidant, forhindrede ikke NA-dannelsen yderligere. Natriumklorid havde begrænset effekt på NA-indholdene i forhold til nitrit og erythorbinsyre. NA blev dannet hurtigt og indholdene forblev relativt stabile selv efter 13 dages opbevaring. Sort peber øgede indholdene af NPIP signifikant. Tilsætning af Fe(III) øgede indholdene af NHPRO, NMTCA and NTCA, hvorimod hæm ikke havde nogen effekt.

En klar positiv sammenhæng blev påvist mellem varmebehandling og stigning i indholdene af NPIP i alle de udførte varmebehandlingseksperimenter. Afhængig af hvilken temperatur der blev opnået i kødet varierede effekten på de øvrige NA. Ved stegning af pølserne fremstillet med forskellige tilsætninger af nitrit til en centrum temperatur på 100°c steg indholdene af NSAR, NTCA og NMTCA. Ved varmebehandling af produkter købt i de lokale supermarkeder og slagtere til højere temperaturer (~250°C) faldt indholdene af NTCA og NMTCA. Både stigning og fald i indholdene af of NPRO, NPYR, *N*-nitrosodiethylamin (NDEA) og *N*-nitrosomethylanilin (NMA) blev observeret afhængig af produkt og varmebehandling.

Udfra de data der blev generet vedrørende forekomsten af NA i forarbejdede kødprodukter på det danske marked, blev eksponeringen for VNA (estimeret som 95 percentilen) via konsum af disse produkter estimeret til at være 0.34 ng kg legemsvægt^{-1} dag^{-1} and 1.1 ng kg legemsvægt^{-1} dag^{-1} for hhv. voksne og børn. "Margins of Exposure" blev ud fra dette eksponeringsniveau beregnet til at være over 10.000 og giver derfor ikke anledning til bekymring. Det kan imidlertid ikke udelukkes at eksponeringen for VNA fra forarbejdede kødprodukter ligger til grund for den stærkere sammenhæng mellem øget risiko for kræft of andre negative effekter på helbredet og indtag af forarbejdet kød end med indtaget af rødt kød. Eksponering for NVNA blev estimeret til at være markant højere (33-90 ng kg legemsvægt^{-1} dag^{-1}). Denne eksponering kan dog ikke risiko vurderes pga. manglende data vedrørende den toksikologiske relevans af disse stoffer.

Samlet set har denne afhandling vist, at hvis den tilsatte mængde af nitrit anvendt ved kødkonservering og/eller farvning øges, øges også indholdet af NA generelt. Eksponering for NA bør pga. de mulige negative helbredseffekter være så lav som muligt. Ud fra den nuværende viden vurderes det at lave indhold af NA i forarbejde kødprodukter bedst opretholdes ved, at så lidt nitrit som muligt tilsættes og ved at nitrit anvendes i kombination med erythorbinsyre (~1000 mg kg^{-1}), ascorbinsyre eller ascorbat. Det forarbejdede kød bør opbevares beskyttet mod ilt for at forhindre at erythorbinsyre bliver opbrugt. Den Europæiske Fødevaresikkerhedsautoritet har konkluderet, at den mikrobielle sikkerhed af kød generelt kan opretholdes ved at tilsætte 50 mg kg^{-1} nitrit. Dette kan skyldes at de relevante precursore ikke er tilstede. Andre midler kan også tages i brug udover tilsætning af nitrit for at sikre den mikrobielle sikkerhed. Forekomsten af de kræftfremkaldende NDMA og NPYR tyder dog ikke på at være relateret til mængden af nitrit eller erythorbinsyre.

Publications

This PhD thesis is based on the following papers and manuscripts:

Paper I:
> **Herrmann, S. S**., Duedahl-Olesen, L., & Granby, K. (2014). Simultaneous determination of volatile and non-volatile nitrosamines in processed meat products by liquid chromatography tandem mass spectrometry using atmospheric pressure chemical ionisation and electrospray ionisation. *Journal of Chromatography A*, 1330(0), 20-29.

Paper II:
> **Herrmann, S. S**., Duedahl-Olesen, L., & Granby, K. (2014). Occurrence of volatile and non-volatile *N*-nitrosamines in processed meat products and the role of heat treatment. *Food Control*, 9 June 2014.
> Available at http://www.sciencedirect.com/science/article/pii/S0956713514002850

Paper III:
> **Herrmann, S. S**., Granby, K. and Duedahl-Olesen, L. (20XX). Formation and mitigation of *N*-nitrosamines in nitrite preserved sausages. Accepted for publication with minor revision in "*Food Chemistry*" Sept. 2014.

Manuscript I:
> **Herrmann, S. S**., Duedahl-Olesen, L., Christensen T., Olesen P.T. & Granby, K. (20XX). Dietary exposure to volatile and non-volatile *N*-nitrosamines from processed meat products in Denmark. Submitted to *"Food and Chemical Toxicology"* in November 2014.

Posters
> **Herrmann, S.S**., Duedahl-Olesen, L. and Granby, K.
> Determination of volatile and non-volatile nitrosamines in nitrite cured meat products
> Presented at the VII International Conference on Chemical Reactions in Food in Prague November 2012.

> **Herrmann, S.S**., Duedahl-Olesen, L. and Granby, K.
> Occurrence of volatile and non-volatile *N*-nitrosamines in processed meat products – Dietary intake
> Presented at the 6th International Symposium on Recent Advances in Food Analysis in Prague November 2013.

Abbreviation

APCI	Atmospheric pressure chemical ionization
Bw	Body weight
EFSA	European Food Safety Authority
Eq	Equation
ESI	Electrospray ionization
LC	Liquid chromatography
LOD	Limit of determination
LOQ	Limit of quantification
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NA	*N*-nitrosamine(s)
NDBA	*N*-nitrosodibutylamine
NDBzA	*N*-nitrosodibenzylamine
NDEA	*N*-nitrosodiethylamine
NDMA	*N*-nitrosodimethylamine
NDMA-d6	*N*-nitrosodimethylamine-*d*6
NDPA	*N*-nitrosodi-n-propylamine
NDPhA	*N*-nitroso-di-phenylamine
NHMTCA	*N*-nitroso-hydroxy-2-methyl-thiazolidine-4-carboxylic acid
NHPRO	*N*-nitrosohydroxyproline
NMA	*N*-nitrosomethylaniline
NMEA	*N*-nitrosomethylethylamine
NMOR	*N*-nitrosomorpholine
NMTCA	*N*-nitroso-2-methyl-thiazolidine-4-carboxylic acid
NPIC	*N*-nitrosopipecolic acid
NPIP	*N*-nitrosopiperidine
NPRO	*N*-nitrosoproline
NPYR	*N*-nitrosopyrrolidine
NPYR-d8	*N*-nitrosopyrrolidine-*d*8
NSAR	*N*-nitrososarcosine
NTCA	*N*-nitrosothiazolidine-4-carboxylic acid
NTHZ	*N*-nitrosothiazolidine
NVNA	Non-volatile *N*-nitrosamines
PIC	Pipecolic acid
RSD_r	Repeatability standard deviation
SIM	Single ion monitoring mode
SPE	Solid phase extraction
TBARS	Thiobarbituric acid reactive substances
VNA	Volatile *N*-nitrosamines

Content

1. Introduction

Nitrite (E 249 – E 250) has been used for preservation of meat products for decades and is still widely used for preservation of products as e.g. bacon, sausages and luncheon meats. Nitrite curing provides an efficient inhibition of the growth of *Clostridium botulinum* (Gibson *et al.*, 1984), and therefore it lowers the risk of toxin formation and botulism. Nowadays nitrite is primarily used because it provides the cured meat with unique colour, flavours and aromas known from products as e.g. bacon (Honikel, 2008). The use of nitrite is however also associated with some risks. The dietary intake of nitrite for Danish children has caused concern because it has been evaluated to be close to the acceptable daily intake (Leth *et al.*, 2008). But this is not the only health concern in relation to preservation of meat with nitrite. Nitrite may react with secondary amines present in the meat to form *N*-nitrosamines (NA), many of which are carcinogenic (IARC, 1998). The possible formation of NA was not considered in the last EU evaluation of the use of nitrite for meat preservation. The health concern is further supported by several epidemiologic studies indicating an association between consumption of processed meat and increased risk of colorectal cancer (Santarelli *et al.*, 2008), stomach cancer (Larsson *et al.*, 2006), pancreatic cancer (Larsson and Wolk, 2012) but also with increased risk of cardiovascular diseases and total causes of death (Rohrmann *et al.*, 2013). The same association has been indicated for red meat though not as strong as for processed meat.

Because of these concerns Denmark has since 1995 obtained National Provision on the use of nitrite for preservation of meat products. The provisions are extended until 2015 (European Commission, 2014). The underlying reason for obtaining the National Provisions is partly the assumption that a positive relationship between the amount of nitrite added to the meat products (ingoing amount) and the levels of NA formed occur. If this assumption is true the levels of NA in processed meat product on the Danish market would be lower than in processed meat products on the market in other European Member States as a result of the National Provisions. The levels of NA in processed meat products on the Danish market have though not been assessed nor has the level of exposure of the Danish population to NA via these products. Thus the National Provisions have been obtained on a principle of caution.

Since the 1970s the occurrence and mechanisms involved in the formation of NA have been studied extensively though in spite of numerous publications there are still areas which are not covered. First of all the number of studies performed on meat or meat products are limited and results obtained for various models may not represent what happens in meat. Furthermore the majority of the available studies deal with only two to five of the so called volatile NA (VNA). This group of NA has received most attention because of their documented carcinogenic effects and because they were amenable with the analytical techniques available in the 1970s and 1980s. Generally the VNA occur at low levels, i.e. <5 µg kg^{-1} to 20 µg kg^{-1} (Hill *et al.*, 1988;Massey *et al.*, 1991). The amount of literature available dealing with the group of non-volatile NA (NVNA) is much more limited. The NVNA include the *N*-nitrosamino acids and occur at significantly higher levels than the VNA, i.e. up to several thousand µg kg^{-1} (Tricker and Kubacki, 1992;Massey *et al.*, 1991). Generally the *N*-nitrosamino acids are considered non-carcinogenic (Habermeyer and Eisenbrand, 2008). Though according to the IARC monographs from 1998 insufficient data occurs in order to perform an evaluation of the carcinogenicity of commonly found NVNA. Thus data on the occurrence of VNA of recent date and the occurrence of NVNA in general are scarce. Also studies on the relationship between the ingoing amount of

nitrite and the levels of NA formed are needed, because the available studies are generally performed with too few levels of nitrite in order to elucidate any possible relationship and/or they only study the relationship for one or a few NA. From going through the literature it has become apparent that the formation of NA is affected by several other factors besides nitrite, which potentially may increase or reduce the formation of NA. These factors include e.g. some commonly used additives and ingredients, heat treatment and fat content. The available studies on the role of various factors on the formation and mitigation of NA have primarily been performed using model systems simpler than meat and/or only for a few VNA.

The methods employed in the majority of the earlier as well as many recent studies on the VNA employ methods based on gas chromatography with detection by thermal energy analyser (Crews, 2010;Rath and Felix Guillermo, 2008;Drabik-Markiewicz *et al.*, 2011;Dunn and Stich, 1984). The TEA detector has limited versatility and is of relatively high cost and this detector is therefore not available in most laboratories. There are also published methods for the determination of NVNA and these are generally based on LC-TEA and extraction procedures consuming large amounts of organic solvent (Massey *et al.*, 1985;Sen and Kubacki, 1987;Tricker *et al.*, 1985). More recently publications of methods for determination of *N*-nitrosamino acids using LC-MS/MS are available, but in matrices as tobacco (Wu *et al.*, 2012) and smokeless tobacco products (Essen *et al.*, 2011). An analytical method allowing for the determination of relevant NA from both the group of VNA and the group of NVNA in the same analytical run would greatly facilitate the work. However such method has to our knowledge not been published. Based on the already described methods it may be possible to include the group of both VNA and NVNA in a LC-MS/MS based method.

Based on the underlying assumption of the National Provisions on the use of nitrite for meat preservation and on the above described deficiencies in the available literature the **aim** of the present thesis was to:

1) Develop an analytical method for the simultaneous determination of VNA and NVNA in meat products (Paper I) which would aid the fulfilment of the remaining aims.

2) Perform a survey on the occurrence of VNA and NVNA in processed meat products on the Danish market (Paper II).

3) Study how the levels of NA are affected by heat treatment relevant for processing performed at home or in the fast food industry (Paper II and Paper III).

4) Study the relationship between ingoing amount of nitrite and the levels of NA formed

5) Study the role of selected processing factors including nitrite on the formation and mitigation of NA in minced meat and cooked pork sausages as representative meat models (Paper III). Because studies have shown that haem and/or iron are involved in the in vivo formation of NA we also included a study on the role of haem and free iron in the NA formation (Paper III).

6) Estimate the exposure to NA from consumption of processed meat products for the Danish population. The estimation will be based on the results of the data on the occurrence of NA and data on consumption of processed meat products by the Danish population. Finally to risk characterise the exposure by estimating the Margins Of Exposure (MOE) to NA, based on the ratio between a bench mark dose level ($BMDL_{10}$) and the estimated dietary exposures (Manuscript I).

2. Background information

2.1 Consumption of processed meats – what is the issue

The variation in processed meat products on the market is huge. In general the term processed meat or cured meat refers to nitrite preserved, salted and in many cases also smoked meat. Common processed meat products includes bacon, ham, sausages (cooked sausages), salamis (raw sausages, fermented and dried). The majority of the processed meat products available and consumed in Denmark are produced from red meat. Red meat is in most cases and in the present work defined as meat of four legged animals. Meat from these animals has a higher content of myoglobin and therefore also appears redder than e.g. poultry and turkey meat which is referred to as white meat.

Curing is basically the addition of sodium chloride and nitrite or nitrate to meat. The amount of nitrite and nitrate allowed to be added to meat products according the common EU legislation and the Danish National Provisions are presented in general terms in Table 2.1-1. As a general rule the EU legislation allows the addition of 150 mg nitrite per kg meat whereas the Danish National Provisions allows the addition of 60 mg nitrite per kg meat. However both in the EU and the Danish legislation there are many exceptions for specialty products and furthermore specific products may be categorised differently in the two legislations. For all details we therefore need to refer the reader to a database available for the EU legislation and to the Appendix 3 of Regulation 542 of 27/05/2013 for the Danish provisions. Links for both of these are presented in Table 2.1-1. Other additives can be added together with nitrite to enhance flavour, storage stability, water holding capacity etc. Curing can be performed by four different methods; dry curing, tank curing, needle stitch curing and emulsion curing. In dry curing the cure mix is smeared over the surface of the meat which is then stored under controlled humidity and temperature during which the curing agents will diffuse into the meat. The curing proceeded faster during tank curing by which pieces of meat is emerged in a brine, i.e. aqueous solution of the curing constituents. The diffusion of the curing agents is not limited by reduced water activity as with the dry curing. The most commonly used curing technique in large scale production for curing of whole pieces of meat is by needle injection. When the brine is injected into the meat with a large number of needles the brine is quickly distributed in the meat. E.g. bacon cured by this technique can be smoked and vacuum packed immediately after preparation and the curing solution will distribute throughout the meat while stored (Santarelli *et al.*, 2008;Fellows, 2009). The process of curing is easier to control by this method than e.g. immersion curing both in regard to sodium chloride content obtained in the meat as well as a good hygiene. Emulsion curing is the technique used for the preparation of sausages in which the curing agents are mixed with minced meat.

Table 2.1-1: Overview of the amounts of nitrite and nitrate allowed to be added to processed meat products according to the common EU legislation and the Danish National Provisions.

	Legal limits. Maximum amount that may be added during manufacturing (mg kg^{-1}). Expressed as sodium nitrite for all			
	Sodium nitrite		Sodium nitrate	
	EU[1]	DK National Provisions[2]	EU[1]	DK National Provisions[2]
Heat treated meat products in general	150	60[3]		
Non-heat treated meat products in general	150	60	150	150

1: The list of authorised food additives and their conditions of use approved for use in food can be consulted via the food additives database available at https://webgate.ec.europa.eu/sanco_foods/main/?event=display

2: Danish National Provisions are laid down in Regulation 542 of 27/05/2013 appendix 3 available at https://www.retsinformation.dk/Forms/R0710.aspx?id=151827.

3: Nitrites may not be added to Danish meat balls and traditional Danish liver pate.

2.1.1 Health concerns

Several epidemiologic studies indicate associations between consumption of red and processed meat and increased risk of e.g. colorectal cancer (Santarelli *et al.*, 2008), stomach cancer (Larsson *et al.*, 2006), pancreatic cancer (Larsson and Wolk, 2012) but also with increased risk of cardiovascular diseases and other causes of death (Rohrmann *et al.*, 2013). The association was stronger for high consumption of processed than for high consumption of red meat in several of these studies. In 2007 the scientifically based evidence leads the World Cancer Research Fund to recommend that consumption of processed meat should be avoided whereas the Danish food authorities recommend that the consumption of processed meat should be limited (www.fvst.dk). Rohrmann *et al.* (2013) estimated that consumption of more than 20 g of processed meat per day increased the mortality rate (Rohrmann *et al.*, 2013). There is no epidemiologic evidence for an association between high intake of chicken and adverse health effects (Norat *et al.*, 2005).

Thus both red and processed meat seems to influence similar processes in humans though the processed meat seems to contain one or more additional factors which add to the observed adverse health effects of red meat. At present there are no clearly demonstrated biologic mechanisms that may explain the risk difference between processed and unprocessed meat. Several factors commonly associated with high intake of red and processed meat has been suggested to contribute to the indicated effects i.e. (1) high fat intake which may promote carcinogenesis via insulin resistance or faecal bile acids; (2) high intake of carcinogenic heterocyclic amines and polycyclic aromatic hydrocarbons formed by cooking meat at a high temperatures; (3) high intake of carcinogenic NA which is formed in the meat and endogenously; (4) intake of nitrosylhaem which may be more toxic than haem (Santarelli *et al.*, 2008). Red meat or haem has in rodents and/or humans been linked to: An increased lipoperoxidation activity; Increased cell proliferation in the large intestine; Increased production

of apparent total *N*-nitroso compounds (ATNC) in both ileal output (Kuhnle *et al.*, 2007) and large intestine (Bingham *et al.*, 2002;Santarelli *et al.*, 2010;Kuhnle *et al.*, 2007) and increased nitrite level in the large intestine (Bingham *et al.*, 2002). The term "ATNC" cover a complex mixture of nitrite-derived products including e.g. NA, S-nitrosothiols, nitrosamides and nitrosoguanidines and iron-nitrosyl species (Hogg, 2007;Bingham *et al.*, 2002). Increased levels of lipoperoxidation and ATNC in the large intestine of humans induced by intake of cured red meat were counter acted by calcium, whereas α-tocopherol only normalised the lipoperoxidation level (Pierre *et al.*, 2013). Allam *et al.* (2011) found that calcium also suppressed the haem toxicity markers induced by haem, haemin and meat (Allam *et al.*, 2011). The hypothesis is, that calcium bind to the haem and results in its precipitation, and thereby inactivation. If there is a link between the presence of nitrosated haem and formation of endogenous and perhaps also exogenous NA, the consumption of calcium with a processed meat meal or addition during processing may prevent the effects. Though according to Allam *et al.* (2011) calcium carbonate and not calcium phosphate should be chosen, because calcium phosphate itself was found to increase the prenoplastic regions in the colon of the rats. Both calcium phosphate and calcium carbonate are used for human dietary supplements.

Thus the adverse health effects are observed both by high consumption of red meat and processed meat and there is some scientific evidence for a role of haem in the biological effects observed. When meat is preserved with nitrite nitrosylhaem and NA are formed. Many NA have been characterised as carcinogenic and nitrosylhaem may have higher catalytic activity and/or stability than haem. A more stable haem complex in the form of e.g. nitrosylhaem could result in a higher fraction of the consumed haem to reach the colon. Nitrosylhaem and/or NA may therefore be the reason for the observed stronger association between adverse health effects and consumption of processed meat than for red meat. The fate of nitrite and the reactions involved in the formation of NA and nitrosylhaem are therefore described in Chapter 2.3.

2.2 Why are NA carcinogenic

The NA are pro-carcinogens, i.e. they need to be metabolically activated before they become carcinogenic. It is enzymes from the family of cytochrome P450 enzymes that catalyse this metabolic activation. It is believed that a key pathway is the hydroxylation of the carbon in the α position to the nitroso group. When for example *N*-nitrosopiperidine (NPIP) is α-hydroxylated an unstable specie is produced which will decompose into a diazohydroxide, a diazonium ion and an oxonium ion. These electrophilic intermediates may react with e.g. DNA and form DNA adducts (Wong *et al.*, 2005)(Figure 2.2-1).
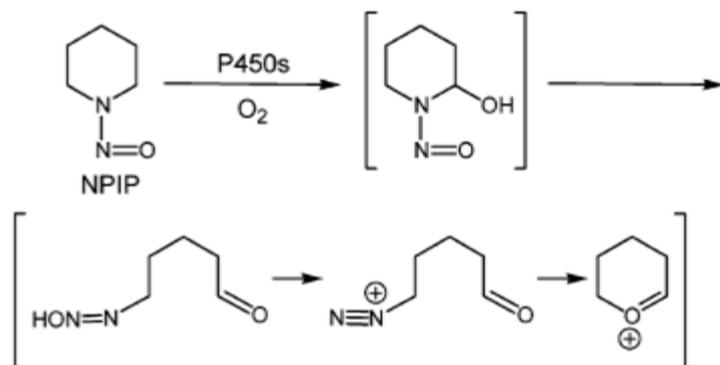
Figure 2.2-1: Metabolic activation of NPIP by α-hydroxylation (modified from (Wong *et al.*, 2005))

The carcinogenic process or development of cancer consists of at least three stages; initiation, promotion, and progression (Figure 2.2-2). In the first stage a genotoxic event needs to occur. This could e.g. be binding of one of the electrophilic intermediates, produced from hydroxylation of NA, to the DNA strand. If the DNA strand is then transcribed or repaired wrongly an error is introduced into the DNA and the cell is initiated. This initiated cell can then be further stimulated to proliferate uncontrollably either by promotion of proliferation or by inhibition of apoptosis. During the last stage, progression, the preneoplastic cells develop into the neoplastic stage and a cancer is produced.

The different cytochrome P450 enzymes, which are also called the phase I enzymes of the detoxification system, have different affinity for different substrates and their expression also vary between tissues and also between individuals. This may explain why the different NA seems to have different target tissues/organs. Enzymes of the cytochrome P450 2A subfamily are important catalyst in the activation of several NA including NPIP and *N*-nitrosopyrrolidine (NPYR). Humans have a high P450 2A activity in the liver (Wong *et al.*, 2005). Reactive agents produced in the liver can either 1) react with e.g. protein or DNA in the liver or 2) they can be excreted directly via urine, if they are polar enough or via the bile into the intestine or 3) they can be further metabolised under catalysis of enzymes belonging to the group of phase II enzymes, which conjugate the reactive agents and thereby deactivate them and generally make them more polar. The longer the reactive agents are allowed to exist before deactivated, by e.g. conjugation, the higher is the risk of these agents reacting with DNA. Thus some balance between the activities of the phase I and the phase II enzymes are needed to ensure that the reactive agents produced by the phase I enzymes are rapidly deactivated. Because the activity of both the phase I enzymes and phase II enzymes vary depending on heritage and environment the susceptibility to NA exposure may vary considerably between individuals.
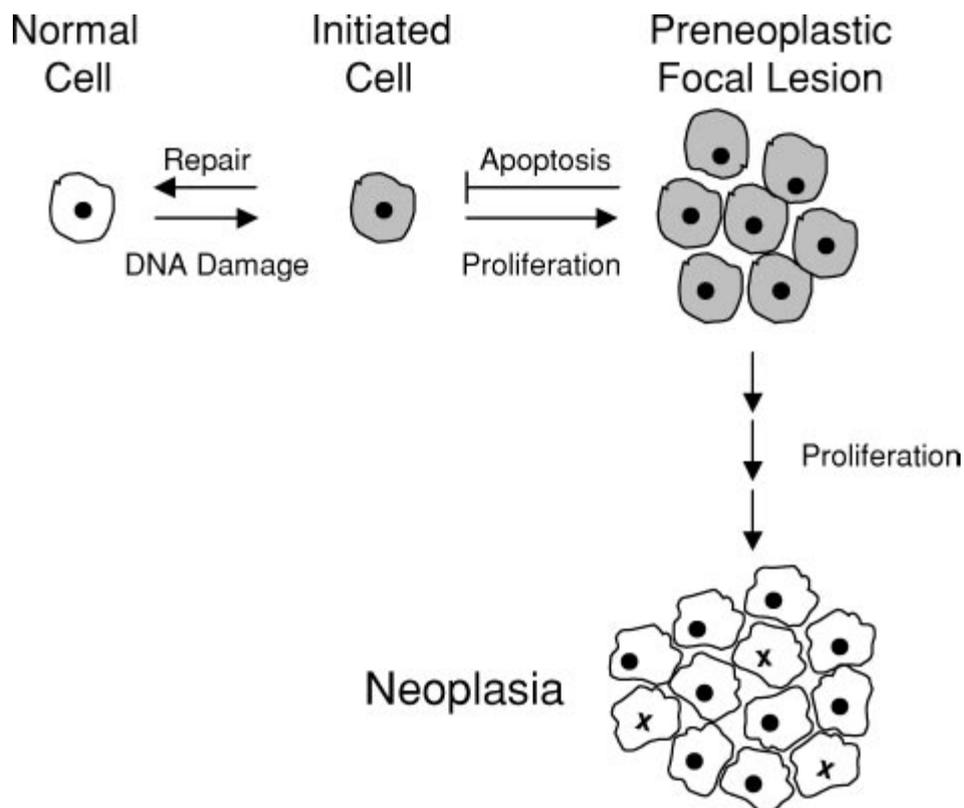
Figure 2.2-2: The stages of carcinogenesis involving a non-lethal mutation in the DNA which is permanent after one round of DNA synthesis (initiation step). For this initiated cell to become cancerous it needs to be stimulated to proliferate and/or the apoptosis of the cell needs to be inhibited. This requires promoting stimuli. The final step is the progression or development of the malignant cell into a tumour (Klaunig and Kamendulis, 2004).

The NA are among the structurally simplest of all groups of carcinogens. Despite their simplicity they are potent carcinogens in many species and organs. The carcinogenicity of the NA vary greatly and blocking of the α-carbon and/or fit with the active site of the oxidative enzyme are suggested to be crucial for the potency of the different NA (Lijinsky, 1987). The IARC has evaluated that e.g. *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) belong to the group of "probably carcinogenic to humans", and *N*-nitrosodibutylamine (NDBA), NPIP and NPYR belong to the group of "possibly carcinogenic to humans" (Yurchenko and Mölder, 2006). It is assumed that the more polar the NA are the more likely is it that they are rapidly excreted unchanged and thereby also less likely to be activated. The relatively high polarity of the hydroxylated and carboxylated NA is assumed to be part of the reason why these NA are less potent carcinogens than their un-substituted NA or non-carcinogenic (Lijinsky, 1987). *N*-nitrososarcosine (NSAR), the carboxylated form of NDMA, is a carcinogen in rat and mice (IARC, 1998), though less potent than NDMA and NDEA. However the very potent NDMA is of comparable polarity with NPRO and more polar than *N*-nitroso-2-methyl-thiazolidine-4-carboxylic acid (NMTCA) and NTCA, which is indicated by the latter two to elute later from a reverse phase LC column than the former. So it is not just a matter of polarity. Studies have shown that the cleavage of the α-carbon-hydrogen bond is a rate-limiting step in carcinogenesis (Lijinsky, 1987). Various substitutions may therefore decrease the carcinogenicity of the NA by strengthening the bond.

2.3 Nitrite induced reactions in processed meats
2.3.1 Nitrite as preservative/colorant

Meat is a very complex matrix and processed meat even more so. Nitrite or nitrate is used for preservation as well as for development of colour and taste. If nitrate is added it can act as a continuous source of low amounts of nitrite. Microbial enzymes can catalyse the reduction of nitrate to nitrite (Tricker and Kubacki, 1992;Honikel, 2008).

The complexity of nitrogen reactivity and therefore also nitrite can be attributed to the fact that nitrogen can change its state of oxidation widely. The nitrogen compounds which have been suggested to be the most important (Honikel, 2008) are shown in Fig. 2.3-1 in their different oxidation states. Especially the nitrogen/oxygen compounds are of interest for the curing process (Honikel, 2008).
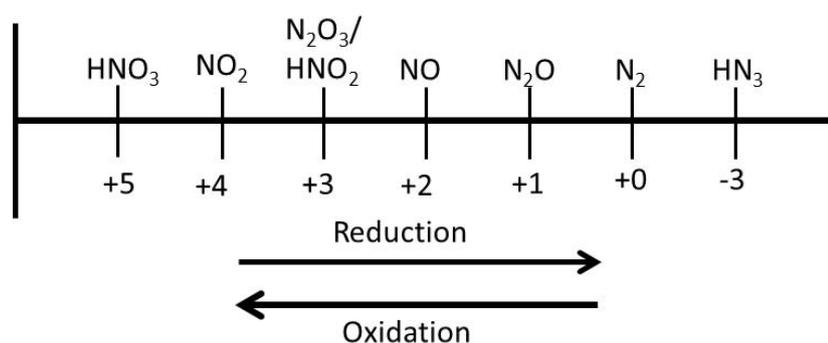
Fig. 2.3-1: Oxidation states of nitrogen compounds (modified from (Honikel, 2008)).

The bactericidal effects of nitrite are due to its derivatives such as nitric oxide which are toxic to *Clostridium* but also to *Listeria* species. The bacteria have numerous possible targets for inhibition including respiratory chains, iron-sulfur proteins and other metalloproteins, membranes and gene expression (Cammack *et al.*, 1999). According to a European Food Safety Authority (EFSA) opinion 50-100 mg kg^{-1} of nitrite is adequate to protect most cooked cured meats against growth and toxin formation by *Clostridium botulinum*. It is further evaluated that a wide range of cured meats that are not cooked, produced under a wide range of circumstances and with varying levels of hygiene may require addition of 150 mg kg^{-1} of nitrite in order to prevent the growth of *Clostridium botulinum* (EFSA, European Food Safety Authority, 2003). Besides nitrite and good hygiene other strategies can be used to ensure the microbial safety of meat products, e.g. salting to reduce the water activity, maintaining low temperature (Leistner, 2000), controlling pH (Pierson, 1982) and adding ascorbate (Gibson *et al.*, 1984).

When nitrite is added to meat, the colour of the meat first turns brown, then with time or stimulated by the presence of a reductant the colour turn into the characteristic red colour of cured meat. These colour changes are attributable to the oxidative state of the haem iron in myoglobin and hemoglobin. The brown colour occur because of the presence of metmyoglobin (MbFe(III)) which is produced by oxidation of the myoglobin (MbFe(II)) when nitrite is reduced to NO (Skibsted, 2011)(eq. 1).

$$MbFe(II) + NO_2^- + H_2O \rightarrow MbFe(III) + NO + 2OH^- \qquad \text{(eq. 1)}$$
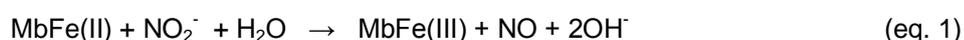
The metmyoglobin can then combine with one of the nitrosating agents, produced in the meat from nitrite, to form an unstable nitrosylmetmyoglobin complex (MbFe(III)NO). This complex auto reduces with time or in the presence of reductants, as ascorbate, to the corresponding relatively stable nitrosylmyoglobin (MbFe(II)NO) (Pegg and Shahidi, 2004), giving the characteristic red colour of cured meat (Skibsted, 2011)(eq. 2).

$$MbFe(III) + NO \leftrightarrow MbFe(III)NO \rightarrow MbFe(II)NO \text{ (bright red colour)} \qquad \text{(eq. 2)}$$

Upon heat treatment the nitrosylmyoglobin will denaturate if heated to 65°C or more by which the heat stable nitrosyl hemochrome (Fox, 1966) is released and the red colour is preserved (eq. 3).

$$MbFe(II)NO + heat \rightarrow \text{ Nitrosyl hemochrome (stable bright red colour)} \qquad \text{(eq. 3)}$$

The evolution of nitrite/nitrate/nitric oxide/nitrosylhaem in mortadella and during heat treatment at different temperature was studied by Barbieri *et al.* 2013. They found that the most efficient colour formation with the lowest resulting levels of nitrite and nitrate was achieved by adding 70 mg kg^{-1} nitrite (tested 40, 70, 100, 150 mg kg^{-1}) to the product and performed the heat treatment at 65°C (55, 60, 65, 70, 72°C tested). The faster the optimum temperature was achieved in the whole product the better it was (Barbieri *et al.*, 2013).

Nitrite or NO may participate in a range of reactions as also indicated in Figure 2.5-1. The fate of nitrite in meat can according to Tricker & Kubacki 1992 (Tricker and Kubacki, 1992) be roughly summarized as follows: 1-5% is lost as nitric oxide gas or becomes bound to lipids, 1-10% is oxidized to nitrate, 5-10% remains as free nitrite, 5-15% reacts with sulfhydryl compounds (mostly peptides and free amino acids) or myoglobin and 20-30% becomes bound to other types of proteins. In addition to these points nitrosation and nitrasation of amines with the formation of NA and nitramines also accounts for a part of the fate of nitrite. An amine is in this context defined as a compound in which one or more of the hydrogen atoms in ammonia have been replaced by an organic functional group. This group of compounds therefore also includes peptides and amino acids.

2.3.2 Formation of *N*-nitrosamines in cured meat

Nitrosation of primary-, secondary-, tertiary amines and the terminal amino groups of peptides and proteins may occur. Primary aliphatic amines can be nitrosated but this reaction does not lead to the production of NA, but to diazo compounds, or a complete replacement of the amino group with a nucleophil, e.g. water or chloride ion (Hill *et al.*, 1988), or the NA of primary amines rearrange and produce water and the corresponding alcohol ($H_2NN=O \rightarrow HN=NOH \rightarrow N_2 + H_2O$, and $RNHNO \rightarrow RN=NOH$)(Zhao *et al.*, 2007).

Tertiary amines can also be *N*-nitrosated, but during the reaction process one group will be cleaved, so the product will be the *N*-nitroso derivative of a secondary amine. The group that cleaves is an aldehyde or a ketone. It has however also been suggested that no NA can be produced from tertiary amines (Honikel, 2008). Nitrosation of secondary amines lead to the formation of relatively stable NA and will be the focus of this report. The general reaction can be summarized as follows:

$$R_2NH + \text{nitrosating agent} \rightarrow R_2N\text{-}N\text{=}O \qquad\qquad (eq.\ 4)$$
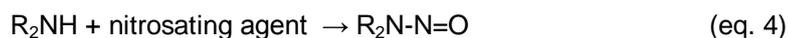
As mentioned whether the formed NA are non-, weakly- or strongly carcinogenic depends on the nature of the amino acid. The nitrite ion itself is not an efficient nitrosating agent without the presence of catalysts, e.g. mineral acids (Hill *et al.*, 1988). The nitrite ion (NO_2^-), formed from the reduction of nitrate will, in aqueous environments as meat, be protonated to the conjugate base of a weak acid, namely nitrous acid (HNO_2), which has a pKa of 3.36.

$$HNO_2 \leftrightarrow H+ + NO_2^- \qquad\qquad (eq.\ 5)$$

The rate of nitrosation of secondary amines at pH of about 3 has been suggested to follow the equation:

$$\text{Rate} = k_3[R_2NH][\text{nitrite}]^2 \qquad\qquad (eq.\ 6)$$

That the nitrite concentration is in second order has been suggested to indicate that the primary reagent at this pH is the anhydride of HNO_2, dinitrogen trioxide, N_2O_3 (Hill *et al.*, 1988).

$$2\ HNO_2 \leftrightarrow N_2O_3 + H_2O \qquad\qquad (eq.\ 7)$$

According to Hill *et al.* (1988), the nitrous acidium ion (H_2ONO^+), dinitrogen trioxide (N_2O_3) and dinitrogen tetraoxide (N_2O_4) are all present at moderate acidity (pH 2-5) and can act as nitrosating agents. The relative proportions of each of the reagents depend on the acidity of the medium. At low pH (<2) H_2ONO^+) becomes most important and is the predominant reagent for reactions with weakly basic amines (e.g. dimethylamine) and peptides. At pH of about 3, N_2O_3 appears to be the primary reagent, at least for secondary amines. The interplay between the three nitrosating agents (indicated by red fonts) is illustrated in Fig. 2 2.

$$NO_2 \underset{}{\overset{+H^+}{\rightleftharpoons}} HONO \underset{}{\overset{+H^+}{\rightleftharpoons}} H_2ONO^+$$

$$\Big\updownarrow +HONO$$

$$NO^\cdot + {}^\cdot NO_2 \rightleftharpoons N_2O_3 + H_2O$$

$$\Big\downarrow O_2 \qquad\qquad \Big\updownarrow O_2$$

$$NO_2 \qquad\qquad N_2O_4$$
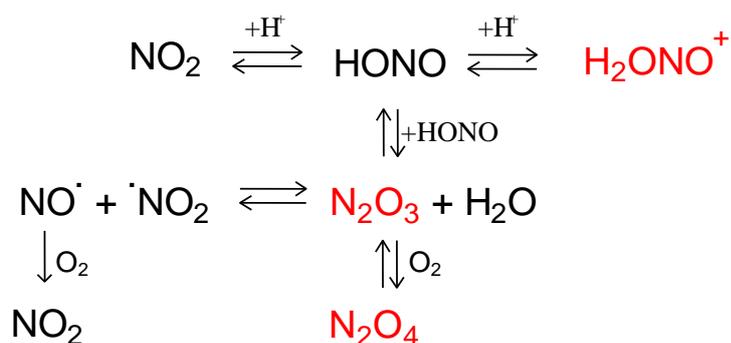
**Fig. 2.3-2**: Formation of nitrous acid (HONO) and subsequent reactions (modified from (Hill *et al.*, 1988).

The pH of meat is usually between 5.5 and 6.5, i.e. well above the pKa of HNO_2, the concentration of this agent will therefore only account for somewhere about 0.1 and 1.0% of the nitrite added to the meat. The primary reactive agents in meat is also believed to be dinitrogen trioxide (N_2O_3)(Pegg and Shahidi, 2004).

Besides having influence on the amount of nitrosating agents pH, also affects the concentration of unprotonated amines (Hill *et al.*, 1988). The pH maximum for the nitrosation of amines depend on the interplay between the increase in rate with increasing pH due to the presence of more unprotonated amine, and the decrease in rate due to the declining production of nitrosating agents. For basic amines like morpholine and N-methylpiperazine, the pH maximum has been shown to be 3.4, whereas secondary amines with a ionizable group (e.g. α-amino acids) near to the nitrosatable amine, showed a different pH maximum, e.g. 2.46 for proline and 2.56 for sarcosine (Hill *et al.*, 1988). All these pH optimum are well below the pH of meat.

Peroxynitrite ($ONOO^-$) has, in neutral and acidic pH, been proposed to result in the production of $\cdot NO_2$ and $\cdot OH$ radicals, which in turn are capable of one-electron oxidation of secondary amines, to form amino radicals ($R_2N\cdot$). These amino radicals are then proposed to react with $\cdot NO$ or $\cdot NO_2$ to yield NA or nitramines. Whether NA or nitramines are yielded has been shown to depend on the pH of the substrate and the pKa of the amine. Masuda *et al.* 2000 (Masuda *et al.*, 2000) have found that unprotonated amines undergo nitrosation more easily than nitration, thus more nitramines than NA are produced if the pH is higher than the pKa of the amine, and vice versa (Masuda *et al.*, 2000). NA can be oxidized to nitramines.

In principle, the *N*-nitrosation is a reversible reaction, which is acid catalysed. However, the presence of the nitroso group dramatically reduces the basicity of the amine nitrogen. Denitrosation of amines at pH >3 therefore require the presence of electron withdrawing alkyl substituent's to weaken the N-NO bond (Hill *et al.*, 1988). Peptides and proteins can also be nitrosated or nitrasated by similar mechanisms as for the amines. Proteins may be considered to be the largest source of potentially *N*-nitrosatable amines (Hill *et al.*, 1988) and following human consumption of foods with nitrosated proteins NA may be released, when the proteins are digested and broken down into peptides and amines. Since nitrite is also present in the human body, participating in essential functions, further nitrosation of meat amino groups may occur in the digestive tract (Joosen *et al.*, 2009).

2.4   Occurrence of *N*-nitrosamines in meat products

N-nitrosamines are found in nitrite or nitrate cured meat products and meat products exposed to other nitrogen sources, e.g. by direct smoking processes or barbecuing (Hill *et al.*, 1988). The highest *N*-nitroso contents are found in products as sausage, smoked meats, bacon and luncheon meats (Stuff *et al.*, 2009), thus meats for which the processing has included nitrite/nitrate addition, smoking and/or heating. NA can be produced in all types of food which contain proteins and which are exposed to a nitrogen source. There is a large amount of published studies on the levels of different NA. The majority of these have examined the levels of a few NA in a few different food items. However, databases on the occurrence and amounts of NA in food products have been set up, collecting the data available in the literature and making it more assessable (Jakszyn *et al.*, 2004;Stuff *et al.*, 2009). The latest being by Stuff *et al.* 2009.

Because of the complexity of the *N*-nitroso compounds produced and because of the unavailability of analytical methodologies for the majority of the non-volatile *N*-nitroso compounds, a lot of research has been directed towards the development of group selective methods (Chapter 3.1.1). The determination of the apparent total *N*-nitroso compounds (ATNC) has been widely used as a measure of the total amount of *N*-nitroso compounds in foods. The amount of actual NA is most likely overestimated by this method. The ATNC levels in cured meats have been shown to occur in amounts up to 7000 µg kg^{-1} (expressed in µg N-NO). For example in bacon the levels of ATNC were found to be in the order of 400 to 7000 µg kg^{-1} meat (expressed in µg N-NO) (Massey *et al.*, 1991). The levels of ATNC in foods invariable exceed the sum of the VNA and NVNA considerably. A part of this difference may be attributed to unknown NA, primarily non-volatile ones (Crews, 2010).

The VNA have been the subject in the majority of the available literature most of which have been generated in the 1970s and early 1980s. The analytical procedures for the analysis of these volatile compounds in foods were well established at this time (Chapter 3.1.2). VNA as NDMA, NPYR, NPIP, NDEA, *N*-nitrosomorpholine (NMOR) and NDBA are commonly assayed (Stuff *et al.*, 2009). The chemical structures of the compounds are presented in Figure 2.4-1. In general the VNA are found in relatively low amounts, i.e. in the order of ≤5 µg kg^{-1}, even in a product like bacon (Hill *et al.*, 1988;Campillo *et al.*, 2011;Yurchenko and Mölder, 2007;Tricker *et al.*, 1991). NDMA and NPYR are the most frequently found VNA in nitrite preseved meat products. For example Spiegelhalder *et al.* (1980) reported 32% and 11% of such products (N=395) to contain detectable levels (>0.5 µg kg^{-1}) of NDMA and NPYR, respectively. Levels above 5 µg kg^{-1} of NDMA and NPYR occurred only in 2% and 6% of the samples, respectively (Spiegelhalder *et al.*, 1980). Other types of VNA were only detected occasionally. According to Stuff *et al.* 2009 the highest levels of NDMA in commonly eaten foods were found in sausages (10.9 µg per 100 g) and for NDBA in bacon (1.3 µg per 100 g); The food sources with the highest levels of VNA were bacon, luncheon meats, sausages and hot dogs. NDMA, NPIP, NPYR, NDEA accounted for the major part of the VNA contents (Stuff *et al.*, 2009). Though others have reported levels of NDMA as high as 17 µg kg^{-1} in baked meat without the addition of nitrite and that the levels vary greatly dependent on the animal species (Rywotycki, 2007) and breeding conditions (Rywotycki, 2003).
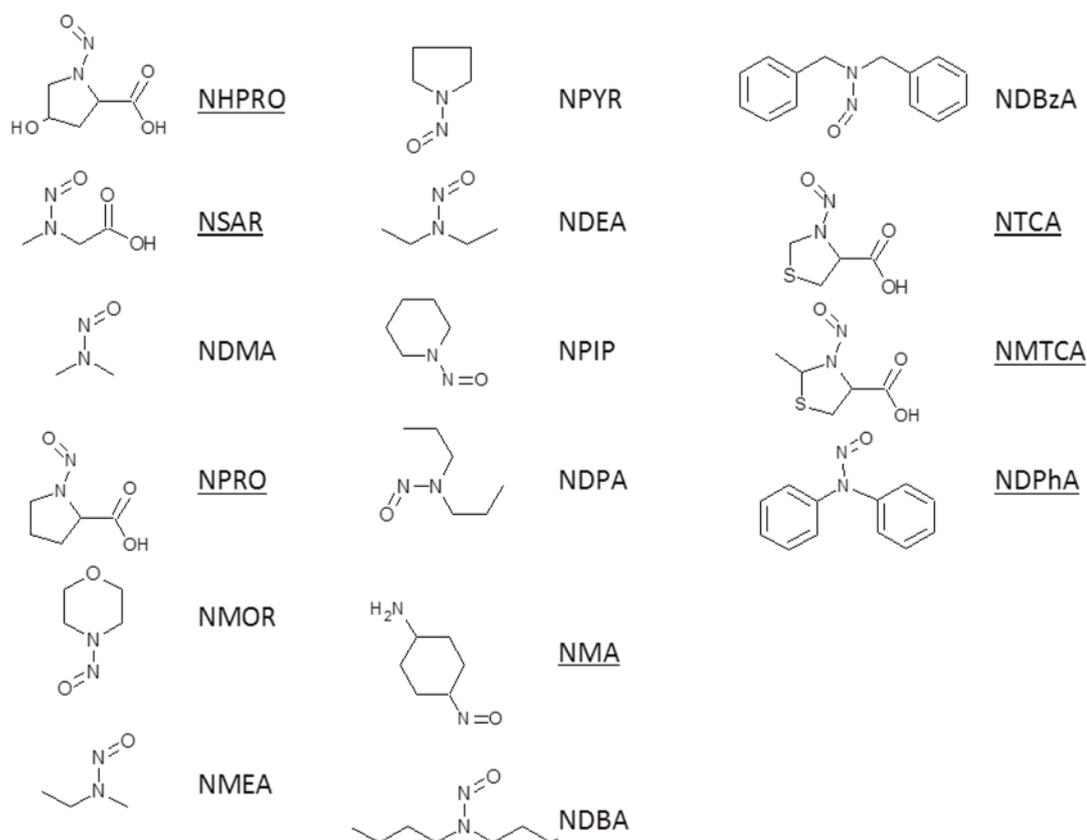
Figure 2.4-1: Structures of the 16 VNA and NVNA relevant for the present study because they have been shown to occur in foods and were available as pure standards. The abbreviations of the NVNA are underlined. NHPRO (*N*-nitrosohydroxyproline), NSAR (*N*-nitrososarcosine), NDMA (*N*-nitrosodimethylamine), NPRO (*N*-nitrosoproline), NMOR (*N*-nitrosomorpholine), NMEA (*N*-nitrosomethylethylamine), NPYR *(N*-nitrosopyrrolidine), NDEA (*N*-nitrosodiethylamine), NPIP (*N*-nitrosopiperidine), NDPA *(N*-nitrosodi-n-propylamine), NMA (*N*-nitrosomethylaniline), NDBA (*N*-nitrosodibutylamine), NDBzA (*N*-nitrosodibenzylamine), NTCA (*N*-Nitroso-thiazolidine-4-carboxylic acid), NMTCA (*N*-Nitroso-2-methyl-thiazolidine 4-carboxylic acid) and NDPhA (*N*-nitrosodiphenylamine).

Knowledge on the occurrence and nature of the NVNA in food is on the other hand limited. The analytical methodologies necessary for the analysis of these compounds, i.e. high performance liquid chromatography coupled with e.g. mass spectrometry, have only recently become standard equipment in most laboratories.

The group of NVNA include 1) Nitrosated amino acids (NPRO, *N*-nitrosohydroxyproline (NHPRO) and NSAR); 2) Nitrosated amino acid/aldehyde condensation products (NTCA, NMTCA); 3) decarboxylation products (e.g. NHPRO to NHPYR and NTCA to *N*-nitrosothiazolidine (NTHz) 4) Nitrosated food contact material migrants as *N*-nitrosodibenzylamine (NDBzA) (Startin, 1996).
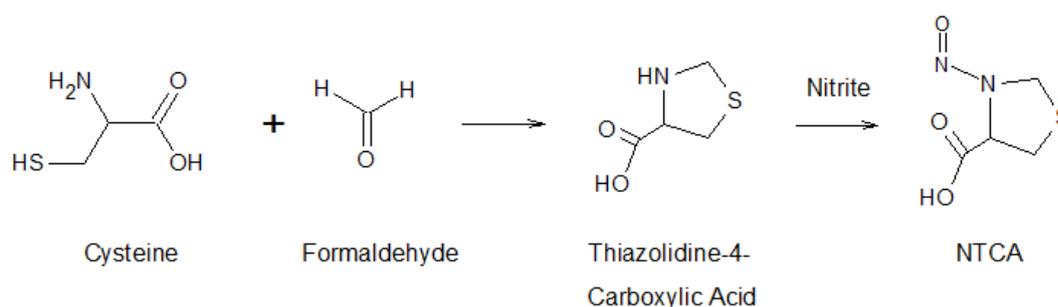
Figure 2.4-2: Formation of NTCA via nitrosation of TCA formed by condensation of the amino-acid cysteine and formaldehyde (modified from (Tricker and Kubacki, 1992)

The most common NVNA in foods are NTCA, NPRO, NSAR, NHPRO (chemical structures presented in Figure 2.4-1) and the decarboxylated derivative of the latter NHPYR (Tricker and Kubacki, 1992;Stuff *et al.*, 2009). The NVNA are often found in higher levels than the VNA, i.e. in the order of 10-2000 µg kg^{-1} (Hill *et al.*, 1988;Stuff *et al.*, 2009). NTCA and NHMTCA were e.g. found in smoked cured meats at concentrations up to 1100 and 600 µg kg^{-1}, respectively (Tricker and Kubacki, 1992). Of commonly eaten foods the highest levels were found for NTCA (142 µg/100 g) in bacon and NHMTCA (118 µg per 100 g) in cheese (Stuff *et al.*, 2009). NTCA accounted for the major part of the characterised NVNA contents in bacon, luncheon meats, sausages and hot dogs. These products were also those with the highest levels of NVNA in total (Stuff *et al.*, 2009).

It has been suggested that *N*-nitrosated amino acids can undergo thermal decarboxylation during cooking to yield the corresponding VNA. Decarboxylation of NPRO, NHPRO and NSAR would then produce NPYR, NHPYR and NDMA, respectively (Tricker and Kubacki, 1992). If this is true the high levels of NVNA found to occur in nitrite preserved meat may be a large source of VNA. More on the reported effects of heat treatment are presented in Chapter 2.5.5).

2.5   Factors affecting *N*-nitrosamine levels

According to eq. 6 in Chapter 2.3.2 the rate of NA formation is only dependent on the concentration of nitrite and the concentration of amine groups. Though any compound which will compete for nitrite or the nitrosating agents derived from it, affect the pH, affect the availability of nitrosable amines, the oxidative status etc. can affect the formation of NA. Processed meat is a complex matrix and can contain several natural constituents and additives which can interact either with nitrite, the nitrosating agents or the amines and thereby prevent or promote the NA formation.

The levels of e.g. antioxidants in meat may be affected by the content of these compounds in the feed, i.e. a feed with high contents of antioxidants may result in meat with higher oxidative capacity. The pH of the meat may vary depending on the stress status of the animal at slaughtering. However we evaluate that the effect those factors may have on the NA formation is limited in comparison with the effects that may be induced by ingredients and/or additives added directly to the meat. Factors as ingredients and additives are also easier to control.

Additives allowed to be added during production of processed meat besides nitrite and nitrate include: preservatives, antioxidants and other additives e.g. those listed in Table 2.5-1. Information on which additives are allowed to be added to processed meat is laid down in the Council directive No 95/2/EC of 20 February 1995 and all of its amendments but a database has been made available which can be searched for specific additives and/or products. The link for this database is presented in Table 2.5-1.

Table 2.5-1: Examples of additives allowed added to processed meat products according to the Council directive No 95/2/EC of 20 February 1995.

Additive group	Additive	E No.	Maximum amount allowed to be added[1]
Preservatives	Sorbic acid – sorbates	E 200 – E203	Needed amount to the surface of dried products
Antioxidants	Ascorbic acid	E 300	Needed amount
	Sodium ascorbate	E 301	Needed amount
	Erythorbic acid or sodium erythorbate	E 315	500 mg kg^{-1} expressed as erythorbic acid
	Extracts of rosemary	E 392	15 or 150 mg kg^{-1} depending on fat content
Others	Phosphoric acid - phosphates – di- tri- and polyphosphates	E338-341, E242 and E 450-452	5000 mg kg^{-1}
	Sucrose esters of fatty acids, sucroglycerides	E 472-474	5000 mg kg^{-1}
	Dextrose, sorbitol, mannitol	E 418, E 420, E 421	Needed amount

1: https://webgate.ec.europa.eu/sanco_foods/main/?event=category.view&pageNo=1&identifier=96

Other additives than those in Table 2.5-1 are allow to be added to meat products, though by studying the ingredients lists of processed meat products available on the Danish market often used additives are sodium chloride, nitrite, antioxidants (most often E300 and E301), sugars

(most often dextrose) and phosphates. As mentioned most processed meat products are also smoked. The fat content of processed meat products vary greatly from about 2% in some luncheon meats to about 50% in products as bacon. All of these ingredients as well as the smoking process and fat have been reported to affect the formation of NA more or less. Ingredients as black pepper and paprika are added to many types of processed meat products in varying amounts and it has been suggested that these spices are sources of precursors for NPIP and NPYR, respectively (Yurchenko and Mölder, 2007).

The majority of the processed meat products that are consumed in Denmark are based on red meat. Red meat has as mentioned a higher myoglobin and iron content than white meat. Haem, from myoglobin or haemoglobin, and/or iron have been suggested to play a crucial role in the endogenous formation of *N*-nitroso compounds, however their role in NA formation in meat has to our knowledge not been studied.

Thus, nitrite, sodium chloride, antioxidants (most often E300 and E301), sugars (most often dextrose), phosphates, smoking, black pepper and paprika are commonly used for processed meat products and all have been reported to have an effect on the NA formation. For the present study it was therefore chosen to focus on these factors. Some of the published literature on the observed effects is presented in the following. The background for the interest in the role of haem and/or iron in the NA formation is also presented.

2.5.1 Antioxidants/reductants

Antioxidants are added to meat products in order to increase the storage stability. The antioxidants protect against reactive oxygen agents. The antioxidants are then oxidised and reduce the compound they react with so they can also be referred to as reductants. Reactive oxygen agents induce oxidative rancidity, which is lipid oxidation, and thereby also unwanted tastes and flavours. Sodium ascorbate, ascorbic acid, sodium erythorbate and erythorbic acid are commonly used antioxidants for meat products. It is common to add 500 mg kg^{-1} to meat products. Going through the available literature there has not been indicated that the four mentioned antioxidants should vary in their function in meat and we will therefore assume that the effect observed for one form is common for all four forms. Rosemary extracts, vitamin E and flavonoids are also used in foods though less commonly in meat products.

The presence of an antioxidant in meat plays an important role in the kinetic aspects of the nitrite reactions. These antioxidants may reduce the nitrous acid formed by protonation of the nitrite ion with a 1:2 stoichiometry and hereby yield nitrogen oxide (NO)(Eq. 8)(Skibsted, 2011).

$$\text{Reductant} + 2\ HNO_2 \ \leftrightarrow \ \text{Dehydro-Reductant} + 2NO \text{ and } 2\ H_2O \qquad \text{(eq. 8)}$$

The reactivity of nitrous acid or nitrite increases with decreasing pH, whilst the reactivity of ascorbate or ascorbic acid decreases (Gray and Dugan, 1975;Skibsted, 2011). Thus the ascorbate anion is more readily nitrosated than ascorbic acid. The lower the pH is the more ascorbate may be needed in order to prevent the nitrous acid to participate in other reactions. The reaction between the dinitrogen trioxide and hydrogen ascorbate has been demonstrated to be quantitatively the most important for (Pegg and Shahidi, 2004) conditions of relevance for meat and the reaction between these are faster than between dinitrogen trioxide and secondary amines as proline (Skibsted, 2011). Thus, the production of NA may be limited by the presence

of ascorbate because it adds to the several reactions that NO from nitrite can participate in (Figure 2.5-1). However, ascorbate should be present in high amounts or combined with low oxygen tension to avoid oxidation of NO, back to the nitrosating agents (Hill *et al.*, 1988).
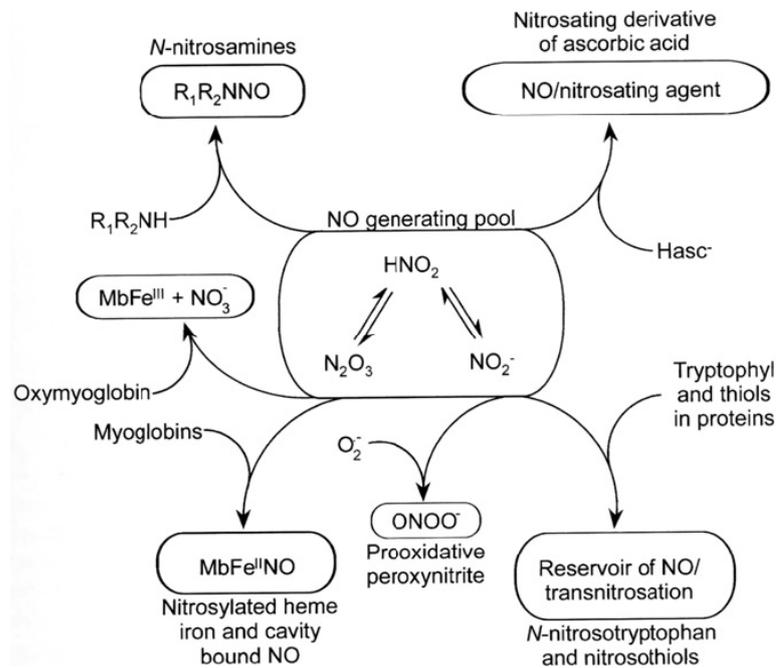
Figure 2.5-1: NO formed from nitrite during meat curing can participate in numerous reactions resulting in formation of *N*-nitrosamines, nitrosylhaem and other nitrosated compounds as nitrosothiols (Skibsted, 2011).

The addition of ascorbic acid may be expected to lower the pH. Though according to (Li *et al.* 2013, 1157-1164), the pH of dry cured sausages was not affected by the addition of 500 mg kg^{-1} of ascorbic acid throughout a ripening period of 28 days. Though, the pH in the sausages decreased from day 0 to day 7 (pH 5.9 as the lowest point) regardless of ascorbic acid had been added or not. Hereafter the pH increased again and stabilised at approximately the same value as on day 0 (6.1-6.2).

A wide range of antioxidants have been tested for their ability to increase the oxidative stability of meat in regard to formation of lipid oxidation products and off taste. Polyphenols from green tea and grapes were e.g. found to decrease the thiobarbituric acid reactive substances (TBARS, by-products of lipid peroxidation) values of dry cured sausages and more effectively so, than ascorbic acid. Though, ascorbic acid resulted in much faster and more efficient reduction of the residual level of nitrite and a reduction in the levels of NDMA and NPYR than several other antioxidants (Li *et al.*, 2013). That ascorbic acid reduce the residual levels of nitrite in meat has also been reported by others (EFSA, European Food Safety Authority, 2003;Li *et al.*, 2013).

Meat products are comprised of a lipid and a lean phase, being a non-polar and a polar phase. Antioxidants as ascorbate and erythorbic acid are polar and their effect on oxidative processes in the lipid phase of meat may therefore be limited. A combination of a non-polar and polar antioxidant may therefore provide better protection against NA formation. Ascorbyl palmitate, the ester of ascorbic acid, is an example of a lipid soluble and hence non-polar antioxidant.

Ascorbyl palmitate and propyl gallate have e.g. been shown to reduce the levels of NPYR formed during frying of bacon more efficiently than sodium ascorbate (Sen *et al.*, 1976).

As described in Chapter 2.3.1 ascorbate favours the formation of nitrosylmyoglobin. Nitrosylmyoglobin may itself have antioxidative effects (Skibsted, 2011). Thus, the addition of antioxidants may to some extent control the processes initiated by the addition of nitrite, because the formation of nitrosylmyoglobin is favoured over the production of the carcinogenic NA. To our knowledge it is not known whether the protective effect of ascorbate is mediated by a reduction in the availability of the nitrosating agents or some other factors e.g. an altered reactivity of nitrosylmyoglobin compared to myoglobin or an altered reactivity and/or stability of the haem moiety resulting in reduced release of iron.

Literature is scarce on the effect of ascorbate on the NA formation in actual meat products. Most of the studies described in the literature are based on tests in different model systems. The studies that we have found only deal with NDMA, NPYR and NDEA. Mottram *et al.* (1975) found that NDMA formation was reduced by the presence of sodium ascorbate in pork slices cured in brine supplemented with DMA. At pH 5.6 a 90% reduction in the NDMA levels was found at a molar ratio of 1:1 of ascorbate to nitrite. Molar ratio of 0.5 or 2.0 resulted in a NDMA reduction of about 80% (Mottram *et al.*, 1975). Rywotycki and Ryszard (2002) found a 24% and 25% reduction in the formation of NDMA and NDEA, respectively, by adding 500 mg kg^{-1} sodium ascorbate to sausages prepared with nitrite (160 mg kg^{-1} nitrite)(Rywotycki and Ryszard, 2002). Li *et al.* (2012) found that 500 mg kg^{-1} ascorbic acid resulted in 28% less NA (total of NDMA, NDEA and NPYR, mostly NPYR) in dry-cured sausages after a 28 days ripening period (Li *et al.*, 2012).

Based on the available literature it seems evident that antioxidants as ascorbate and ascorbic acid have pronounced effects on the NA formation (Tricker and Kubacki 1992, - 39). It has though been suggested that ascorbic acid can act as a catalyser of NA formation in the presence of fat. This was indicated because of the observations that under acidic conditions, simulating the human proximal stomach, the presence ascorbate limited the formation of NDMA, NDEA and NPIP, whereas if lipid was also added ascorbate stimulated the formation of the three VNA (Combet *et al.*, 2007).

2.5.2 Sodium Chloride

Sodium chloride is added to meat products for preservation as well as for taste enhancement. The preservative effect is mediated by reduction of the water activity. In general the amount of sodium chloride added to meat products are about 2% (www.foodcomp.dk) though levels as high as 7% sodium chloride typically for dry cured ham is applied for some products (Tulip, a large Danish producer of processed meat, 2012, pers. com. 4 Sept.). Because high intake of sodium chloride has been associated with increased risk of cardiac diseases there is a general interest to reduce the levels of sodium chloride in our foods. It has though been indicated that the preservative effect of nitrite is enhanced by the presence of sodium chloride and vice versa (Pegg and Shahidi, 2004). Sodium chloride may therefore also interact with the nitrite induced formation of NA.

The catalytic effect of sodium chloride may be mediated by anions as I$^-$, Br$^-$, Cl$^-$, acetate, phthalate and weak acids (Tricker and Kubacki 1992, - 39). These substances can result in the

formation of specific nitrosating agents with higher reactivity (Hill *et al.* 1988). With increasing sodium chloride concentrations, it has been suggested that, nitrous acid may transform into nitrosyl chloride (NOCl) by the reaction in eq. 9.

$$HNO_2 + H^+ + Cl^- \quad \rightarrow \quad NOCl + H_2O \qquad \text{(Eq. 9)}$$

NOCl is more reactive than N_2O_3, though less reactive than the nitrous acidium ion, H_2ONO^+ (Skibsted, 2011). Thus the addition of sodium chloride may therefore affect the NA formation because of altered levels of reactive nitrosating agents capable of nitrosating amines. Though under acidic conditions as in the stomach, it has been suggested that the presence of nucleophiles ($I^- > Br^- > Cl^-$) can catalyse the denitrosation of *N*-nitroso compounds (Douglass *et al.* 1978, 581-605).

Sodium chloride may have prooxidative effects which were recognized in meat systems at concentrations from 0.5 to 2.5%. However at higher levels (>2.5%) it was indicated to have antioxidative effect because it was found to inhibit lipid oxidation processes. This inhibition may arise if sodium chloride displaces iron from the binding sites. Though, other results indicate that it is inorganic iron that plays a role in the initiation of lipid oxidation processes (Gheisari *et al.*, 2010).

Sodium chloride may affect the levels of NAs in meat products. E.g. Rywotycki (2002) found that the levels of NDMA and NDEA were lower in nitrite preserved meat if besides nitrite sodium chloride and/or sodium ascorbate was added. The reducing effect of adding sodium chloride and ascorbate was not additive (Rywotycki, 2007;Rywotycki and Ryszard, 2002), which may indicate that they influence a common mechanism, thus in agreement with sodium chloride being an antioxidant/reductant as ascorbate. Sodium chloride in the range from 0 to 4% sodium chloride was as sodium ascorbate found to increase the rate of NO formation in nitrite-antioxidant mixtures with a linear relationship (Sebranek and Fox 1985, 1169-1182). Sodium chloride was also found to exhibit a concentration dependent inhibition of the NPYR formation in fried slices of minced pork meat sausages. Significant reduction in the NPYR level was observed by adding 0.1% sodium chloride compared to no sodium chloride. A similar trend though not statistically significant was found by increasing the sodium chloride contents further to 0.5 and 1.0%. A sodium chloride content higher than 1.5% had no significant effect on the NPYR formation. A 50% higher level of NPYR was found if no sodium chloride was added compared to when 1.5% was added (Theiler *et al.*, 1981).

Thus the available literature indicates that addition of sodium chloride to nitrite preserved meat may reduce the formation of NA. Some studies indicate that there may be a concentration dependent effect whereas others indicate that only minor effects are obtained by increasing the sodium chloride levels above 1.5%.

2.5.3 Sugars

Sugars are added to several types of meat products. For fast and medium-fermented sausages as salami sugar serves as food for starter cultures and helps to offset the sourly and tangy flavour and acts as a minor hurdle in lowering water activity. Sugar are however also added to non-fermented heat treated products as wiener sausages and meat sausages (luncheon meat)("www.foodcomp.dk"). Sugars are added for taste enhancement, to increase water holding

capacity, for their contribution to browning during frying and in some cases for their ability to disguise high levels of sodium chloride (McArdle and Hamill, 2011). Dextrose (D-glucose) is commonly used by industry and commonly used levels are in the order of 1 to 3% (Tulip, a large Danish producer of processed meat, 2012, pers. com. 4 Sept.).

Pensabene and Fiddler (1993) found that the carbohydrates sucrose, sorbitol, fructose and polydextrose added to frankfurters with surimi increased the levels of NDMA by from 12 µg kg^{-1} up to 22 µg kg^{-1}, highest effect observed for sorbitol. They also showed that there was no relationship between the amount of DMA and NDMA formation (Pensabene and Fiddler, 1993). In contrast Theiler *et al.* found no effect on the NPYR levels having 0.4 to 0.8% sucrose in fried slices of minced pork meat sausages (Theiler *et al.*, 1981). They did however find a reduction in the NPYR level if adding 0.1 to 1% reducing sugars as glucose, maltose and lactose (Theiler *et al.*, 1984). The reducing sugars may reduce the levels of the nitrosating agents as ascorbic acid does (Bailey *et al.*, 1980).

The effects of some sugars may be mediated by a decrease in the pH of the meat product. The pH drop induced by sugar in sausages depends on the type and amount of sugar utilized. Introduction of more sugar generally leads to lower pH. About 1 g (0.1%) of dextrose per 1 kg of meat lowers pH of meat by 0.1 pH. This means that 10 g of dextrose added to meats with an initial pH value of 5.9 will lower pH by one full unit to 4.9. Sugar levels of 0.5% - 0.7% are usually added for reduction of the pH level to just under 5.0. When using acidification as a main safety hurdle, salami is microbiologically stable at a pH of 5.2 or lower (Bailey *et al.*, 1980). pH seems to affect the formation of the different NA differently depending on the pKa values of the amines, less NPYR was e.g. formed in fried bacon at a pH 7.5 than at pH 5.5 (Bailey *et al.*, 1980). Whereas less NDMA (50% reduction) was formed by changing the pH from 5.6 to 4.5 in cured pork slices fortified with dimethylamine (Mottram *et al.*, 1975).

So whether or not adding sugar affects the NA formation may depend on the type of sugar used, the type of product it is added to, the presence of other additives or ingredients and on which NA is studied.

2.5.4 Fat content and phosphates

Phosphates bind the meat fibres together by stabilising emulsions and by solubilising the muscle proteins. If too much phosphate is added the meat will get a rubbery consistency and perhaps unwanted state (Boles, 2011). As part of the solubilisation phosphates also increase the water holding capacity of the meat (Rogers, R.W.. Edited by Hui,Y.H. *et al.*, 2005). Phosphates also increase the pH. Though 2000 to 5000 mg kg^{-1} only increases the pH of meat by about 0.1 to 0.3 units. Since it affects the pH it can also affect the reactivity of nitrite. Polyphosphates can act as a metal chelator and may therefore interact with iron and/or haem (Chapter 2.5.6). Different effects have been reported in the literature. Rywotycki & Ryszard (2002) found polyphosphates to enhance levels of NDMA and NDEA in nitrite cured canned meat, whereas Theiler *et al.* (1981) (model system) did not find any effect of polyphosphate on the formation of NPYR in fried slices of minced pork meat sausages.

Because the phosphates stabilise the emulsions of e.g. sausages there may be an interaction between the phosphate and the lipid in the product. The formation of NDMA and NPYR seems to be associated with the lipid phase as found for bacon by Mottram *et al.* (1977) and for fried

pork by Yurchenko and Mölder (2007). The latter also found higher levels of both NDMA and NPYR if the pork had been spiced with paprika (Yurchenko and Mölder, 2007). Release of nitrosating agents from the lipid phase during frying was suggested as the reason for the increase in NPYR during frying of bacon observed by e.g. Sen *et al.* (1976). This group also found that the lipid soluble antioxidants propyl gallate and ascorbyl palmitate inhibited the NPYR formation more efficiently than sodium ascorbate (Sen *et al.*, 1976). Several mechanism for this preferential formation in the lipid phase has been presented; higher temperature during frying than in the lean part with a higher water content, a different chemical environment favouring nitrosation (Mottram *et al.*, 1977), higher solubility of both nitrogen oxide (NO) and oxygen in the lipid phase (Liu *et al.*, 1998) resulting in higher levels of nitrosating agents as e.g. N_2O_2.

Thus phosphates may affect the NA formation by altering the pH, chelate metal ions and/or by increasing the contact between the lipid and the lean aqueous phase.

2.5.5 Heat treatment and smoking

Meat products can be exposed to elevated temperatures during e.g. ripening, drying processes, smoking and production of canned meat but also during frying or other types of further processing e.g. at home or in the fast food industry. Drying and smoking of meat products has been used for preservation for centuries. It increases storage stability by reduction of the water activity and by precipitation of smoke constituents with antibacterial effect (Sunen *et al.*, 2001).

Application of heat during processing as frying and baking has been shown to affect the levels of NA in processed meat (Drabik-Markiewicz, Van den Maagdenberg, De Mey, Deprez, Kowalska and Paelinck, 2009,Rywotycki, 2007,Drabik-Markiewicz, Dejaegher, De Mey, Kowalska, Paelinck and Vander Heyden, 2011,Yurchenko and Mölder, 2006,Yurchenko and Mölder, 2007). Especially the levels of NDMA and NPYR have been shown to be affected by e.g. frying (Yurchenko and Mölder, 2007). Some of the minor effects reported may though be the result of concentration of the product when water evaporates from the product during the cooking process. The formation of NDMA and NPYR was optimum at a frying temperature of 150°C (Mottram *et al.*, 1977) to 200°C (Drabik-Markiewicz *et al.*, 2011). Though, at 150°C about 80% of the NDMA and NPYR produced were recovered in the vapour (Mottram *et al.*, 1977). The heat treatment may speed up processes in the meat including nitrosation or result in release of nitrogen oxide or other nitrosating agents bound to lipids and hereby give rise to the production of NA (Hotchkiss *et al.*, 1985). It has also been suggested that e.g. NDMA and NPYR can be produced during frying from decarboxylation of the NVNA, NSAR and NPRO, respectively. Though, this has to our knowledge not been documented experimentally.

As described in Chapter 2.3.1 a temperature around 65°C may optimal for the formation of the cured meat meat colour, thus the kinetics of nitrite and thereby also the thereof derived nitrosating agents are affected also at these lower temperatures. So called warm smoking is performed at a temperature of 60-80°C whereas cold smoking is performed at temperatures <33°C (Fellows, 2009). Cold smoking primarily contribute with flavour and colour, whereas warm smoking also preserve the product by reduction of the water activity and bacteria and enzymes are destroyed (Fellows, 2009).

During smoking the meat is exposed to wood smoke, which means smoke from incomplete combustion of organic matter. This smoke contains a large number of compounds of which

acids, phenols and carbonyls add to the texture, taste and appearance (Simko, 2011). Formaldehyde is also a component of wood smoke (Sen *et al.*, 1986) and as described earlier formaldehyde and other aldehydes can condensate with cysteine and result in the formation of NA. Phenols from the smoke may on the other hand prevent the NA formation. Significantly higher levels of NTCA have been reported for smoked meat than for non-smoked meat (Sen *et al.*, 1986;Tricker and Kubacki, 1992). The smoke itself seems to have little or no effect on the levels of NDMA and NPYR. Constituents in the smoke may even cause an inhibition in the formation of NDMA and NPYR. Liquid smoke was found to reduce the levels of NPYR in fried nitrite cured minced pork meat (Theiler *et al.*, 1984). The reason for this observed effect may be the occurrence of some compounds in the smoke which prevent the NA formation but may also be the result of increased competition for the nitrosating agents from TCA, which is very easily nitrosated to NTCA.

Thus elevated temperatures and/or smoke can affect the levels of both VNA and NVNA. From the above described literature it would be expected that heat treatment would increase the levels of VNA and decrease the levels of NVNA containing carboxylic acid groups. Smoking seems to favour the formation of NTCA and other NVNA derived by condensation of aldehydes and cysteine.

2.5.6   Haem and iron

As described in Chapter 2.1.1 haem has been suggested to play an essential role in the endogenous formation of *N*-nitroso compounds in both man and rodents. There are also some epidemiologic studies which have found evidence for an association between cancer and the consumption of red meat as beef and pork but not with the consumption of white meat as chicken. The observed effects in vivo may be counteracted by α-tocopherol and/or calcium, which may support a role of iron and/or haem (Chapter 2.1).

The highest levels of total iron are found in beef meat, i.e. about 2 mg per 100 g. Beef also has the largest fraction of the total iron accounted for by haem iron (90%). The mean total iron level of pork meat is about 1 mg/100 g of which 75% is accounted for by haem bound iron. For chicken meat the total iron content is about 0.6 mg per 100 g and approximately 50% of this is accounted for by haem iron (Carpenter and Clark, 1995)(www.foodcomp.dk). Thus the dietary intake of haem iron is about 2.5 times higher per gram of beef meat than per gram of pork meat and about 2.5 times higher per gram of pork meat than per gram of chicken meat.

When haem and other proteins are ingested they will to some extent be degraded into amino acids. If haem is digested the iron of the haem moiety is be released. The effects that have been observed in vivo could therefore be caused by free iron and not by haem. These observations may indicate an essential role of haem and/or iron in the formation of NA in meat products. Though, haem has been found to have no effect on the degree of lipid oxidation in meat (Gheisari *et al.*, 2010). If there are shared mechanisms between lipid oxidation and NA formation, which is indicated by the fact that both processes are inhibited by e.g. erythorbic acid, iron and/or haem may also affect the NA formation in meat. To our knowledge the role of haem and iron on NA formation in meat has not been studied.

2.6 Summary

Based on this brief introduction to some of the current knowledge in the field on NA formation in meat, it can be concluded that the addition of nitrite to meat results in formation of NA. The primary nitrosating agent in meat is believed to be N_2O_3. Exposure to NA via consumption of processed meat may contribute to the adverse health effect associated with consumption of these products. Many NA are carcinogenic though they need to be metabolically activated before they become carcinogens.

The total amount of nitroso compounds, the ATNC, can in processed meat products be in the order of up to 7000 µg kg^{-1} (expressed as N-NO). The individual VNA typically occur at levels of ≤5 µg kg^{-1} whereas the individual NVNA occur at levels up to 2000 µg kg^{-1}. Many NVNA are considered non-carcinogenic or less carcinogenic than the VNA. However, some NVNA may during cooking be decarboxylated to the corresponding VNA. Thus, the content of NVNA in foods may significantly contribute to the carcinogenic effects connected with dietary consumption of processed meats.

Because of the complex chemistry of nitrite and thereby also the NA formation several factors may affect the degree to which nitrite additions leads to formation of NA. These factors include e.g. antioxidants, sodium chloride, sugars and polyphosphates. Some of the effects observed by sugars and polyphosphates may be mediated by their effect on the pH in the meat. NA formation has been suggested to occur more readily in the lipid phase of meat and polyphosphates, an emulsifier, may interact with the lipid dependent NA formation. The presence of antioxidants (e.g. ascorbate) has consistently been shown to reduce the levels of NA in nitrite preserved meats and instead promote the formation of nitrosylhaem. Spices as black pepper and/or paprika may increase the levels of NPIP and NPYR.

The knowledge on NA formation and the effect of varies factor on their formation is almost solely based on studies on the levels of NDMA and NPYR and in a few cases NDEA. It is relevant to study the effect of the mentioned additives, fat content and heat treatment on the levels of other NA as well. The likelihood of unknown NA to occur in meat products is high. Gaining more knowledge on how the formation of other NA, besides NDMA and NPYR, respond to relevant factors will improve the general picture on how to avoid or mitigate formation of NA.

3. Analysis of *N*-nitrosamines in meat products

As described in the introduction further data is needed on VNA and especially the NVNA. Thus a method for the simultaneous determination of NVNA and VNA in processed meat products would facilitate the fulfilment of the overall aim of the present thesis. Though such a method has not been described in the literature and the development and validation of such a method was therefore included as an aim of the present study. The NA chosen as target analytes in the developmental work (see Figure 2.4-1) have previously been reported to occur in processed meat products and were commercially available as pure standards.

The following chapters is a brief description of the principles of methods for NA analysis described in the literature (Chapter 3.1) and a description of the developmental work performed to obtain an analytical method for the simultaneous determination of both VNA and NVNA in meat products (Chapter 3.2 and 3.3 as well as Paper I).

3.1 Methods described in the literature for NA analysis
3.1.1 Analysis of apparent total nitroso compounds

Since the discovery of NA in foods in the 1970s a wide range of methods have been published for the determination of these compounds in different food matrices. Basically three different approaches have been applied. Either the total amount of compounds that can release NO, selected number of VNA or NVNA have been determined.

The total amount of compounds that can release NO, also referred to as the Apparent Total Nitroso Compounds (ATNC) has in several studies been measured by nitrogen chemiluminescence detection. The extract is injected directly into a Thermal Energy Analyser (TEA). In the TEA detector the nitric oxide radicals are cleaved from the nitroso compounds by treating with hydrogen bromide in glacial acetic acid, the gas is then oxidized using ozone into activated nitrogen dioxide, which emits light in the near infrared region as it decays. The emitted light is then detected and quantified. More recently (Kuhnle *et al.*, 2007;Santarelli *et al.*, 2010;Pierre *et al.*, 2013;Bingham *et al.*, 2002) determined the ATNC levels in faces or ileal output of rodents and humans as possible biomarker for cancer initiation and/or promotion (Oostindjer *et al.* 2014, 583-596).

When determining the ATNC it is not possible to differentiate between NA and other compounds as nitrite, S- and C-nitroso compounds, ironnitrosyls and dinitrosyl iron complexes (Hogg, 2007) and nitramines (Hotchkiss *et al.*, 1978). Only NA are carcinogenic and not the latter. Thus determination of ATNC will most likely result in an overestimation of the NA level (Tricker and Kubacki, 1992). However if the ATNC increase, either in the meat or in vivo, this is most likely also connected to formation of some NA which are carcinogenic.

In order to evaluate the significance of the nitroso compounds found to occur in foods in regard to human health more specific methods were needed by which the individual nitroso compounds could be identified and quantified.

3.1.2 Analysis of volatile *N*-nitrosamines

The analysis of VNA has in several studies been undertaken by separation by GC and detection by the TEA detector both previously (Tricker *et al.*, 1991) and more recently (Drabik-Markiewicz *et al.*, 2009;Yurchenko and Mölder, 2007). Previous studies have also applied GC coupled with MS for the analysis of VNA, however at that time the TEA detector provided better sensitivity and specificity (Crews, 2010). The TEA detector however is seldom available because of its relatively high cost and because its use is not very versatile. It has become increasingly common to perform NA analysis by GC or LC with detection by tandem mass spectrometry (MS/MS). Nowadays tandem mass spectrometry is commonly available and can provide good sensitivity and specificity. GC-MS/MS methods have been applied for the analysis of VNA in meat products in a few publications (e.g. (Sen *et al.*, 1988)) whereas several publications are available using this technique for analysis of VNA in water (e.g. (Planas *et al.*, 2008)) and tobacco products (e.g. (Xiong *et al.*, 2010)).

Publications are also available describing the application of LC-MS/MS for the analysis of VNA in water (Topuz *et al.*, 2012;Ripollés *et al.*, 2011) rubber (Sung *et al.*, 2010) and cosmetics (Schothorst and Somers, 2005). Eerola *et al.* (1998) and Ripollés *et al.* (2011) have published LC-MS/MS for the analysis of VNA in dry sausages (NDMA, NPYR, NDEA and NPIP) and water, respectively. Both groups used an atmospheric pressure chemical ionization (APCI) interphase instead of the commonly applied electrospray ionization (ESI) interphase. Ripollés *et al.* found that greater sensitivity of the method could be obtained by using the APCI instead of the ESI for several VNA (Ripollés *et al.*, 2011). This has also has been described in other fields of analytical chemistry for analysis of low polarity molecules (e.g. (Japelt *et al.*, 2011)). A description of the principle difference between APCI and ESI is included in Chapter 3.2.2. Higher sensitivity and lower background noise when using acetonitrile as the organic solvent in the mobile phase compared to when using methanol has been reported for VNA analysis (Sung *et al.*, 2010;Ripollés *et al.*, 2011). The addition of low amounts of formic acid to the mobile phase improved the peak intensities by promoting the ionization of the analytes.

The extraction of VNA from meat or meat products has commonly been performed by extraction with dichloromethane followed by concentration by a Kuderna-Danish apparatus (Crews, 2010;Massey *et al.*, 1991;Sen *et al.*, 1979) in order to avoid loss of analytes during the concentration step. Though acceptable recoveries have been obtained for VNA using standard concentration equipment (Eerola *et al.*, 1998;Yurchenko and Mölder, 2007). Dichloromethane solvent is known as an effective and versatile extraction solvent. Dichloromethane is however an unwanted solvent in the Danish laboratories because of safety issues. Other solvents have been used for the extraction of VNA. Better extraction efficiencies of tobacco specific NA from snuff were e.g. obtained using ethyl acetate than when using dichloromethane (Jansson *et al.*, 2003). The same may however not be the case for a matrix like meat because of the high protein and fat content.

In order to remove fat from the extract several apply a liquid/liquid partitioning step by which the crude extract is washed with hexane or heptane which is then removed after phase separation (Yurchenko and Mölder, 2007;Ozel *et al.*, 2010;Eerola *et al.*, 1998). Solid phase extraction (SPE) has also been used for the clean-up of extract of prior to GC (Yurchenko and Mölder, 2007;Crews, 2010) and LC separation (Eerola *et al.*, 1998;Ripollés *et al.*, 2011) of NDMA, NDEA, NPYR, NPIP and NDBA.

3.1.3 Analysis of non-volatile *N*-nitrosamines

In the past the focus has primarily been directed towards the occurrence and concentration of VNA. This has largely been due to the absence of suitable selective and sensitive HPLC detectors for *N*-nitroso compounds but most likely also because of the documented carcinogenic effect of the VNA and an assumption of the NVNA being of no toxicological relevance. Some attention has been directed towards the NVNA that can be derivatised and analysed by GC-TEA (e.g. (Massey *et al.*, 1991)). When analysing NVNA an obvious choice of chromatographic method would be LC.

HPCL has also been coupled with the TEA detector for the analysis of both VNA and NVNA though this requires the use of cold traps to remove organic solvent vapours prior to detection and they are unworkable with aqueous mobile phases because of freeze-up and blockage of the cold traps (Eerola *et al.*, 1998). The methods which have been applied for the analysis of NVNA over the years have in general involved work and solvent extensive extraction and clean-up procedures followed by GC-TEA (e.g. (Pensabene and Fiddler, 1990;Massey *et al.*, 1991)) or LC-TEA analyses (e.g.(Sen *et al.*, 1993)). The NVNA have often been extracted with an acidified aqueous extract after which the analytes were transferred to an organic solvent by liquid/liquid extraction then cleaned up and analysed by the same procedure as for the analysis of VNA.

3.1.4 Simultaneous analysis of VNA and NVNA

To our knowledge only one example of a method including both VNA and NVNA is a method developed for the simultaneous determination of five volatile and non-volatile NA in biological fluids and cosmetic product. The method used was based on LC with Photodiode array detection (Wang *et al.*, 2006). The NVNA included in the study belonged either to the group of NA that are non-volatile and of low polarity or to the group of non-volatiles of high-polar and non-ionic NA. A method for simultaneous determination of NA in meat however needs to include the ionic NVNA since several of the NVNA relevant for meat fall into this category.

Since the VNA have been shown to be applicable with LC-MS/MS methods there should be a potential for the development of a common method which could allow for the determination of both VNA and NVNA by the same method.

3.2 Development of an analytical method for the determination of both VNA and NVNA in processed meat products

Chromatographic separation and detection of both VNA and NVNA using the same method is a challenge because of relatively large differences in the physical and chemical characteristics of these compounds. The VNA have traditionally been separated by gas chromatography whereas the NVNA are incompatible with GC unless they are derivatized into volatile compounds. As described in the previous Chapters analysis of VNA has however been found possible by LC-MS/MS systems. Such a system would also be the obvious choice for an analytical platform for NVNA analysis. Because some of the NA are non-ionic and relatively non-polar a system on which ionisation by both APCI and ESI was chosen, this system would make it possible to apply the ionisation technique that provide the greatest sensitivity and/or to be able to include more NA in the method by using both ionisation techniques. Choosing a system based on MS/MS detection will allow for good sensitivity and specificity.

The system available best fulfilling these requirements and needs were an Agilent 1200 Series HPLC (Agilent Technologies, Santa Clara, CA, US) coupled to an Agilent 6460 Series Triple Quadrupole (Agilent Technologies) with the possibility of both Jet Stream ESI and APCI though in separate runs. Collision gas on this system was nitrogen. The software installed for instrument control, data acquisition and for processing of the qualitative and quantitative data was MassHunter Workstation software (version B.01.04, Agilent Technologies).

As part of a method for simultaneous determination of VNA and NVNA in processed meat products a generic extraction procedure was also needed. There are no previous recordings of an extraction procedure allowing the extraction of both VNA and NVNA from meat products.

3.2.1 Chromatographic method

If both the VNA and the NVNA are to be separated on the same LC-column it is a requirement that it is compatible with aqueous mobile phase and high organic content in mobile phase because of wide range in polarity of the NA. The nitrosamino acids are strong organic acids, with pKa of 3.2 for NSAR and for carboxylic acid groups generally around pKa 4-5. Because of their small molecular mass and their polar group they are also highly polar and it was therefore expected that these NA would be difficult to retain on a standard reversed phase column. By lowering the pH of the mobile phase these acids will be protonated which in turn can result in better retention as well as less ionic interactions with free silanol groups or metal ions in the stationary phase resulting in improved peak shapes. The stationary phase should therefore also be compatible with eluents of low pH. Formic acid at a concentration of 0.1% is a common additive in many LC applications and it was, as mentioned earlier, found to increase the instrumental response for several NA.

For the development of the chromatographic method the following were tested: several analytical columns, methanol and acetonitrile as organic modifier of the mobile phase, different levels of formic acid in the mobile phase, different gradient programs and different injection volumes.

Several columns were tested for their ability to retain the highly polar NA and in general provide a good separation. The major challenge was found to retain and separate the early eluting NA as NHPRO, NSAR, NDMA, NMOR and NPRO. These NA eluted relatively quickly and were not

base-line separated on columns as the Genesis, PFP, Kinetex, Kinetex XB-C18, Acclaim PA2 column. The best separation of the NAs was obtained using a Zorbax eclipse Plus C8 and a Poroshell Phenyl-hexyl column (Agilent Technologies, Santa Clara, CA, US). These two columns performed similarly, though the latter provided generally more narrow peaks and the peaks of NHPRO and NSAR, occurring as both R- and S-form, did not split, as was the case on the other types of columns including the Zorbax eclipse Plus C8 column (Figure 3.2-1). The Poroshell Phenyl-Hexyl column was therefore chosen for the further method development.
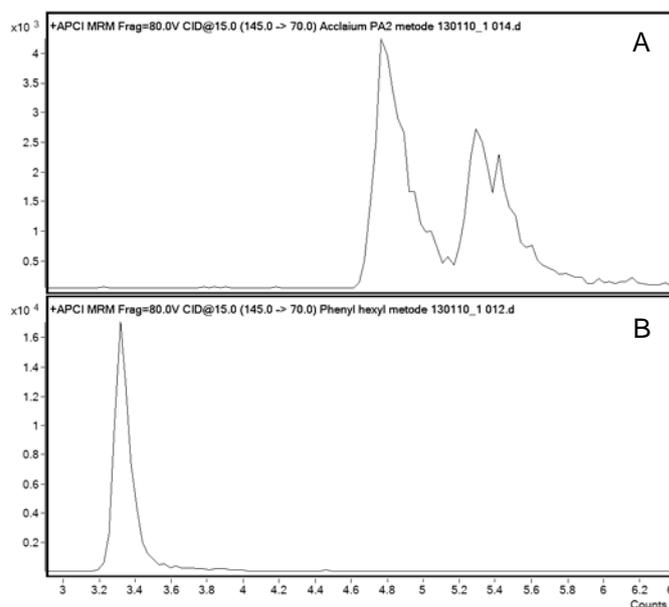
Figure 3.2-1: Observed peak shape for NPRO on the Acclaim Polar Advantage 2 column (A) and the Poroshell 120 Phenyl-Hexyl Column (B) using the same eluent programme.

Acetonitrile was initially used as the organic modifier of the mobile phase because this is the first choice in many applications because it results in less back pressure than e.g. methanol. As mentioned Sung *et al.* (2010) and Ripollés *et al.* (2011) reported that higher sensitivity was obtained when using acetonitrile instead of methanol when analysing VNA by LC(ESI)MSMS and LC(APCI)MS/MS, respectively. However low retention times for especially NHPRO and NSAR rendered us to test methanol as organic modifier and the retention times were found to increase slightly for the NA with carboxylic acid groups (e.g. NHPRO and NSAR) and that only minor differences in the instrumental responses were observed by using methanol or acetonitrile as organic modifiers.

In accordance with Sung *et al.* (2010) and Ripollés *et al.* (2011) we also found that the addition of formic acid to the mobile phase (0.01, 0.1 and 1%) increased the instrumental response for most of the NA and increased the retention of the acidic NA slightly. The responses increased with increasing content of formic acid, though the benefit of increasing the formic acid concentration from 0.1 to 1% was limited and considering the stability of the stationary phase we chose to elute with 0.1% formic acid.

A large amount of effort was put into optimising the gradient programme in order to have the very polar NHPRO eluting later than the solvent front, having the NA separated enough in order for them to be divided into segments with an appropriate number of compounds in each and

having the non-polar compounds eluting within a reasonable time. This was best achieved by an eluent programme starting with a high content of water (95%), with a relatively slow increase in the organic content in the first five minutes in order to have optimal separation of the very polar NA. After this point a relatively step increase in the organic content was needed in order to have the other NA eluting within acceptable time. The highly polar NA could be retained more if the initial content of the organic modifier was lowered (e.g. 0 or 2%). However the ionisation efficiency was reduced if the content of the organic modifier the compounds entered the interface with was reduced. It was found optimal to equilibrate the column for 5 minutes with 2% organic modifier between each run and then upon loading of the sample to change the content of the organic modifier to 5%. This allowed for almost maximum retention as well as increased ionization efficiency.
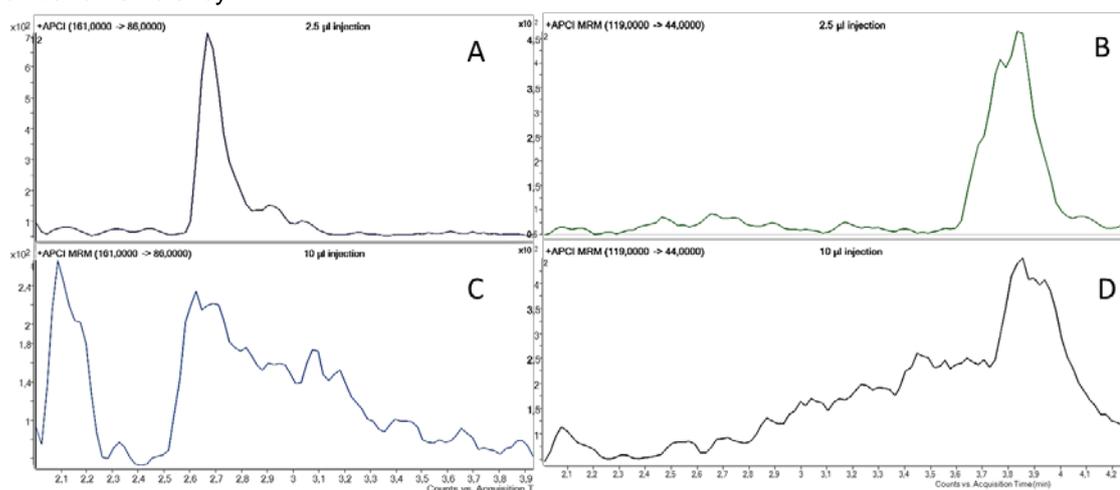
Figure 3.2-2: Example of how the chromatography of NHPRO (A and C) and NSAR (B and D) improved by optimizing the injection volume. With high injection volume of 10 µl (C and D) the column was overloaded resulting in poor chromatography and increased baseline whereas the peak shapes were significantly improved and the baseline reduced by instead injecting 2.5 µl (A and B).

The Poroshell Phenyl-hexyl column provided the best chromatography however one drawback of this column is that it is not a high capacity column and it can therefore relatively easily get overloaded especially when injecting "dirty" extracts. We tested different injection volumes of a standard solution mixed 1:1 with a sample extract to determine the injection volume providing the best signal to noise ratio. We found that by injecting 5 or 10 µl the chromatography of NHPRO, NSAR, NDMA and NPRO was compromised resulting in increased base line and very broad peaks (Figure 3.2-2 C and D) and therefore also low signal to noise ratios. If the injection volume was reduced to 2.5 µl the chromatography of the mentioned NA was improved (Figure 3.2-2 A and B), however the responses of the latter eluting NA were diminished. The optimal comprise between these two effects were therefore found to be with an injection volume of 3.5 µl.

The final eluent programme and other relevant chromatographic conditions are described in Paper I and following optimization of the mass spectrometry settings (Chapter 3.2.2) the chromatography of the target NA appeared as in Figure 3.2-3.
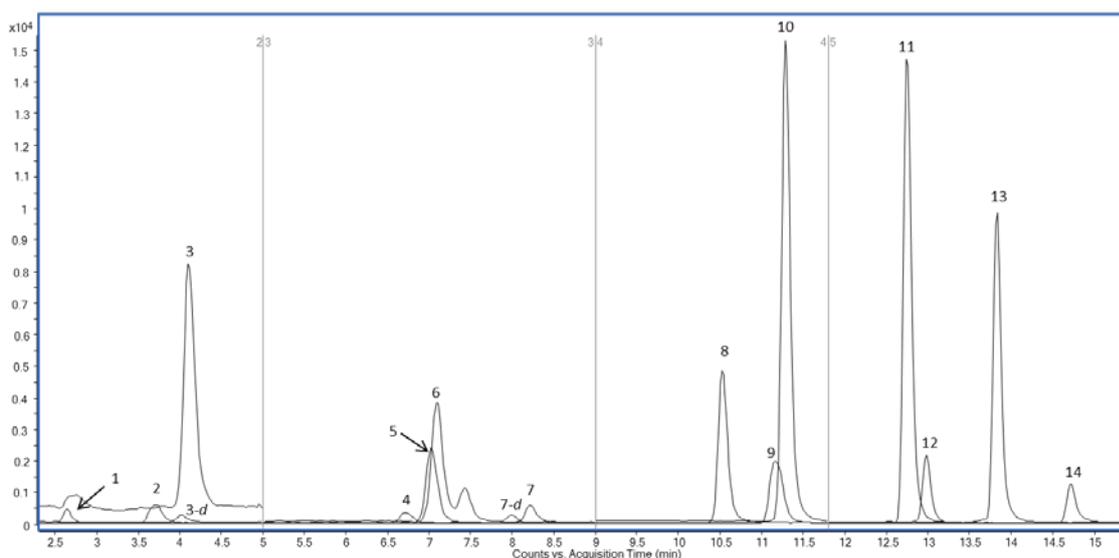
Figure 3.2-3: HPLC(APCI)MS/MS chromatogram for of standard mixture solution of 100 ng mL-1 of the VNA and NVNA ionized by APCI : 1) *N*-nitrosohydroxyproline (NHPRO), 2) *N*-nitrososarcosine (NSAR), 3) *N*-nitrosodimethylamine (NDMA), 3-d) NDMA-d6, 4) *N*-nitrosoproline (NPRO), 5) *N*-nitrosomorpholine (NMOR), 6) *N*-nitrosomethylethylamine (NMEA), 7) *N*-nitrosopyrrolidine (NPYR), 7-d) NPYR-d8, 8) *N*-nitrosodiethylamine (NDEA), 9) *N*-nitrosopipecolic acid (NPIC), 10) *N*-nitrosopiperidine (NPIP), 11) *N*-nitrosodi-n-propylamine (NDPA), 12) *N*-nitrosomethylaniline (NMA), 13) *N*-nitrosodibutylamine (NDBA) and 14) *N*-nitrosodibenzylamine (NDBzA).

3.2.2   Detection method

The VNA are generally relatively non-polar and do not contain ionisable groups whereas several of the NVNA are polar and have ionisable groups in form of a carboxylic acid groups.

MS/MS is in general the choice for quantitative analysis because so far it has been the technique which provides maximum sensitivity and selectivity. The TEA detector can also provide good sensitivity and selectivity for NA though it is an expensive instrument with limited application possibilities and is therefore an instrument which is rarely available.

The ESI interphase is the straight forward choice in LC-MS/MS and is for most applications a reliable choice. Though the not so commonly used APCI interface can in some cases provide better sensitivity and a wider application range. Both ESI and APCI are so called soft ionization techniques that operate at atmospheric pressure. In both ESI and APCI the mobile phase is forced through a capillary needle with a nebulizer gas and voltage gradient applied so that a spray is produced. When ionization is performed by ESI the analyte entering the ionization chamber needs to be charged in advance. In positive mode the positively charged analyte ions are repelled from the capillary needle and will therefore dominate in the formed droplets. The ions will be packed closely together with other positively charged compounds, e.g. matrix compounds. Organic acids as formic acid can enhance the analytical response probably by limiting the number of analyte ions binding to e.g. matrix compounds. With APCI it is not a requirement that the analyte is ionised in advance and application of this interface may therefore be an advantage in the development of a generic NA method. The APCI generally

also apply better for the ionisation of relatively small molecules than the ESI interface. This fact also indicates that the APCI may apply better for the VNA than ESI.

In the APCI interface the production of ions is initiated by the corona-needle discharge which produces a plasma region with a high density of charges, e.g. electrons and protons, which can interact with the molecules in the ionization chamber. The solvent molecules are high in abundance and are therefore also the primary charge carrier. Thus in APCI the analytes are ionised when they travel through the plasma region. At this point the solvent has been evaporated by the drying gas and heat and the ionisation processes therefore occur in the gaseous phase.

In both APCI and ESI the highest sensitivity is obtained if only one ion is produced in the ion source, e.g. the protonated parent ion $[M+H]^+$. Ripolles *et al.* (2011) found that when analysing water samples using APCI the dominant ion was the $[M+H]^+$ and that only negligible amounts of e.g. sodium adducts were formed. However by using ESI the formation of sodium adducts was dominant (Ripollés *et al.*, 2011).

Test of ionization method
Initially it was tested if the targeted NA were ionisable (producing an MS signal) by ESI and/or APCI both in negative and/or positive mode. For this task we used default settings for the ESI and APCI and had the NA entering the interphase with the same composition of the mobile phase and no chromatographic separation. Two pairs of parent and daughter ions (transitions) were selected whenever possible using both interfaces and then the optimal collision energy was determined for each transition. Hereafter the MS settings were optimized for each NA after chromatographic separation so that each NA entered the interphase with the relevant composition of the mobile phase. If the NA were ionisable in both interphases the MS setting were also optimised on both interphases.

Thirteen out of sixteen targeted NA were ionisable by APCI in positive mode. *N*-nitroso-di-phenylamine (NDPhA), NTCA, and NMTCA were only ionisable by ESI, and NTCA and NMTCA only in negative mode. NPRO, *N*-nitrosomethylaniline (NMA), NHPRO, NMOR, NDBA, NPIP and NPYR were ionisable by both APCI (positive) and ESI (positive). Most of the NA ionisable by both interfaces the ESI interphase gave the highest instrumental response when analysing pure standards. However, when analysing matrix matched calibration standards the differences in sensitivity was negligible, indicating less matrix induced ion suppression in the APCI interface and/or less formation of adducts. Ionisation by APCI was therefore chosen for all of the NA that were compatible with this interphase. We also found it as an advantage to be able to analyse the majority of the NA in the same analytical run. Only NTCA, NMTCA and NDPhA were analysed using ESI. We thereby had the possibility only to run sample analysis using APCI if NTCA, NMTCA and NDPhA were not found. However NTCA and NMTCA were found in almost all types of processed meat products (Paper II) and also in the sausages produced in connection with the formation and mitigation studies (Paper III).

It is generally assumed that APCI is less prone to matrix induced ion suppression than ESI because the ionization processes occurs in the gas phase having the analyte molecules separated more in space from the matrix molecules then if the ionisation occurred in the liquid phase as in ESI.

It was not possible to specify two product ions of the [M+H]$^+$ for NDMA, *N*-nitrosopipecolic acid (NPIC) and NMTCA. For NDMA, it was possible to determine two set of precursor and product ion, though one of the product ions was of low mass, making it unspecific. NDMA was therefore quantified by collecting the molecular ion in the first quadrupole, having the collision energy at null in the second, and collecting the molecular ion again in the third quadrupole. This approach allowed for analysis of NDMA in the MRM acquisition mode. Only one product ion could be determined for NMTCA by ESI in negative mode however no interfering peaks were observed in any of the matrices analysed and the specificity was therefore found satisfactory. No interfering peaks were observed for NPIC for any of the analysed matrices either. NPIC was not a target compound, but used for the monitoring of nitrosation processes occurring during the extraction, and it was therefore found acceptable only to have one set of precursor and product ion.

Details on choice of interphase, interphase settings and MS settings are presented in Paper I.

3.3   Development and optimization of extraction procedure

An extraction procedure should preferably be as simple as possible requiring only basic laboratory equipment and consume small amounts of organic solvents which preferably should not be dichloromethane or other solvents of high toxicity. The method should be suitable for the extraction of NA (polar, less polar, ionic (basic and acidic) and neutral) from processed meat with high levels of protein, fat in levels ranging from about 2% to about 50% and sodium chloride in levels up to about 2%, spices and a range of additives. Meat and even more so processed meat will be categorised as a so-called "dirty" matrix.

Acetonitrile has been proven a generic solvent for several purposes in our laboratory (Poulsen *et al.*, 2012;Rasmussen *et al.*, 2010) as well as by others (Mol *et al.*, 2008;Przybylski and Segard, 2009). A wide range of analytes can be extracted with acetonitrile and less matrix, including fat/lipids, are co-extracted compared to extraction with e.g. methanol, acetone (Mol *et al.*, 2008) or ethyl acetate (Lehotay *et al.*, 2010). Acetonitrile can also be used for protein precipitation. Acidification of the extraction solvent may further facilitate the extraction of acids and slightly basic compounds from different matrixes, among here meat (Mol *et al.*, 2008). Protonation of amino functionalities and acidic functionalities on the matrix material at low pH preventing both acidic and basic analytes to bind to the matrix was suggested as a reason for the observed effect. Acidified extraction solvent has also been shown to be necessary for an efficient extraction of the mycotoxin fumonisin, which is a compound with carboxylic acid groups, as the *N*-nitrosoamino acids (Sewram *et al.*, 2003). Acidic condition may however stimulate the nitrosation processes and therefore also artefact NA production. The artefact formation can either be hindered by the addition of e.g. propyl gallate (Eerola *et al.*, 1998) or the artefact production can be monitored by adding pipecolic acid (piperidine-2-carboxylic acid) prior to extraction and analyse for content of *N*-nitrosopipecolic acid (Tricker *et al.*, 1985).

When performing extraction with acetonitrile it has become common to include a partitioning step in order to lower the amount of water in the acetonitrile phase. However this partitioning step was found to reduce the recoveries of the very polar compounds (Mol *et al.*, 2008). Thus if a truly generic extraction method is needed the partitioning step should be omitted.

Based on the experience obtained in our laboratory and from the literature on generic extraction methods a simple acetonitrile extraction procedure was defined as the point of departure and it was found relevant to study the role of seven factors, presented in Table 3.3-1, on the extraction efficiencies of NA from samples of chicken sausage prepared without the addition of nitrite spiked at 50 µg kg^{-1}.

A 2-level fractional factorial design of 8 runs/experiments (resolution III) was setup to systematically evaluate the importance of 1) sample size (2.5 versus 5 gram), 2) the volume of extraction solvent (5 versus 15 ml), 3) adding water and performing a second extraction or not, 4) extracting with neutral or acetonitrile acidified with 1% formic acid, 5) extraction time (10 minutes versus 2 hours), 6) cleaning the extract by liquid/liquid extraction with heptane or not, and 7) diluting the final extract water (1:1 versus 1:4).

Using a fractional factorial design the importance of more factors can be tested without increasing the number of experiments. A fractional factorial design is an incomplete factorial design. The results of a fractional factorial design can either indicate that it is worthwhile to perform the complete factorial design or indicate that some factors can be evaluated as less significant and a more detailed study of the other factors can be performed. There are two reasons why a factorial design is to be preferred over the classical approach in which the response is investigated for each factor in turn while all other factors are held constant: firstly, the factorial experiment detects and estimates any interaction between the studied factors, which the classical approach cannot; secondly, if the factors are additive the factorial design needs fewer measurements than the classical approach in order to give the same precision. In general, if k factors are under study, the classical approach involves k times as many measurements as a factorial approach of the same precision.

Table 3.3-1: Design of in the 2-level fractional factorial experiment and the seven factors each at two levels under study for their effect on recovery of NA from spiked meat samples.

RunOrder	Sample size (2.5 or 5 gram)	Volume of extraction solvent (7.5 or 15 ml)	A second extraction after addition of 0 or 5 ml water	Acidified extraction solvent or neutral	Extraction time after acetonitrile addition	Wash with heptane (0 or 2.5 ml)	Dilution of final extract with water (1:0 or 1:1)
1	2.5	7.5	5	Acidic	10 min	0	1:1
2	2.5	7.5	5	Neutral	60 min	2.5	1:0
3	5	7.5	0	Acidic	10 min	2.5	1:0
4	5	15	5	Neutral	10 min	0	1:0
5	2.5	15	0	Acidic	60 min	0	1:0
6	5	7.5	0	Neutral	60 min	0	1:1
7	2.5	15	0	Neutral	10 min	2.5	1:1
8	5	15	5	Acidic	60 min	2.5	1:1

By analysing the extract obtained in accordance with the specifications in Table 3.3-1 it was found that: 1/2) The relation between sample and acetonitrile should be around 1:3 in order to have efficient protein precipitation resulting in clear extracts; 3) The extraction efficiency of the

highly polar NAs was not increased by introducing a second extraction after addition of water and resulted in cloudy extracts probably due to solubilisation of proteins; 4) Addition of formic acid to the extraction solvent increased the extraction efficiency especially for the *N*-nitrosamino acids (NHPRO, NSAR). The improved extraction efficiency by addition of formic acid to the extraction solvent is in accordance with results obtained by Mol *et al.* (2008). Furthermore, 5) an extraction time of 10 min. was as efficient as 60 min. and 6) washing with heptane had no significant effect on the signal intensities. 7) Dilution of the final extracts 1:1 with water significantly improved the peak shapes of the highly polar NAs, i.e. NHPRO, NSAR and NPRO, which eluted very early on several of the tested columns.

Based on the results of this first setup four supplementary setups were performed studying in more detail the effect of acidifying the acetonitrile (factor 4), performing a second extraction after water addition (factor 3) and the effect of removing the ACN by evaporation leaving an aqueous residue. This was done with a factorial design experiment similar to the experiment presented above, though only including two factors in each setup.

From these studies it was found that the observed improved chromatography and higher response of performing a second extraction after addition of water was primarily due to improved chromatography caused by a lower proportion of organic solvent in the extract. It was therefore tested if the acetonitrile could be removed from the crude extract by evaporation leaving a small aqueous residue, without losing the VNA. No significant loss due to the vaporization step was observed, however the solubility of the most non-polar NAs (as NDPhA) were perhaps compromised. The recoveries remained in general at an acceptable level, i.e. within the range of 70 to 120%, and the chromatography of the early eluting NA was improved as exemplified in Figure 3.3-1. When removing the organic phase the solubility of several constituents are decreased including fats. A freezing out step prior to the evaporation step was therefore included in the extraction procedure in order to reduce the risk of analytes being precipitated together with fats and other constituents of low solubility during the evaporation step. Freezing of the crude acetonitrile extract resulted in precipitation of some low soluble constituents, as e.g. lipid. However precipitates were still observed when the acetonitrile phase was evaporated. When the aqueous residue was filtrated (using a 0.45 µm filter vial) a clear extract was obtained.

Throughout the method development NPIC was included as a target NA. Pipecolic acid (PIC) was added to the spiked test samples and this did not result in the occurrence of NPIC in the analysed extracts. The absence of NPIC indicates that no significant NA production had occurred during the extraction.

Based on these results the procedure presented in Figure 3.3-2 was defined. Because only basic analytical equipment, single use extraction tubes, few steps and small amount of organic solvent were used the developed extraction method is evaluated as fast, simple, coast effective and environmentally sound. This extraction procedure was employed for the extraction of both VNA and NVNA from spiked samples for the validation of the method (see Chapter 3.3.2)
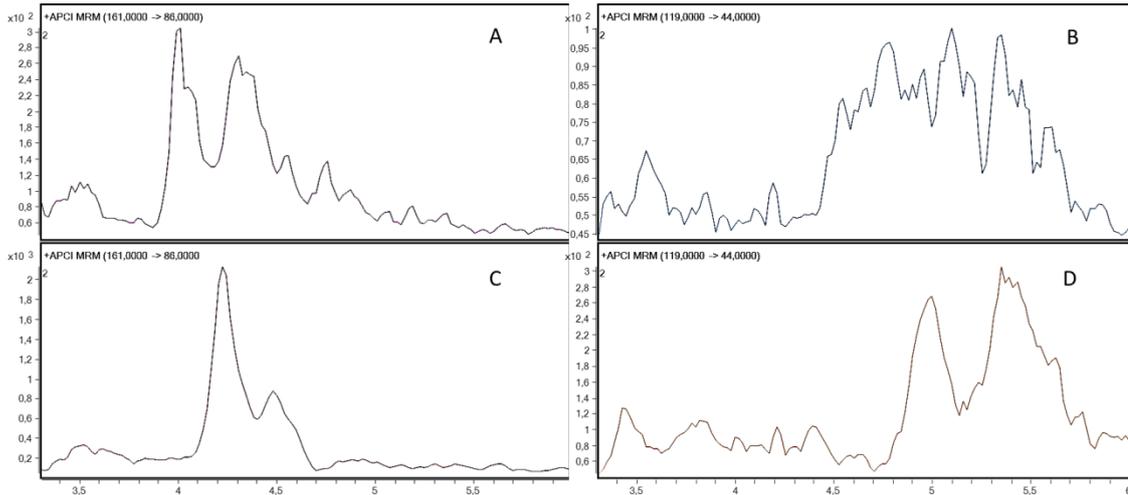
Figure 3.3-1: Example of how the chromatography of NHPRO (A and C) and NSAR (B and D) appeared for a standard solution mixed 1:1 with a meat extract before removal of the acetonitrile from the crude extract (B and D) and after removal of the acetonitrile by evaporation under a stream of nitrogen until 1 ml of primarily aqueous extract was left (A and C).
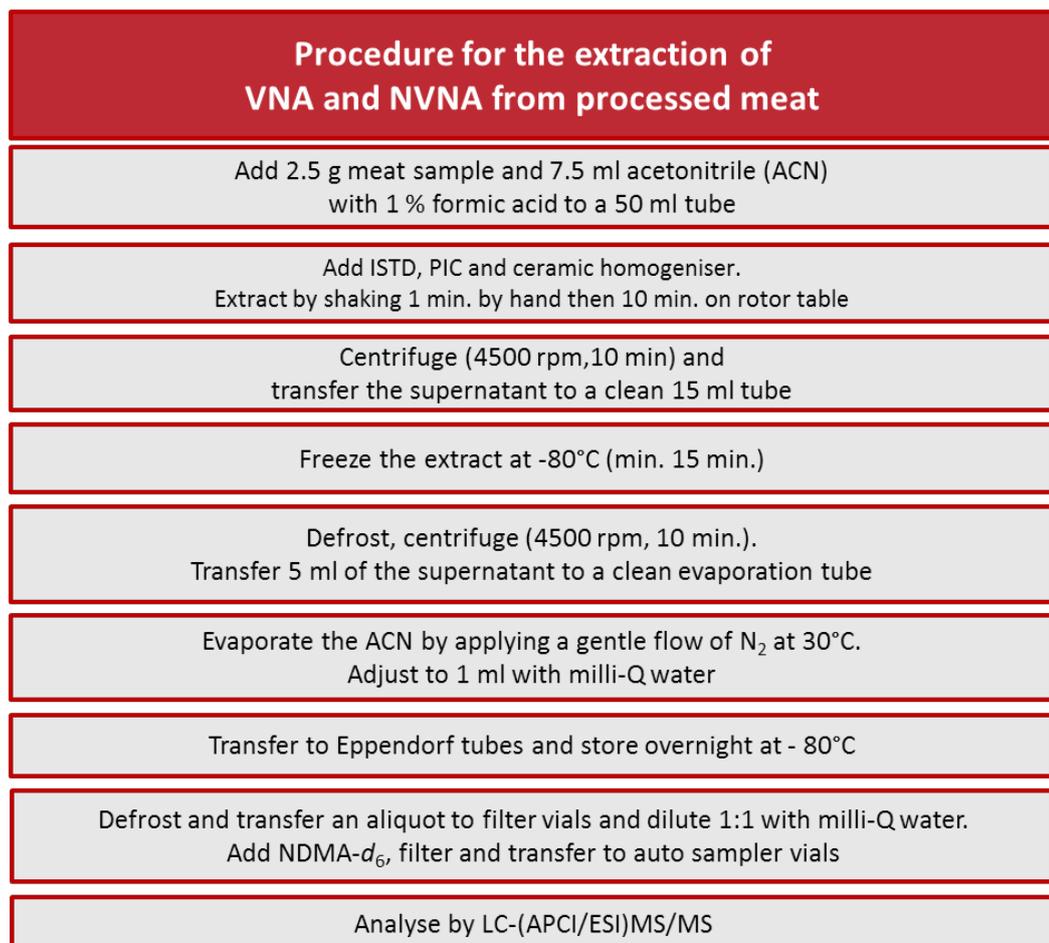
Figure 3.3-2: Outline of the generic extraction procedure developed for the extraction of VNA and NVNA from processed meat products.

3.3.1 Clean-up of crude extract

The occurrence of co-extracted matrix can lead to general bad performance of a method. Matrix can result in poor ionisation efficiency because a large amount of matrix ions are introduced into the source and this may then result in poor sensitivity. Matrix can also result in poor chromatography because the matrix competes with the analytes for the available binding sites on the stationary phase, resulting in peak broadening. Matrix may also precipitate and accumulate in the ion source and in the first part of the MS leading to increased need for maintenance in order to maintain good performance. Some suppression of the analytical responses as well as matrix peaks was observed when analysing the final aqueous extracts. We therefore considered and tested if clean-up by Solid Phase Extraction (SPE) could be included in addition to the already included freezing out step.

SPE is a common choice of method for clean-up of extracts. By this method the sample extract is loaded on a solid phase in a tube. First the impurities or the matrix are wash off with an appropriate solvent which does not disrupt the binding of the analyte(s) to the solid phase. Then the analytes are eluted off with a solvent that is just strong enough to disrupt the binding of the analytes but not the bindings of matrix that bind stronger to the solid phase. There is a large amount of different pre-packed solid phases commercially available.

Despite the large number of different SPE cartridges to choose from it can be a challenge to find a SPE cartridge which will separate your analytes from the matrix, especially if the method includes several analytes with different physical and chemical characteristics. Several attempts were made to find an SPE product that was able to retain the highly polar NA, as NHPRO, NSAR and NDMA, but still able to release the less polar NA as NDBzA and NDBA by eluting with an appropriate amount of organic solvent. The experiences made with SPE clean-up is described in the following.

Clean-up by SPE

First three types, i.e. Oasis HLB (Waters, Milford, US), HybridSPE-Phospholipid and Bond Elute Plexa SPE (Agilent Technologies, Santa Clara, CA, US) were tested for their ability to retain the target NA dissolved in water. None of these cartridges were found applicable. The Oasis HLB and the Bond Elute Plexa SPE did not retain the highly polar NA, NHPRO and NDMA, and bound the relatively apolar NA, *N*-nitrosodi-n-propylamine (NDPA), NDBA and NDBzA to strongly to the cartridge material so that they could not be eluated even with 100% acetonitrile. The HybridSPE-Phospholipid is developed to bind phospholipids and therefore act more as a selective filter. This cartridge allowed several of the NA to pass through the filter as intended whereas NHPRO, NSAR, NPRO and NPIP needed to be washed off with acetonitrile and it was not possible to wash NDPA, NDBA and NDBzA of the cartridge even with 100% acetonitrile.

As a last attempt an SPE cartridge that has a more polar solid phase, than those tested previously, was tested. By choosing a more polar column the highly polar NA were expected to bind stronger and the more non-polar NA to bind less to the solid phase. For this purpose we tested Bond elute ENV SPE (Agilent Technologies, Santa Clara, CA, US).

As can be seen from the results presented in Table 3.3-2 this SPE cartridge could not retain the highly polar NA either and the non-polar NA could not be eluted. NHPRO and NSAR were mainly recovered in the wash (milli-Q water). The more non-polar NA in the right side of the table could again not be eluted. Only a fraction of the loaded amount was recovered in the

eluates for these non-polar NA (2nd elution was with 100% acetonitrile). The results indicate that only about five of the target NA could be applicable with SPE clean-up.

Table 3.3-2: Analytical responses obtained and calculated recoveries for a matrix matched standard solution of the target NA for the un-cleaned solution and for the solution after clean-up on Bond elute ENV SPE (1st and 2nd elute). The cells shaded in green indicate that the analytical response for the eluates accounted for 75% or more of the total recovery (sum of run-through, wash, 1st and 2nd elute) (Carpin, 2013).

		Nitrosamines													
		NHPRO	NSAR	NDMA-d6	NDMA	NPRO	NMOR	NMEA	NPYR-d8	NPYR	NDEA	NPIC	NPIP	NDPA	NDBA	NDBzA
Non-matrix matched	Uncleaned	9539	40237	6736	747603	114503	143170	386038	5772	31952	70947	91343	596356	603947	571614	1226588
	Run-through	1575	3036	69	18818	1908	556	975	19	111	0	61	0	0	3351	326
	Wash	4324	14241	783	157668	14141	6750	12744	139	1114	147	478	0	0	7276	171
	1st Elute	2617	16338	761	159568	36847	23315	29442	396	5380	793	8829	7828	0	3318	104
	2nd Elute	543	7982	766	166947	56447	88658	143797	1637	20044	18861	66294	258801	61789	27765	73872
	Recovery in Elutes	33	60	23	44	81	78	45	35	80	28	82	45	10	5	6
	Total Recovery	95	103	35	67	95	83	48	38	83	28	83	45	10	7	6
Matrix matched	Uncleaned	27791	28568	5693	645345	110126	132969	355464	5629	32409	66452	61768	541848	612004	651035	1249523
	Run-through	9990	4350	226	46850	10532	3855	8376	64	804	163	798	583	0	2693	0
	Wash	32203	15847	1312	280681	50702	27593	55631	486	6003	1664	6431	5235	0	3525	0
	1st Elute	11010	13185	1209	233368	66037	49820	71536	1044	11323	2158	15602	15672	0	2890	59
	2nd Elute	368	2425	281	69365	22961	58214	117035	1118	14266	24909	50727	298805	117054	52529	97342
	Recovery in Elutes	41	55	26	47	81	81	53	38	79	41	107	58	19	9	8
	Total Recovery	193	125	53	98	136	105	71	48	100	43	119	59	19	9	8

Based on the result obtained from testing the four different SPE products we chose to continue without the use of SPE clean-up because the aim was to develop a generic method allowing for the determination of as many NA as possible.

In order to have satisfactory recovery of the analytes and still have some of the matrix removed it would be necessary to clean aliquots of the same extract by different methods resulting in several fractions for each sample increasing analysis time considerably. In order to keep the amount of matrix entering the ion source at a minimum, the LC flow was not directed to the MS until shortly before the first eluting analyte.

3.3.2 Validation of the developed method

To demonstrate the specificity, precision, accuracy and lower limit of detection (LOD) the developed method was validated, using spiked samples and determining the recoveries, Relative Standard Deviation for repeatability (RSD_r) and LOD for each of the 16 NAs (incl. NPIC). Because no standard material was available the validation was performed on three representative processed meat products (kassler, bacon and salami) spiked with 1, 3 or 30 µg kg^{-1} of each of the targeted NA. Details of the setup and the results of the validation are presented in Paper I.

The developed method had satisfactory recovery and precision (RSD ≤23%) for at least one of the tested spike levels for all three matrices (kassler, bacon, salami), except for NDPhA and NDBzA. Low precision was observed for NDBzA in salami (≥30%), however clear peaks were observed at all three spike levels. The low precision may be caused by interfering matrix. The instrumental response of NDPhA also varied greatly, either because of large variations in the extraction efficiencies but more likely because of incompatibility with the developed MS method. NDPhA is thermally instable (Zhao *et al.*, 2006), and the low and unstable responses could be the result of degradation caused by the relatively high temperature in the source (275-300°C). The recovery of NHPRO was found to decrease with increasing spike levels and therefore not be validated. Though, the recoveries from spiked quality control samples analysed since have been found more acceptable.

The LODs obtained with the described method were generally <1 µg kg^{-1}, though with some exceptions. LOD for NDBA and NTCA in kassler were 1.7 and 2.1 µg kg^{-1}, respectively; LOD for NDMA and NTCA in bacon were 3.3 and 4.9 µg kg^{-1}, respectively; and LOD for NPYR, NTCA and NMTCA in salami were 17.1, 18.4 and 9.4 µg kg^{-1}, respectively. Relatively high LODs were obtained for NTCA and NMTCA, because the "natural" content in the unspiked salami samples were relatively high, i.e. 69 and 18 µg kg^{-1}, respectively. The high LOD for NPYR salami resulted from a too high variation of the results (relative standard deviation of 38%) though the recoveries were around 100% even at a spike level of 1 µg kg^{-1}. The validation results are presented in detail in Paper I. Thus it was not possible to obtain equally low LODs for the individual analytes for all three types of matrices. Furthermore, the validation show that results obtained for one matrix is not always possible to extrapolate to all other e.g. processed meat matrices. Whether this is specific for the present method or is also true for some of the already published methods for analysis of NA is difficult to evaluate because these are generally validated on only one matrix and in some cases only at one relatively high spike level (e.g. 5-10 µg kg^{-1}). The LODs obtained with the present method are though comparable with the LODs obtained by others (Massey *et al.*, 1991;Drabik-Markiewicz *et al.*, 2009) even though it offers a wider scope, are simpler and do not require the use of a TEA detector. If however the extracts were more extensively cleaned before analysis the differences between the validation results obtained for the different matrices may have been smaller. The occurrence of co-extracted matrix in different levels or of different nature may affect the method performance resulting in the observed differences between the matrices. The matrix effects observed were on the other hand generally minor for the different analyte/matrix combinations. The matrix effects were evaluated by comparing the instrumental responses obtained by analysing an aqueous standard solution with that obtained by analysing the same standard solution matched 1:1 with meat extract. An exception was e.g. the analytical response for NHPRO which were significantly enhanced by kassler, bacon and salami co-extracts. Slight suppression or enhancement of the signals for NSAR, NDBzA, NMA and NDBA were also observed and are described in more detail in Paper I.

3.3.3 Evaluation of the developed method

The aim was to develop a generic method which would allow for the simultaneous analysis of several VNA as well as several NVNA in processed meat. No such method has to our knowledge previously been described in the literature. The point of departure for the method development was therefore an analytical platform consisting of a LC-MS/MS system and a generic extraction procedure with as little clean-up as possible in order not to narrow the analytical scope of the method. Having no or only limited clean-up may however result in the occurrence of co-extracted matrix in the extracts for which the levels and nature may vary depending on the type of processed meat product. If the levels and nature of the co-extracts vary then the method performance can also vary. This was most likely the reason for the observed differences in the LOQs that could be obtained for the three representative meat products used for the validation. However if more extensive clean-up were to be included it would most likely result in some NA being lost during the procedure or that the crude extracts needed to be divided into several fractions each cleaned by different procedures. This strategy would result in the need for several analytical runs for each sample.

Had the primary focus been analysis of the traditional VNA, i.e. NDMA, NPYR, NPIP and NDEA because of the documented carcinogenicity of these NA, several other methods which have already been developed and published would have been a more obvious point of departure. Some of these more targeted methods, include more extensive clean-up of the extracts and analysis by GC-MS(/MS), might provide lower detection limits for these particular VNA than our new method.

Considering the wide scope, the simplicity and that only basic laboratory equipment is required we find that the method developed in the present work contribute significantly to the wide range of already developed methods for NA analysis.

4. The occurrence of NA in meat products on the Danish market

A survey of the occurrence of NA in processed meat products on the Danish market was undertaken as described in Paper II. The aim of the survey was to obtain data on the occurrence of both VNA and NVNA in meat products typically preserved with nitrite. No such survey has previously been undertaken for meat products on the Danish market.

As mentioned in the introduction Denmark has National Provisions regarding the regulations for the use of nitrite for meat preservation which in general allow the addition of 60 mg kg^{-1} nitrite to most processed meat products. All products available on the Danish market should be produced in accordance with the Danish provisions even though they have been produced in another EU Member state. According to the common EU regulation it is in general allowed to add 150 mg kg^{-1} to processed meat products.

Assuming an association between the amount of ingoing nitrite and the amount of NA formed then the levels of NA in the products on the Danish market are expected to be lower than the levels reported by others (Chapter 2.4). We therefore analysed processed meat products from the Danish market (N=70), as well as from the Belgian market (N=20) for comparison, for their content of eight VNA and five NVNA. The results of the survey will be described here in brief but are presented in detail in Table 1 Paper II.

As expected the levels of VNA in the Danish products were low with a mean of ≤0.8µg kg^{-1} for the individual VNA, highest for NPYR. In contrast the NVNA occurred in considerably higher levels resulting in mean levels of ≤118 µg kg^{-1} for the individual NVNA, highest for NTCA. Levels of up to 2000 and 4000 µg kg^{-1} of NTCA occurred in the Danish sample of ham and a Belgian sample of salami, respectively. Slightly higher mean levels were indicated for the Belgian products (i.e. VNA ≤1.5µg kg^{-1} and NVNA ≤270 µg kg^{-1}). However the differences in the mean levels were not found to be statistically significant (Student t-test). NHPRO was detected in about 60% of the Danish samples. The quantification of NHPRO was however not always reliable, probably due to interfering matrix, and the specific results are therefore not reported. NDBA was not detected in any samples and NDPA was detected in a few samples at very low levels (≤0.2 µg kg^{-1}) i.e. below LOD. The results for NDPA are not reported because the low levels result in uncertainty in the identification and quantification. The levels of VNA found in the present survey are comparable with those reported in previous and recent studies (Chapter 2.4), however the frequency in which they are found may be lower and thereby also the mean contents.

There are no Danish maximum limits for NA. The US-EPA has set a maximum limit of 10 µg kg^{-1} for the sum of VNA for cured meat products (Crews, 2010). Only three samples analysed in the present survey contained more than 10 µg kg^{-1} of VNA in total. One Danish sample of smoked filet contained 15 µg kg^{-1}. Two Belgian samples of chorizo contained 11.4 and 10.2 µg kg^{-1} of VNA.

Analysis of the residual nitrite and nitrate was also performed (Paper II). No relationship between residual amounts of nitrite or nitrate and the NA levels could be demonstrated. This

was expected because lack of this relationship has been observed by others (EFSA, European Food Safety Authority, 2003) and it has been the underlying reason for the legislation being altered to include maximum levels for ingoing amounts and not only maximum levels for residual amounts.

Thus the survey showed that the majority of processed meat products available on the Danish market contain NVNA. NTCA was found to be almost ubiquitous in processed meat products. Because NTCA were found in as high amounts as 4000 µg kg^{-1} it may be speculated that this NA account for a significant part of the ATNC. As mentioned ATNC in bacon was found at levels up to 7000 µg kg^{-1} (expressed in µg N-NO)(Massey *et al.*, 1991). The survey also showed that, even though less nitrite is allowed to be added to products intended for the Danish market, than for the common European market, VNA still occur in some samples. There were no obvious differences in the levels found in the samples from the Danish and the Belgian market. It is expected that higher levels of nitrite generally have been added to the products from the Belgian market than from the Danish market but no significant differences in the NA levels could be demonstrated. As described in Chapter 2.5 a wide range of factors besides nitrite can affect the levels of NA formed and these may overshadow any possible association between the levels of NA and nitrite added. Controlled studies, in which all other factors are held constant and only the added nitrite levels vary, are probably needed in order to elucidate any possible association between ingoing amount of nitrite and the levels of NA formed. Studies on minced meat and on cooked pork sausages were therefore undertaken studying the role of nitrite and other factors in NA formation. These studies will be the subject of the following chapters.

5. Formation and mitigation of *N*-nitrosamines in meatballs – preliminary studies

In order to elucidate any possible association between ingoing amount of nitrite and the level of NA formed in meat products as well as the role of often used additives, in the formation and mitigation of NA a range of studies were performed. Preliminary trials were performed on minced meat.

Several types of setups were tested in order to find a meat model which was as simple as possible however still allowing for the production of NA. The most promising and workable of these models involved preparation of meatballs made from minced pork loin. The meat balls were wrapped in sterile gauze used for hanging during storage (10°C). This meat model was employed in four preliminary trials carried out to study the effect of ingoing amount of nitrite, erythorbic acid, sodium chloride, sodium polyphosphate, dextrose, liquid smoke and storage time on the formation of NA. These preliminary studies have not been described in any of the papers or manuscripts but will be described briefly in the following chapters (Chapter 5.1-5.4)

5.1 Role of ingoing amount of nitrite

In this trial the minced meat was only mixed with sodium chloride and different levels of nitrite (150, 300 and 600 mg kg^{-1} meat). The gauze wrapped meat balls were stored at 10°C for 2 or 13 days. In this setup only four NVNA (NHPRO, NPRO, NTCA and NMTCA) were dectecable at all nitrite levels and one VNA (NPIP) was detectable at quantifiable levels at the highest nitrite level (600 mg kg^{-1}). The results are presented in Appendix A. An association between added amount of nitrite (150-600 mg kg^{-1}) and the levels of NHPRO and NPRO were demonstrated. However the levels of NTCA and NMTCA seemed to be independent of the nitrite concentration since approximately the same levels of these two NVNA were produced regardless of the amount of nitrite added. This may indicate that the formation of these two NVNA is limited by another factor than nitrite, e.g. the availability of the relevant precursor.

Similar levels of NA were found in the meatballs stored for 2 days and 13 days, indicating that the NA are formed relatively fast and that no further production occur during storage for up to 13 days. An exception was though the levels of NMTCA which decreased with increased storage time. NDMA were also detected in some of the meatballs but could not be reliably quantified because of the low levels (<0.5 µg kg^{-1}). However the results indicated that the NDMA levels decreased with increased storage time and that the levels slightly increased with increasing levels of nitrite (for the meat balls stored for 2 days but not for 13 days).

The meatballs produced in this setup were also analysed for nitrite and nitrate. As shown in Figure 5.1-1 the levels of nitrite had decreased to approximately one third of the added level after two days of storage at 10°C and decreased further by further storage. The levels of nitrate increased slightly due to the addition of nitrite but could far from account for the large decrease in nitrite. If ascorbate/erythorbic acid had been added to the meatballs the nitrite levels would most likely have been reduced even faster because its presence favours the formation of NO. Generally it is said that less than 50% of the added nitrite can be detected in a nitrite analysis the first day after production and it may be reduced to less than 10% after a week (Gry *et al.*, 1983). The results also demonstrate why the residual level of nitrite cannot be used as an

indicator of the amount of nitrite added, especially not for products that are stored for more than a few days after production.
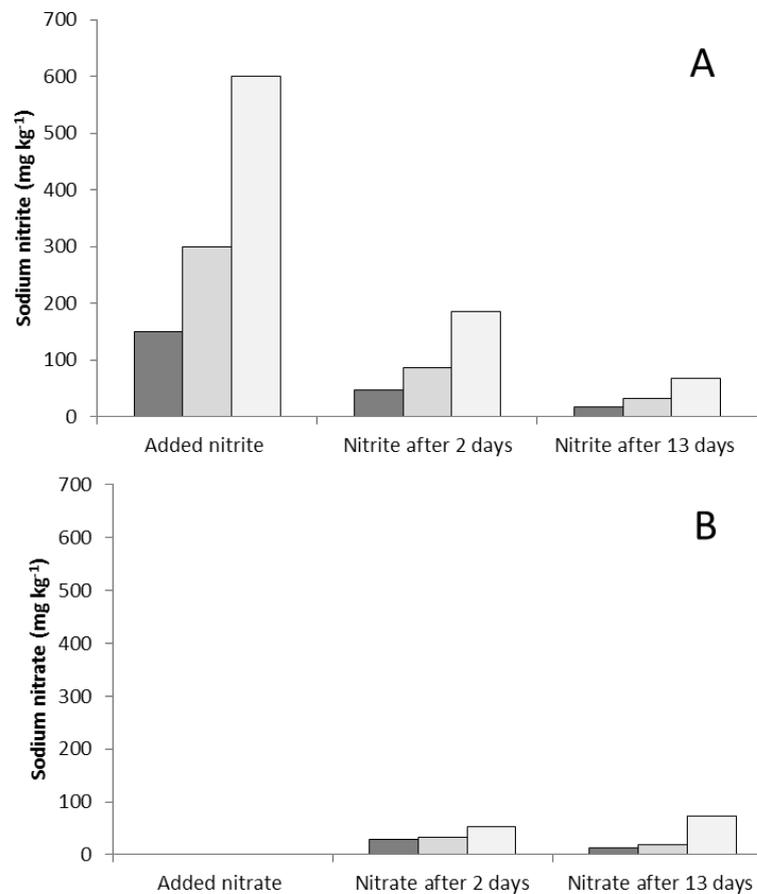
Figure 5.1-1: Nitrite (A) and nitrate (B) levels (mg kg^{-1}) in meatballs measured 2 and 13 days after addition of 150, 300 or 600 mg kg^{-1} nitrite (day 0). Modified from (Träger, 2013).

5.2 Screening of the importance of seven factors (sodium chloride, sodium erythorbic acid, sodium polyphosphate, dextrose, smoke and storage time)

The meatball model was also employed for preliminary trial on the role of seven factors, evaluated as highly relevant for the production of most meat products, in the formation and mitigation of NA. Liquid smoke was chosen over an actual smoking process because it was easier to handle and the role of heat treatment during the smoking process was eliminated. Development in the NA formation with time was included as on additional factor.

A 2-level 1/8 fraction factorial design (resolution IV) was setup which defined 16 combinations of the seven factors, i.e. 16 meatballs with each their combination of the seven factors in high or low level were prepared, stored for 1 or 5 days and analysed. The high and the low levels chosen for each factors have been evaluated as relevant for industrial production and are presented in Table 5.2-1. For nitrite however the chosen high level of 300 mg kg^{-1} is only relevant for some very specific regional products; though we wished to use a relatively high level in order to increase the chance of NA formation. A table of the experimental design as well

as Main Effects Plots and Interaction Plots generated with Minitab are to be found in Appendix B.

Quantifiable levels of NHPRO, NPRO, NTCA and NMTCA were found in the meatballs. Also low levels of NDMA (≤ 1.2 µg kg^{-1}) were detected in half of the meatballs added 300 mg kg^{-1} nitrite. For the four NVNA the high level of nitrite (300 mg kg^{-1}) resulted in significantly higher concentrations at 95% confidence level compared with the low level of nitrite (60 mg kg^{-1}) which is indicated by an asterisk on the Main Effects Plots in Appendix B. The plots were not generated for NDMA because it was only detected in some meatballs. There was however no indication of NDMA occurring in the meat balls of a specific composition.

Besides the significant increase in the NVNA induced by the higher nitrite level no other statistically significant effects were demonstrated. Some effects were though indicated. Sodium chloride and erythorbic acid seemed to reduce the levels of NHPRO and NPRO, whereas it increased the levels of NTCA and had no effect on the NMTCA levels. Sodium tripolyphosphate seemed to reduce the levels of NHPRO, NPRO and NTCA but increase the levels of NMTCA. The liquid smoke resulted in an increase in the level of NTCA as would be expected from the literature. Otherwise only minor effects were observed as indicated by overlapping or only slightly separated lines in the Interaction Plots (Appendix B).

Table 5.2-1: Factors chosen to be studied in a fractional factorial design setup for their role in the formation and mitigation of NA in meat balls prepared from minced pork loin.

Factor	Low level	High level
Sodium nitrite	60 mg kg^{-1}	300 mg kg^{-1}
Sodium chloride	1 %	7%
Erythorbic acid	0 mg kg^{-1}	500 mg kg^{-1}
Sodium tripolyphosphate	0 mg kg^{-1}	5000 mg kg^{-1}
Dextrose	0 mg kg^{-1}	5000 mg kg^{-1}
Liquid smoke	0 mg kg^{-1}	2000 mg kg^{-1}
Storage time	1 day	5 days

Based on these results it was chosen to perform further studies on the effects of nitrite, erythorbic acid and sodium chloride.

5.3 Role of nitrite, sodium chloride and sodium erythorbic acid

Based on the results from the seven factor study it was chosen to further study the effects of nitrite, erythorbic acid and sodium chloride in a trial setup providing higher resolution. The two former factors were chosen because these induced the greatest differences in the NA levels. Sodium chloride was chosen because it induced a relatively large decrease in the levels of NHPRO and because some interaction with erythorbic acid was indicated for all four NVNA as indicated by non-parallel lines in the Interaction plots (Appendix B). For NTCA and NMTCA it was indicated that the interaction could lead to higher NA levels if the sodium chloride level was high and the erythorbic acid was absent.

A 2-level full factorial design in duplicate requiring the preparation of 16 meatballs prepared with 8 different combinations of high and low levels of the three factors was setup. The three factors and the chosen high and low levels are presented in Table 5.3-1. A table of the experimental

design as well as Main Effects Plots and Interaction Plots generated by Minitab appear from Appendix C.

Table 5.3-1: Levels of the three factors under study for their effect on the NA formation in minced pork meatballs.

Factor	Low level	High level
Sodium nitrite	60 mg kg^{-1}	300 mg kg^{-1}
Sodium chloride	1%	7%
Erythorbic acid	0 mg kg^{-1}	500 mg kg^{-1}

Also in this 3 factor setup it was only the levels of NHPRO, NPRO, NTCA and NMTCA that were quantifiable. Low levels of NDMA were detected in a few meatballs but at levels (≤0.5 µg kg^{-1}) too low to quantify. Nitrite was the factor which affected the NA levels most, as indicated by a steeper slope in the "Main effect plots" and well separated lines in the "Interaction Plots" representing the high and low nitrite level. The increase induced by the high nitrite level was statistically significant for all four NVNA. Erythorbic acid induced a significant reduction in the levels of NHPRO, NPRO and NMTCA but had virtually no effect on the level of NTCA. No significant major effects were observed for sodium chloride.

An interaction between nitrite and erythorbic acid was found significant for NHPRO, NPRO and NMTCA. This interaction indicated that erythorbic acid had little or no effect on the NA levels if combined with the low level of nitrite, whereas it inhibited the NA formation if combined with the high nitrite level. Significant interaction between nitrite and sodium chloride were also indicated for NTCA and NMTCA. The high sodium chloride content seemed to reduce the level of NTCA if in combination with the high nitrite level whereas it had no effect if combined with the low nitrite level. For NMTCA it seemed that sodium chloride resulted in increased level if combined with the low nitrite level but had virtually no effect if combined with the high nitrite level.

This study showed again that nitrite stimulates the formation of NA whereas erythorbic acid prevents the formation except for NTCA. Interaction between nitrite and sodium chloride was again indicated for NTCA and NMTCA which at least for NMTCA results in increased formation at the low nitrite level of 60 mg kg^{-1}, which is the maximum amount allowed to be added to most processed meat products on the Danish market. A curved relationship between the amount of sodium chloride and the levels of NA formed has been indicated by others (Theiler *et al.*, 1981). We therefore chose to study the effect of a range of sodium chloride on the NA formation.

5.4   Role of sodium chloride

It was in 5.3 indicated that sodium chloride may both inhibit and promote the formation of NA. We therefore chose to specifically study the effect of sodium chloride. An argument for studying the role of sodium chloride in more detail is also that it is a priority of the Danish Veterinary and Food Administration to lower the intake of sodium chloride and therefore also the content of sodium chloride in processed meat products. It is therefore relevant to know whether a reduction in the content of sodium chloride may lead to an increased risk of NA formation.

Table 5.4-1: Level of NA produced in meatballs prepared with different levels of sodium chloride (0-14%). The results are average of duplicate determinations except for the results shaded in grey which are based on single determination.

	Sodium chloride content (%)									
	0	1	2	3	4	5	6	7	10	14
NHPRO	4.1	4.0	2.9	1.9	2.5	<1.5	<1.5	<1.5	<1.5	<1.5
NPRO	41	40	36	38	32	26	20	22	22	17
NTCA	24	29	37	31	45	31	35	24	26	21
NMTCA	8.2	5.9	5.4	6.0	5.4	5.3	6.3	6.4	6.0	5.8

As can be seen from Table 5.4-1 minor changes in the NVNA levels were observed by changing the sodium chloride content a few percent. The general trend is however that the levels decrease with increasing amount of sodium chloride except for NTCA. The highest level of NTCA was observed at a sodium chloride content of 4%. Lower levels of NTCA were found for both higher and lower levels of sodium chloride than 4%. NDMA was observed in some of the samples (<1µg kg^{-1}) and no relationship with the sodium chloride content was indicated. The levels were small compared to LOQ are the data therefore not shown.

5.5 Summary and conclusions on the findings of the preliminary formation and mitigation trials

Nitrite was in these preliminary trials found to affect the formation of the NA in the minced meat more than erythorbic acid, sodium chloride, sodium tripolyphosphate, dextrose, liquid smoke and time. Erythorbic acid was the factor which second most affected the NA formation though resulting in a reduction in the NA levels. The remaining five factors did affect the NA levels but only to a minor extent and induced no significant effects. The studies does not indicate that lowering the sodium chloride contents in processed meat products will significantly affect the levels of NA.

Based on the studies we concluded that several factors can affect the NA levels to a minor extent and it was relevant to study the role of ingoing amount of nitrite and erythorbic acid further.

6. Formation and mitigation of volatile and non-volatile *N*-nitrosamines in cooked sausages

Based on the results and experience gained from the preliminary trials described in Chapter 5 it was chosen to do further studies on the role of nitrite and erythorbic acid. Though, only NVNA (NHPRO, NPRO, NTCA and NMTCA) were formed in the minced meat even at high levels of nitrite. If considering the available literature on occurrence of NA in actual meat products and on the occurrence in laboratory produced "meat products" it seems that often much lower levels and fewer types of NA are found in the laboratory produced "meat products". Reasons for this may be that the real meat products 1) have a higher bacterial activity, 2) contain more additives and ingredients making it a more complex matrix, 3) contain more fat, 4) that they have been stored for longer and/or 5) have been heat treated. It was therefore chosen to perform additional formation and mitigation studies using a more complex model. Sausages prepared from a recipe for wiener sausages were chosen as a model. A deviation from the recipe was though extra high black pepper and paprika content. Sausages do not require maturation time, it is relevant to heat treat and to produce with different contents of fat and it is the processed meat product which account for the largest fraction of the total consumption of processed meats in several European Member States (Chapter 8). Extra black pepper and paprika were added because it has been suggested that NPIP and NPYR may occur because of these spices. Using this more complex model we examined the role of ingoing amount of nitrite, the effect of heat treatment, the role of erythorbic acid, fat content, sodium tripolyphosphate, black pepper, drying and frying and the role of iron and myoglobin on the formation of NA. All of these studies have been described in Paper III but will also be briefly described in Chapter 6.1, 6.2, 6.3 and 6.4, respectively.

6.1 Role of ingoing amount of nitrite

Studies have indicated that there is a positive though not necessarily linear relationship, between the amount of nitrite added and the amount of NA formed (Drabik-Markiewicz, Van den Maagdenberg, De Mey, Deprez, Kowalska & Paelinck, 2009, Drabik-Markiewicz, Dejaegher, De Mey, Kowalska, Paelinck & Vander Heyden, 2011, Robach, Owens, Paquette, Sofos & Busta, 1980, Sen, Iyengar, Donaldson & Panalaks, 1974, Yurchenko & Mölder, 2007). These studies also indicate that the formation of the different NA may have different dependencies on the ingoing amount of nitrite. The effect of nitrite on the NA levels may also vary between test systems/meat products. The majority of the available publications only deal with the VNA, i.e. typically NDMA, NDEA, NPYR and NPIP, which typically account for a small fraction of the ATNC. Thus, data on a possible relationship between ingoing amount of nitrite and both the formation of VNA and NVNA in a meat product are scarce or non-existing.

We therefore studied the occurrence of VNA and NVNA in sausages prepared in accordance with a recipe for wiener sausages though with varying levels of nitrite, i.e. 0, 60, 100, 150, 250 and 350 mg kg^{-1}. The sausages were heat treated at 70°C for 50 minutes and then stored for 24 hours at 5°C before they were stored at -60°C until analysis.

As appears from Figure 6.1-1 1A and 1C an approximately linear relationship between the amount of nitrite added and the levels of NHPRO, NPRO, NPIP and NTCA was indicated though with NPRO and NTCA exhibiting a much steeper slope than for NHPRO and NPIP. A

steep increase in the level of NMTCA was observed by adding 60 mg kg^{-1} but only a slight increase was observed by increasing the nitrite level further, indicating that another factor than nitrite is limiting the formation of NMTCA. In sausages prepared with 150 mg kg^{-1} nitrite, which is the amount of nitrite allowed to be added to sausages for the common European market, NPIP (Figure 6.1-1 1C), NHPRO, NPRO, NTCA and NMTCA (Figure 6.1-1 1A) were found in levels of approximately 2, 10, 40, 70 and 25 µg kg^{-1}, respectively. The levels of the commonly examined VNA, NDMA and NPYR remained at or below LOQ regardless of the amount of nitrite added. Poor relationship between the ingoing amount of nitrite and the formation of NDMA was also indicated by studies performed by (Drabik-Markiewicz *et al.*, 2011) and (Gry *et al.*, 1983). The formation of NDMA may be more dependent on factors as type of processing, i.e. curing time, duration of smoking and the temperature with which the smoking was performed (Gry *et al.*, 1983) as well as microbial activity (Rywotycki, 2003). NDMA also occurs in raw meat and at higher levels in meat from conventionally bred animals than from organically bred animals (Rywotycki, 2003). If the relevant precursor had been present in the sausages a positive relationship may have been observed.
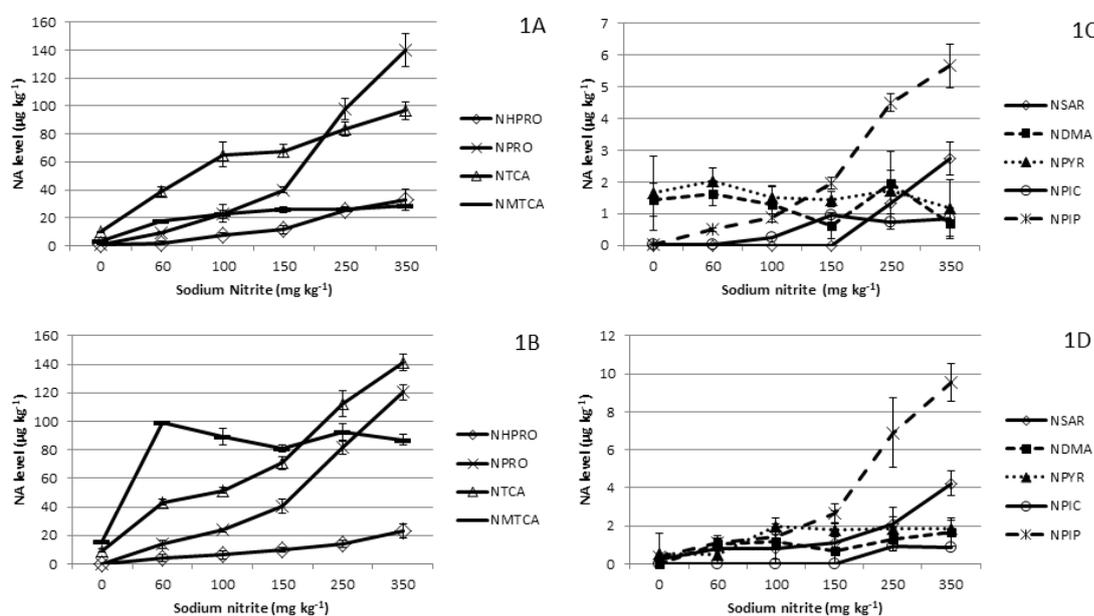
Figure 6.1-1: Levels of VNA and NVNA measured in sausages prepared with 0, 60, 200, 150, 250 or 350 mg kg^{-1} nitrite (1A and 1C) and sausages which were fried after preparation (1B and 1D). Most NVNA occurred at high levels (1A and 1B), whereas the VNA and NSAR occurred at lower levels (1C and 1D). The levels found in the fried sausages have been corrected for weight loss during frying, i.e. presented in µg kg^{-1} sausage before frying (from Paper III)

In contrast to the minced meat model NPIP and small though consistently low levels of NDMA and NPYR were produced in the sausages. Higher levels of both NTCA and NMTCA were also produced in the sausages than in the minced meat model. Thus some ingredient(s), the heat treatment (70°C for 50 minutes) or the higher fat content (~25%) contributes to the formation of the NA. The occurrence of NPIP and NPYR may, as mentioned earlier, be associated with the content of black pepper and/or paprika in the sausages. Precursors for NTCA and NMTCA may have been added via some of the sausage ingredients. NPYR also occurs more frequently and

in higher levels in products containing spices, especially those containing paprika (Yurchenko and Mölder, 2007).

To study the effect of heat treatment (70°C for 50 minutes) we also analysed sausages that were put into the -60°C freezer without heat treatment (t_0) and immediately after heat treatment (t_1) in addition to the sausages analysed after heat treatment and 24 hours of storage at 5°C (t_2). No significant differences were observed in the NA contents resulting from these three treatments, except for NTCA which increased by the heat treatment (Paper III).

As can be seen from Figure 6.1-1 1C and 1D NPIC occur at small levels in the extract of sausages prepared with more than 60 mg kg^{-1} for the non-fried sausages and in sausages prepared with more than 150 mg kg^{-1} for the fried sausages. This indicates that some nitrosation has been occurring during the extraction but also that this excess in nitrosating agents is not present if the added amount of nitrite is 60 mg kg^{-1} or less. That this excess of nitrosating agents does not occur in the fried sausages, unless more than 150 mg kg^{-1} of nitrite is added, is in good agreement with the fact that the levels of several NA increase as a result of frying and use up some the nitrosating agents. The samples were re-extracted with the addition of ascorbate prior to the extraction (~500 mg kg^{-1}) and re-analysed. This did however not prevent the formation of NPIC. The results of for the other NA were also similar.

The levels of nitrite and nitrate were also determined for the sausages (Figure 6.1-2). Even though the longest storage time was 24 hours it seems quite clear that the nitrite level is decreasing much slower than the nitrite levels in the minced meat model (Figure 5.1-1), which were reduced to about a third of the added level in 2 days. Thus the heat treatment (70°C for 50 minutes) seems to disrupt some process or processes leading to the decrease in nitrite. The same relationship was demonstrated experimentally by Gibson *et al.* (1984) using a slurry of pork meat as model (Gibson *et al.*, 1984).
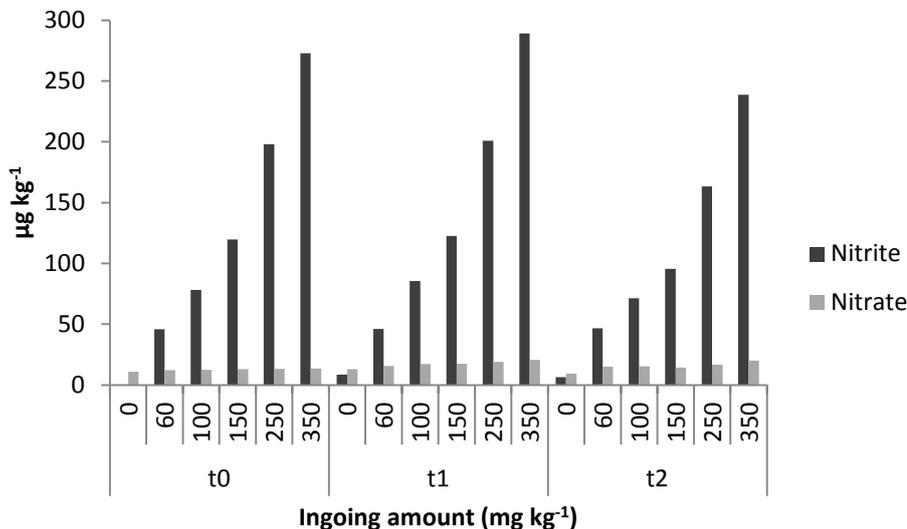
Figure 6.1-2: Nitrite and nitrate levels measured in sausages prepared with 0, 60, 200, 150, 250 or 350 mg kg^{-1} nitrite and analysed) without drying (t_0) with drying for 50 minutes at 70°C (t_1) and further storage for 24 hours at 5°C (t_2).

6.2 Effect of heat treatment

Studies by others have shown that the levels of NA in processed meat increase during e.g. frying or baking of nitrite preserved meat products (Drabik-Markiewicz, Van den Maagdenberg, De Mey, Deprez, Kowalska and Paelinck, 2009,Rywotycki, 2007,Drabik-Markiewicz, Dejaegher, De Mey, Kowalska, Paelinck and Vander Heyden, 2011,Yurchenko and Mölder, 2006,Yurchenko and Mölder, 2007). The underlying reason for this observed increase is not known but it has e.g. been suggested to be decarboxylation of NVNA as e.g. NSAR and NPRO to NDMA and NPYR, respectively. Others have suggested that the heat treatment simply speeds up the processes in the meat including nitrosation or that the heat result in release of nitrogen oxide or other nitrosating agents bound to lipids and hereby give rise to the production of NA (Hotchkiss, Vecchio and Ross, 1985). It is of relevance to know how heat treatment affects the NA levels in order to perform a well-founded evaluation of the levels of NA in the products as consumed.

The effect of frying and baking on the NA levels in six different products purchased at the local supermarkets and butcher stores was therefore studied. These included; brine cured bacon; needle-pumped cured bacon; ham boiled and smoked; serrano ham dry cured; pepperoni; and chorizo. The two types of ham, pepperoni and chorizo were baked in an oven preheated to 250°C until light brown whereas the two bacon types were fried on a pan until brown and crispy. This study is described in detail in Paper II and the results presented in Figure 1 in the paper. The overall findings were that the levels of NPIP increased by frying and baking, whereas varying impacts were observed for NPRO, NDMA, NPYR, NDEA and NMA, depending on the type of product and/or the heat treatment. For four of the six products a decrease in the NDMA level was observed. For NPYR an increase in the levels were observed for four of six products. The levels of NTCA and NMTCA decreased by frying and baking. This decrease may be caused by heat induced decarboxylation of NTCA and NMTCA to NTHz and NMTHz, respectively. However the levels of NTHz may also decrease during frying of bacon (MANDAGERE, GRAY, IKINS, BOOREN & PEARSON, 1987) indicating that NTHz is not produced from the decarboxylation of NTCA.

Thus the present study did not clearly show that the levels of NDMA and NPYR increase during heat treatment as has been reported by others. It has however been shown by others that approximately 80% of the NDMA and NPYR which is formed during frying are found in the vapour and that was at a frying temperature of 150°C (Mottram *et al.*, 1977). With a baking temperature of 250°C it is very likely that the net production of NDMA and NPYR in our study were positive but that it could not compensate for the loss via the vapour. Furthermore the products used for the heat treatment study were from the Danish market and therefore obligated to be produced in accordance with the more strict Danish National Provisions regarding the use of nitrite and we speculated that there might be an association between the potential of heat induced NA formation and the availability of nitrite or nitrosating agents.

The effect of pan frying on the NA levels in the sausages prepared with 0, 60, 100, 150, 250 or 350 mg kg^{-1} of nitrite (Chapter 6.1) was therefore studied. These sausages were pan fried until a centre temperature of 100°C, i.e. approximately 10 minutes of frying time. A picture of the fried and un-fried sausages is presented in Figure 6.2-1. The study and the results are described in detail in Paper III and will briefly be summarised in the following.

Figure 6.2-1: Picture of sausages pan fried until a centrum temperature of 100°C was achieved which took approximately 10 minutes and the same type of sausages without frying.

Frying of the sausages did not induce any significant differences in the levels of NDMA and NPYR (Figure 1D Paper III) which remained at or below 2 µg kg^{-1} regardless of the amount of ingoing nitrite. This may indicate that the relevant precursors are not present in the meat model we have applied. Sen, Iyengar, Donaldson and Panalaks (1974) also found that increasing level of nitrite had limited effect on the formation of NDMA in fried bacon. They did however find good relationship between nitrite level and NPYR level (Sen, Iyengar, Donaldson & Panalaks, 1974).

The levels of NSAR, NPIP (Figure 1D Paper III), NTCA and NMTCA (Figure 1B Paper III) increased by a factor of up to 2, 2, 1.5 and 4, respectively. Though for NTCA an increase was only observed for the fried sausages prepared with the three highest levels of nitrite (150, 250 and 350 mg kg^{-1}). This resulted in a more linear relationship between added nitrite and the level of NTCA and with a steeper slope than found for the non-fried sausages. Thus the higher levels of NTCA reported for smoked than for not smoked products (Massey, Key, Jones & Logan, 1991) may also be at least partly attributed to the heat treatment (60-80°C) which the traditional hot smoking processes is associated with (Fellows, 2009). The levels of NHPRO and NPRO decreased though only slightly. In the fried sausages prepared with 150 mg kg^{-1} nitrite the levels of NPIP, NHPRO, NPRO, NTCA and NMTCA amounted to 2.6, 10, 40, 70 and 80 µg kg^{-1}. If comparing these with the levels found in the non-fried sausages (i.e. 2, 10, 40, 70, 25 µg kg^{-1}) only for NPIP and NTCA are an increase observed.

Even though the risk of evaporation of NDMA and NPYR ought to be significantly lower in this study because of the lower temperature (100°C) we still did not see any increase in the levels of those NA. The levels of NTCA and NMTCA increased by heat treatment which is in contrast to the results obtained for the other heat treatment study performed. This may indicate that degradation of NTCA and NMTCA occur if the temperatures are high enough (~250°C).

6.3 Role of erythorbic acid, ascorbyl palmitate, fat content, sodium tripolyphosphate and black pepper

In order to study the role of erythorbic acid, ascorbyl palmitate, fat content, sodium tripolyphosphate and black pepper a 2-level fractional factorial design for the five factors (16 runs, resolution V) was set up. The details and results of this study are presented in Paper III. Erythorbic acid was included as a factor again because it was found to have large effect on the NA formation in the preliminary trials. Different fat contents were included because it has been

suggested that processes occurring in the lipid layers can promote the NA formation (Chapter 2.5.4). It was also chosen to include sodium tripolyphosphate because this may, as an emulsifier, increase the contact between the lipid and the aqueous lean meat phase and thereby interact with the possible effect of the increased fat content. Again if nitrosating agents or NA are formed in the lipid phase then a lipid soluble reductant as ascorbyl palmitate may reduce the NA formation. Black pepper were expected to be the reason for the occurrence of NPIP in the sausages prepared for the study described above and not in the minced meat model employed in the preliminary trials. We would therefore like to study if there is a concentration dependency between the amount of black pepper added and the amount of NPIP produced. The chosen low and high levels are presented in Table 6.3-1. The design prescribed the preparation of sausages with 16 different combinations of these five factors and levels. The amount of nitrite added to all sausages was 150 mg kg^{-1}. After preparation and heat treatment for 50 minutes at 70°C the sausages were packed in plastic bags which were then sealed. Two identical sets of sausages were prepared and one set was then stored for 24 hours and the other set for 5 days at 5°C before freezing to -60°C until analysis.

Table 6.3-1: Factors chosen to be studied in a fractional factorial design setup for their role in the formation and mitigation of NA in sausages.

Factor	Low level	High level
Erythorbic acid	250 mg kg^{-1}	1000 mg kg^{-1}
Ascorbyl palmitate	0 mg kg^{-1}	250 mg kg^{-1}
Fat content	~12%	~25%
Black pepper	1250 mg kg^{-1}	5000 mg kg^{-1}
Sodium tripolyphosphate	0 mg kg^{-1}	5000 mg kg^{-1}

Based on this study we conclude, that the high level of erythorbic acid significantly reduced the levels of NHPRO, NPRO, NTCA, NMTCA and perhaps NPYR compared to the low level of erythorbic acid. Only low levels of NSAR, NDMA and NPYR were produced and any effects on the levels of these would be difficult to register because of the high uncertainties on the quantitative determinations. Main effect plot and interaction plots for NSAR, NDMA and NPYR were therefore not included in Paper III but are included in Figure 1 in Appendix D. Ascorbyl palmitate was also found to significantly reduce the levels of NPRO, NTCA and slightly the levels of NHPRO and NMTCA. Having a water soluble and fat soluble antioxidant in combination may result in an even higher inhibition of the NA formation and the study did show that in the sausages prepared with the low levels of erythorbic acid the addition of ascorbyl palmitate the results indicated a further inhibition, though it seemed to have little or no effect in the sausages prepared with the high level of erythorbic acid.

The levels of NPIP increased by a factor of about 4 (0.1 to 0.4 µg kg^{-1}) when the amount of black pepper added to the sausages was increased by a factor of 4. The black pepper also induced higher levels of NMTCA and perhaps NDMA. Higher levels of NPYR and NDMA were indicated for the sausages prepared with the high fat content which is in agreement with what is reported in the literature (e.g. (Mottram *et al.*, 1977))

In this setup erythorbic acid and ascorbyl palmitate were the factors with the greatest effect on the NA levels. Ascorbyl palmitate may contribute to the inhibition of NA but further studies are needed in order to examine whether a combination of the two antioxidants can provide better

inhibition of the NA formation than erythorbic acid alone. It was therefore chosen to further examine the effect of combining the two antioxidants at different levels. This study is also described in Paper III but will be briefly summarised below.

By preparing sausages with five different concentrations of both erythorbic acid (396, 500, 750, 1000 and 1104 mg kg^{-1}) and ascorbyl palmitate (26, 150, 450, 750 and 874 mg kg^{-1}) we tested the concentration dependent effect of each of the antioxidants and the two in combination. The results are illustrated as surface plots (Figure 4 in Paper III). These plots demonstrate that the levels of NA in sausages can be decreased by adding erythorbic acid and that the degree of inhibition increase with increasing amount of erythorbic acid, at least up to the highest tested level of 1104 mg kg^{-1}. The effect of adding ascorbyl palmitate however seems to have the opposite effect, i.e. to stimulate the formation of NA or to counter act the effect of erythorbic acid. The plots for NSAR, NDMA and NPYR were not included in Paper III but are included in Figure 2 in Appendix D.

6.4   Role of iron and myoglobin

Haem is suggested to play an essential role in the endogenous formation of NA linked with high consumption of red and processed meat (Chapter 2.1.1). A role of haem in NA formation was also indicated in a study by Lunn *et al.* (2007). They found that in an aqueous solution no nitrosation of morpholine occurred even at elevated levels of nitrite, however if haem iron was added NMOR was produced (Lunn *et al.*, 2007). Calcium was by Pierre *et al.* (2013) found to prevent a haem induced increase in endogenous formation of nitroso compounds in humans (Pierre *et al.*, 2013). Iron play an essential role in lipoperoxidation processes (Igene, King, Pearson & Gray, 1979, Ladikos & Lougovois, 1990) occurring in meat; and antioxidants as ascorbate/erythorbic acid inhibit these unwanted processes. Because antioxidants also inhibit the NA formation a role of iron may be assumed. We therefore wanted to examine whether haem and/or iron also affect the formation of NA in meat products. Calcium and erythorbic acid was included as factors to have a secondary indication of the roles of haem and iron. By including erythorbic acid in this setup it was also possible to test the effect of this factor again.

Figure 6.4-1: Sausages during heat treatment for 50 min at 70°C) prepared with different combinations of four factors (haem, Fe(III), erythorbic acid and calcium). Supplementing with haem (left side) resulted in sausages of a darker red colour.

No significant effects on the levels of NA were observed by supplementing the sausage meat with haem (myoglobin)(Figure 5 A1-E1 Paper III). Though supplementing the sausages with haem turned the colour of the sausages more dark red (sausages on the left side in Figure 6.4-1). However a slight reduction in the levels of NPIP (Figure 5 C1 Paper III), NSAR and NPYR were indicated (Figure 3 Appendix D). This reduction may be the result of an increased competition for the nitrosating agents because more NO was bound to the added haem or because of the antioxidative effect of nitrosylhaem (Figure 3 Appendix D).

Addition of Fe(III) on the other hand significantly increased the levels of NHPRO and NMTCA (Figure 5 A1 and E1 Paper III). An increase was also indicated for NTCA (Figure 5 D1 Paper III) and NPYR (Figure 3 Appendix D). For the remaining NA no effect was observed by adding Fe(III). Erythorbic acid reduced the NA levels also in this setup (Figure 5 A1-E1 Paper III) except for NDMA and NPYR. Though, again it should be mentioned that the changes in the levels of NSAR, NDMA and NPYR were small and all detected levels were at close to the level of LOQ.

Interaction between Fe(III) and erythorbic acid was indicated for NTCA and NMTCA, though only significantly for NMTCA (Figure 5 E2 Paper III). The inhibitory effect of adding erythorbic acid on NTCA and NMTCA was not observed if it was combined with the addition of Fe(III) (Figure 5 D2 and E2 Paper III). That this interaction is not seen for the other NA indicate that different mechanisms are involved in the formation NTCA and NMTCA than the other NA. NTCA and NMTCA seem to be closely linked with oxidation processes. Increased levels of Fe(III) are linked with stimulation of lipid oxidation processes which in turn will result in the formation of aldehydes. The formation of NTCA and NMTCA may then be stimulated by the presence of aldehydes.

Thus the present study does not indicate that haem is a catalyst for the formation of NA in meat products as has been suggested for endogenous formation. It does however indicate that free iron can stimulate the formation of NA in meat and that the effect of adding antioxidants as erythorbic acid which normally reduces the levels of NA is reduced or prevented by the elevated iron levels. This effect was especially clear for NTCA and NMTCA. The formation of NTCA and NMTCA was also prevented to a lesser extent by just the presence of erythorbic acid than was the formation of NHPRO, NPRO and NPIP. The levels of these three NA were reduced by approximately 60-75% by the addition of the 1000 mg kg^{-1} erythorbic acid.

6.5   Summary and conclusions

In the sausages prepared the occurrence of nitrite was the most important factor for the NA formation. The levels of NPIP, NHPRO, NPRO, NTCA and NMTCA increased with increasing amounts of nitrite (0, 60, 100, 150, 250 and 350 mg kg^{-1}) added to sausages. The levels of the commonly examined VNA, NDMA and NPYR remained at or below LOQ, indicating e.g. that the relevant precursors were not present in the applied meat model.

Of the tested factors, besides nitrite, erythorbic acid was the factor which affected the NA levels the most. It inhibited significantly the formation of NHPRO, NPRO, NPIP, NTCA and NMTCA. This inhibition was for NTCA and NMTCA reduced by addition of free iron (Fe(III)). Haem was found to have no effect on the NA formation. Non-significant reduction in the NSAR levels by addition of erythorbic acid was also indicated. Ascorbyl palmitate had less effect than erythorbic

acid and a combination of the two provided no further protection than erythorbic acid alone. The inhibitory effect of erythorbic acid increased with increasing concentration (up to 1000 mg kg^{-1}). An approximate linear relationship between the amount of black pepper added and the levels of NPIP formed was found. The formation of NMTCA was also stimulated by the addition of black pepper. Only slight effects of increased fat content and addition of tripolyphosphate were observed.

For sausages prepared with different levels of nitrite pan frying to a centre temperature of 100°C resulted in increased the levels of NSAR, NPIP, NTCA and NMTCA. The levels of NTCA only increased in the sausages prepared with 150, 250 and 350 mg kg^{-1} of nitrite. The NTCA levels in the other sausages were unchanged. Pan frying or baking (250°C) of different purchased processed meat products increased the levels of NPIP. The levels of NTCA and NMTCA decreased and both increases and decreases were observed for NPRO, NDMA, NPYR, NDEA and NMA, depending on the type of product and/or the heat treatment.

7. Protein bound *N*-nitrosamines

Release of NA as a result of protein degradation/digestion has been suggested as a source of endogenous NA. Dunn and Stich (1984) found that treatment of processed meat with proteolytic enzymes increased the amount of NRPO available for the analytical method, indicating that protein bound NA occur. They also found that the level of NPRO determined after treatment with the proteolytic enzymes correlated better with the amount of NRPO excreted in the urine of human subjects following ingestion than with the levels of NPRO determined without the enzyme treatment. Especially a protease Type XIV digestion released significant amounts of NPRO, i.e. 124 ng g^{-1} meat compared to 9 ng g^{-1} meat without enzyme digestion. A level of about 60 ng g^{-1} would correspond to the amounts excreted via urine. By providing ascorbic acid with the ingested portions of nitrite cured meat did not significantly reduce the amount of NPRO excreted in the urine, which the author takes as an indication of the NPRO already being available in the meat but bound to protein (Dunn and Stich, 1984).

We therefore considered it relevant to measure the levels of VNA and NVNA before and after enzymatic treatment of representative nitrite preserved meat products. The study is not included in any of the papers or manuscript but will be described in the following.

We chose to perform a pilot study in which two representative meat products, i.e. serrano ham and salami, were digested with two types of proteases. Serrano and salami were chosen as representative products because these products had been found to contain NA. The enzymes chosen were Type XIV and subtilisin (Sigma-Aldrich Co. St Louis, MO, USA). The former was chosen based on the results obtained by Dunn and Stich (1984) and the latter because it has a broad substrate specificity and can therefore be an efficient meat protein digesting enzyme. 1g of meat product was digested with 2ml of buffer containing Protease Type XIV (Streptomyces griseus ≥3.5 units mg^{-1} solid) or subtilisin (Bacillus licheniformis Typ VIII, 7-15 units mg^{-1} solid) dissolved in tris(hydroxymethyl)aminomethane (6 g protease per 400 ml) adjusted to pH 8.8 for the type XIV and to pH 9.5 for subtilisin. 1 g homogenised meat was mixed thoroughly with 2 ml of protease solution, incubated for 30 minutes at 37°C and then for a further 120 minutes at 50°C. The obtained aqueous mixture was then analysed by the method presented in Paper I.

The results of this pilot test did not give a clear indication of increases in the NA levels (data not shown). The two types of enzymes also seemed to vary in effect. Though if an increase in the NA levels had been observed it would not prove that protein bound NA are formed during enzymatic digestion. Digestion of the proteins also increase the levels of amines available for nitrosation, myoglobin which may be degraded resulting in the release of iron and perhaps NO which is then able to react with free amines instead. Thus an increase in the NA levels as a result of enzymatic digestion would not necessarily justify the occurrence of protein bound NA. Though regardless of the underlying mechanism, for the increase in the NPRO levels observed by Dunn and Stich (1984), it is interesting that the levels of NA may increase when the proteins in processed meat are enzymatically digested because this would also mean that NA are released during digestion in humans. Further studies into the effect of protein digestion on the NA levels were not performed because the underlying causes of any observed changes in the NA levels may be multiple. Instead we chose to study the role of iron and haem levels on the NA formation as described in Chapter 6.4.

8. Exposure – risk assessment

Several estimates of different populations or subgroups exposure to VNA via processed meat products have been published (Tricker *et al.*, 1991;Keszei *et al.*, 2013). Very few of these publications are of more recent date and most of them only estimate the exposure to NDMA and in a few cases also NPYR and NPIP. In general the exposure levels to the assessed VNA has been found to be low and of no or little concern. Because of lack of data and perhaps also because it has been assumed that the NVNA are of no toxicological relevance no estimation of the exposure to NVNA has so far been published. No estimation of the exposure to NA has previously been performed for the Danish population. We therefore found that an estimation of the Danish population's exposure to VNA and NVNA via processed meat products was relevant in order to evaluate whether the levels of NA found in the present study (Chapter 4) may be of concern for the public health.

Based on the results of the survey (Paper II) and consumption data from the Danish National Survey of Diet and physical Activity (DANSDA) (Dietary habits in Denmark 2003-2008) an estimation of the exposure of the Danish population to NA via consumption of processed meat products was estimated. The estimated exposure levels were then risk assessed by calculating the the margin of exposure (MOE). The MOE is the ratio between a bench mark dose level ($BMDL_{10}$) and the estimated dietary exposures. The $BMDL_{10}$ has been estimated by others from toxicological studies on NDMA using total number of liver tumours as endpoint (Zeilmaker *et al.*, 2010)(Manuscript I). The results of the study including the estimated exposure level and which types of processed meat products that contribute to the exposure for children (4-6 years) and adults (15-75 years) are presented in Figure 1 and 2 in Manuscript I.

The consumption of processed meat products per day, at the 95th percentile, is 20 g for adults and 16 g for children and adults. It is primarily sausages, salami, pork flank (spiced and boiled) and ham that are consumed. This consumption of processed meat results in an exposure to NVNA of 33 and 90 ng kg bw^{-1} day^{-1} for adults and children, respectively. As it would be expected from the levels of NA found in the survey the exposure to VNA is significantly lower amounting to 0.34 and 1.1 ng kg bw^{-1} day^{-1} for adults and children, respectively. The classical VNA (NDMA, NPYR, NPIP, NDEA) accounted for >90% of the exposure to VNAs. The exposure levels estimated in the present work is 0.03 µg day^{-1} for adults with an average body weight of 75 kg. This is lower than the exposure level estimated for meat products (~0.1 µg day^{-1} for the sum of NDMA, NPYR, NPIP) and food (0.1 µg day^{-1} for NDMA) by Keszei *et al.* (2013) and Tricker *et al.* (1991), respectively (Table 3 Manuscript 1). The estimated exposure to NVNA though is in the low end of the exposure range (10-100 µg day^{-1}) suggested by Tricker and Preussmann (1991). Though other NVNA than those included in the present study has been identified in processed meat (Sen *et al.*, 1993;Janzowski *et al.*, 1978;Tricker and Kubacki, 1992) so the estimated exposure may be underestimated.

Based on a $BMDL_{10}$ of 29,000 ng kg bw^{-1} day^{-1} a MOE value of ≥17000 is derived for the exposure to the sum of VNA and NSAR, indicating an exposure of low concern. Though, a MOE value of ~200 is derived when including the NVNA. Because the $BMDL_{10}$ is based on toxicological studies on NDMA the risk associated with the NVNA will most likely be highly over-estimated. Though, data on the toxicological significance of NVNA is lacking.

There may be several underlying reasons for the estimated lower exposure to VNA for the Danish population from processed meat products. First of all, the consumption of processed meat products is lower than e.g. for the German population. Secondly, the levels of VNA may be lower in the meat products on the Danish market. The survey described in Chapter 4 and Paper II in fact indicated, but not demonstrated, that the mean levels of NA in processed meat products from the Danish market were lower than the mean levels found in samples taken from the Belgian market. A generally lower level of NA in meat products on the Danish market may partly be attributable to the National Provision allowing for the addition of less nitrite than the common EU regulation. Tough NDMA and NPYR, for which poor relationship with the ingoing amount of nitrite has been indicated (Chapter 5.1, Paper 3), account for the largest fraction of the estimated exposure to VNA. Lower levels of NDMA and NPYR may be more related to factors as e.g. general meat quality, smoking technique and temperatures during storage, bacterial activity, the animal species and breeding conditions. Based on the results obtained in the present work it is expected that if the ingoing amount of nitrite is increased the levels of NA in the meat products and thereby also the exposure level to NA will generally increase except for NDMA and NPYR.

9. Main findings and conclusions

The **aim** of the present thesis was to study the role of ingoing amount of nitrite as well as factors relevant for industrial processing of meat and the effect of heat treatment on the formation and mitigation of VNA and NVNA in meat. Knowledge is needed in this area in order to define strategies for limiting the formation of NA in general and not only for the few commonly assayed VNA. Secondly, data on the occurrence of VNA and NVNA in processed meat products on the Danish market were to be generated and used for an evaluation of the exposure level resulting from consumption of processed meat products.

For the purpose a fast, simple and environmentally rational method based on acetonitrile extraction and LC-(APCI/ESI)MS/MS was developed. The method was successfully validated for 13 NA, eight VNA and five NVNA, as well as NPIC. The LODs for the validated NAs were between 0.2 and 1 µg kg^{-1}, with only few exceptions. The method was then applied for the studies on occurrence, formation and mitigation of NA in processed meat products. From these studies the following conclusions can be drawn:

Occurrence of volatile and non-volatile *N*-nitrosamines in processed meat products and the role of heat treatment
Both VNA and NVNA were found to occur in nitrite preserved meat products on the Danish market. The mean levels of the VNA were generally low, whereas the mean levels of the NVNA were considerably higher. Although lower levels of nitrite are allowed to be added to processed meat products for the Danish market, than for the common European market, the majority of the Danish processed meat products still contain NA. There was no relationship between the residual amounts of nitrite and the levels of NA.
Samples from the Belgium market also contained both VNA and NVNA. Higher, though non-significant, mean levels were generally found for the Belgian samples than for the Danish samples. Generally the levels of NA found in the present study are comparable with those reported in previous and recent studies; however the frequency with which they were found may be lower and thereby also the mean contents.

Role of ingoing amount of nitrite on the NA formation
Positive relationship between the amount of ingoing nitrite and the levels of the NHPRO, NPRO, NTCA, NMTCA, NSAR, and NPIP was demonstrated in sausages. The levels of NDMA and NPYR increased slightly by the presence of low levels of nitrite but did not increase further by increasing the amount of nitrite. This may occur because of absence of the relevant precursors. The residual levels of nitrite were reduced slower in the heat treated sausages than in raw minced meat.

Effect of heat treatment
A clear positive effect of heat treatment on the levels of NPIP was demonstrated in all the heat treatment experiments performed. When sausages prepared with different levels of nitrite were fried until a centre temperature of 100°C also the levels of NSAR, NPIP, NTCA and NMTCA increased, whereas the levels of NHPRO and NPRO decreased slightly and the levels of NDMA and NPYR were unchanged. When various products purchased at the local supermarkets and butchers were baked or fried to a higher temperature (250°C), the levels of NTCA and NMTCA

decreased, whereas the levels of NPRO, NDMA, NPYR, NDEA and NMA both increased and decreased depending on type of product and/or heat treatment.

Role of commonly used additives and fat content
The commonly used additives under study in the preliminary trials were besides nitrite; erythorbic acid, sodium chloride, sodium tripolyphosphate, dextrose and liquid smoke. The importance of time was also studied. From these studies, performed on minced meat, it was concluded that all of the factors under study could affect the NA levels but that, the ingoing amount of nitrite and the presence of erythorbic acid had the most significant effect on the NA levels. Sodium chloride was found to affect the levels of NA but again the importance of nitrite and erythorbic acid were of higher importance. Only minor changes were found in the NA levels at different times of storage.

The role of erythorbic acid, ascorbyl palmitate, fat content, sodium tripolyphosphate and black pepper on the NA formation were studied in sausages prepared in the laboratory. Besides nitrite was erythorbic acid found to affect the NA levels most, also in the sausages. The levels of NHPRO, NPRO, NPIP, NTCA and NMTCA were inversely related to the amounts of erythorbic acid (396-1104 mg kg^{-1}). By preparing the sausages with 1000 mg kg^{-1} erythorbic acid the levels of the different NA were reduced by 20 to 75%. Adding ascorbyl palmitate, a fat soluble antioxidant, provided no additional protection from NA formation than a high level of erythorbic acid (1000 mg kg^{-1}) alone.

The assumption that the occurrence of NPIP is related to the contents of black pepper was confirmed and a linear relationship between the amount of pepper and NPIP was indicated. No significant effects were observed by increasing the fat content of the sausages. Slight non-significant increases in the levels of NDMA and NPYR were though indicated.

Role of iron and haem
Free iron (Fe(III)) and not haem significantly increased the levels of NMTCA and NHPRO and non-significantly increased the levels of NTCA in the sausages. The fact that the NA are affected differently by e.g. increased levels of iron indicate that different mechanisms are involved in their formation. Addition of Fe(III) counteracted the other ways inhibitory effect of erythorbic acid on the formation of NTCA and NMTCA. The fact that the formation of these two NVNA was positively affected by increased levels of Fe(III) and inhibited by erythorbic acid may indicate a link to lipid oxidation processes, which are also stimulated by iron and inhibited by e.g. erythorbic acid.

Dietary exposure to volatile and non-volatile *N*-nitrosamines from processed meat products in Denmark
The consumption of processed meat products per day, at the 95th percentile, is 20 g for adults and 16 g for children, consisting primarily of sausages, salami, pork flank (spiced and boiled) and ham. The exposures to VNA by the consumption of processed meat were low, i.e. 0.34 ng kg bw^{-1} day^{-1} for adults and 1.1 ng kg bw^{-1} day^{-1} for children. The exposures to NVNA were estimated to be considerably higher, i.e. 33 ng kg bw^{-1} day^{-1} for adults and 90 ng kg bw^{-1} day^{-1} for children. The calculated MOE (≥17000) for the VNA exposure indicate that this is of low concern. The exposure to the VNA alone may be of low concern, but it may be high enough to account for the stronger association between processed meat and adverse health effects, than

between red meat and adverse health effects, found in some epidemiologic studies. The risk associated with the much higher exposure to the NVNA is not known and cannot be evaluated until appropriate toxicological studies have been performed.

Further knowledge on several areas is needed before it can be concluded whether or not consumption of nitrite preserved meat products can pose a health risk and which factors in the processed meat that may be responsible for any such health risk. These areas include not only the toxicological significance of the NVNA but e.g. also whether there are differences in the biological activity of nitrosylhaem and haem, or perhaps some unknown factor.

Based on the literature and data presented here it can be concluded that the use of nitrite for meat preservation and/or colouration generally increases the NA levels. Because of the possible adverse health effects of NA the exposure level ought to be kept at a minimum. Based on the present knowledge low levels of NA in processed meat are best achieved by adding as little nitrite as possible and only in combination with erythorbic acid, ascorbic acid or ascorbate. In order not to deplete the anti-oxidative effect of e.g. erythorbic acid the meat should be stored without access to oxygen. EFSA has concluded that microbiological safe meat products generally can be produced by the addition of 50 mg kg^{-1} of nitrite. Other means besides nitrite addition can insure the microbiological safety. The occurrence of the carcinogenic NDMA and perhaps NPYR seems however not to be related to the levels of nitrite nor the levels of erythorbic acid.

In brief we have found that:
- Both VNA but especially NVNA occur in nitrite preserved meat products on the Danish market.
- There were no relationships between residual amount of nitrite and the levels of NA.
- The levels of several NA increase with increasing amount of ingoing nitrite.
- Of the tested factors the presence of nitrite and erythorbic acid had the most significant effects on the formation and mitigation of NA, respectively.
- The levels of the commonly assayed VNA, NDMA and NPYR, were not affected significantly by increasing levels of nitrite or by erythorbic acid.
- Heat treatment increased the levels of NPIP, whereas varying effects were observed for other NA depending on temperature and product.
- Higher levels of Fe(III), but not haem in the form of myoglobin, induce the formation of NHPRO, NTCA and NMTCA.
- The exposure to VNA characterised as human carcinogens does not give cause for concern when comparing with a BMDL factor for NDMA.
- The exposure to NVNA is considerably higher but their toxicological relevance is not known.
- Consumption of ham, salami and sausages are the primary sources of NA from processed meat products.
- The exposure to NA via processed meat may explain the stronger association between cancer and consumption of processed meat than for red meat.

10. Perspectives

Whether the occurrence of NA in processed meat products are accountable for the, in some epidemiologic studies observed, stronger association between adverse health effects and consumption of processed meat than of red meat is not known. Because several of the NA are known carcinogens the exposure to these ought to be limited as much as possible, even if there is doubt about their role in the observed adverse health effects of high consumption of processed meat. Thus, limiting the use of nitrite and making it a requirement to use ascorbic acid, ascorbate, erythorbate or erythorbic acid in combination with nitrite seems recommendable.

Though, when the NA formation is prevented by the addition of erythorbic acid, ascorbic acid or ascorbate the formation of nitrosylhaem is favoured. It is possible that nitrosylhaem or nitrosyl hemochrome have a different stability and/or biological activity than haem. Thus the occurrence of nitrosylhaem and/or nitrosyl hemochrome in processed meat may be accountable or partly accountable for the adverse health effects of high intake of processed meat. Thus the role of nitrosylhaem and/or nitrosyl hemochrome needs to be elucidated.

Thus even though the amount of literature on NA is large there is still many areas that needs to be studied further.

11. References

Allam, O., D. Bahuaud, S. Tache, N. Naud, D. E. Corpet, & F. H. F. Pierre (2011). Calcium carbonate suppresses haem toxicity markers without calcium phosphate side effects on colon carcinogenesis RID B-3544-2008. *British Journal of Nutrition*, 105(3), 384-392.

Bailey, M. E., A. K. Mandagere, & European Meeting of Meat,Research Workers (1980). Nitrosamine analysis and inhibition studies. *Proceedings of the European Meeting of Meat Research Workers*, Vol. II, M-8, 237-240.

Barbieri, G., M. Bergamaschi, G. Barbieri, & M. Franceschini (2013). Kinetics of nitrite evaluated in a meat product. *Meat Science*, 93(2), 282-286.

Bingham, S. A., R. Hughes, & A. J. Cross (2002). Effect of white versus red meat on endogenous N-nitrosation in the human colon and further evidence of a dose response. *Journal of Nutrition*, 132(11), 3522-3525.

Boles, J. A. (2011). Use of cold-set binders in meat systems. *In Processed meats - Improving safety, nutrition and quality*, edited by Kerry, J.P & Kerry, J.F., Woodhead Publishing, Cambridge, UK.

Campillo, N., P. Viñas, N. Martínez-Castillo, & M. Hernández-Córdoba (2011). Determination of volatile nitrosamines in meat products by microwave-assisted extraction and dispersive liquid–liquid microextraction coupled to gas chromatography–mass spectrometry. *Journal of Chromatography A*, 1218(14), 1815-1821.

Carpenter, C. E., & E. Clark (1995). Evaluation of Methods Used in Meat Iron Analysis and Iron Content of Raw and Cooked Meats. *Journal of Agricultural and Food Chemistry*, 43(7), 1824-1827.

Carpin, M. A. (2013). Nitrosamines in nitrite preserved meat products. Master Thesis project report, National Food Insitute, Technical University of Denmark.

Crews, C. (2010). The determination of N-nitrosamines in food. *Quality Assurance and Safety of Crops & Foods*, 2(1), 2-12.

Drabik-Markiewicz, G., B. Dejaegher, E. De Mey, T. Kowalska, H. Paelinck, & Y. Vander Heyden (2011). Influence of putrescine, cadaverine, spermidine or spermine on the formation of N-nitrosamine in heated cured pork meat. *Food Chemistry*, 126(4), 1539-1545.

Drabik-Markiewicz, G., K. Van den Maagdenberg, E. De Mey, S. Deprez, T. Kowalska, & H. Paelinck (2009). Role of proline and hydroxyproline in N-nitrosamine formation during heating in cured meat. *Meat Science*, 81(3), 479-486.

Dunn, B. P., & H. F. Stich (1984). Determination of free and protein-bound N-nitrosoproline in nitrite-cured meat products. *Food and Chemical Toxicology*, 22(8), 609-613.

Eerola, S., I. Otegui, L. Saari, & A. Rizzo (1998). Application of liquid chromatography-atmospheric pressure chemical ionization mass spectrometry and tandem mass spectrometry to the determination of volatile nitrosamines in dry sausages. *Food additives and contaminants*, 15(3), 270-279.

EFSA, European Food Safety Authority (2003). Opinion of the Scientific Panel on Biological Hazards on the request from the Commission related to the effects of Nitrites/Nitrates on the

Microbiological Safety of Meat Products. Available at http://www.gencat.cat/salut/acsa/html/ca/dir2948/pdf/14.pdf (accessed 23-06-2014).

Essen, S., H. Kimpton, C. Wright, & K. McAdam (2011). Determination of selected N-Nitrosoamino acids in smokeless tobacco products. Poster presented at the CORESTA Smoke Science and Product Technology Joint Study Groups Meeting, Graz, Austria, (08/2013).

European Commission (2014). Final report on a desk study to monitor the implementation of Directive 2006/52/EC in the EU Member States as regards the use of nitrites by the industry in the different categories of meat products and the organization of national controls. Health and Consumers Directorate-General, Safety of the Food chain, Chemicals, contaminants, pesticides. Not available online (23-06-2014).

Fellows, P. J. (2009). *Food processing technology - Principles and practice*. Third edition. Woodhead Publishing, Cambridge, UK.

Fox, J. B. (1966). Chemistry of Meat Pigments. *Journal of Agricultural and Food Chemistry*, 14(3), 207-210.

Gheisari, H. R., J. K. S. Moller, C. E. Adamsen, & L. H. Skibsted (2010). Sodium Chloride or Heme Protein Induced Lipid Oxidation in Raw, Minced Chicken Meat and Beef. *CZECH Journal of Food Sciences*, 28(5), 364-375.

Gibson, A. M., T. A. Roberts, & A. Robinson (1984). Factors controlling the growth of Clostridium botulinum types A and B in pasteurized cured meats. VI. Nitrite monitoring during storage of pasteurized pork slurries. *Journal of Food Technology*, 19(1), 29-44.

Gray, J. I., & L. R. Dugan (1975). Inhibition of N-nitrosamine formation in model food systems. *Journal of Food Science*, 40(5), 981-984.

Gry, J., N. J. D. Rasmussen, W. K. Jensen, I. G. Brandt, & B. Fabech (1983). Investigations on effects of nitrite in commercially prepared Danish cured meat products. The Federation of Danish Pig Producers and Slaughterhouses and The National Food Agency of Denmark. (Not available online)

Hill, M. J., R. C. Massey, D. E. G. Shuker, S. Leach, A. R. Tricker, R. Preussmann, & J. R. Rowland (1988). *Nitrosamines. Toxicology and microbiology*. 169pp. Ellis Horwood Ltd., UK. ISBN 0-89573-605-0.

Hogg, N. (2007). Red meat and colon cancer: Heme proteins and nitrite in the gut. A commentary on "Diet-induced endogenous formation of nitroso compounds in the GI tract". *Free Radical Biology and Medicine*, 43(7), 1037-1039.

Honikel, K. (2008). The use and control of nitrate and nitrite for the processing of meat products. *Meat Science*, 78(1-2), 68-76.

Hotchkiss, J. H., J. F. Barbour, L. M. Libbey, & R. A. Scanlan (1978). Nitramines as thermal energy analyzer positive nonnitroso compounds found in certain herbicides. *Journal of Agricultural and Food Chemistry*, 26(4), 884-887.

Hotchkiss, J. H., A. J. Vecchio, & H. D. Ross (1985). N-Nitrosamine formation in fried-out bacon fat: evidence for nitrosation by lipid-bound nitrite. *Journal of Agricultural and Food Chemistry*, 33(1), 5-8.

IARC (1998). IARC monographs on the evaluation of carcinogenic risks to humans. Some N-Nitroso Compounds. *IARC monographs on the evaluation of carcinogenic risks to humans.*, 17.

Jakszyn, P., A. Agudo, R. Ibánez, R. Garcí¬a-Closas, G. Pera, P. Amiano, & C. A. González (2004). Development of a Food Database of Nitrosamines, Heterocyclic Amines, and Polycyclic Aromatic Hydrocarbons. *The Journal of nutrition*, 134(8), 2011-2014.

Jansson, C., A. Paccou, & B. Osterdahl (2003). Analysis of tobacco-specific N-nitrosamines in snuff by ethyl acetate extraction and liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, 1008(2), 135-143.

Janzowski, C., G. Eisenbrand, & R. Preussmann (1978). Occurrence of N-nitrosamino acids in cured meat products and their effect on formation of N-nitrosamines during heating. *Food and cosmetics toxicology*, 16(4), 343-348.

Japelt, R. B., D. Silvestro, J. Smedsgaard, P. E. Jensen, & J. Jakobsen (2011). LC-MS/MS with atmospheric pressure chemical ionisation to study the effect of UV treatment on the formation of vitamin D(3) and sterols in plants. *Food Chemistry*, 129(1), 217-225.

Joosen, A. M. C. P., G. G. C. Kuhnle, S. M. Aspinall, T. M. Barrow, E. Lecommandeur, A. Azqueta, A. R. Collins, & S. A. Bingham (2009). Effect of processed and red meat on endogenous nitrosation and DNA damage. *Carcinogenesis*, 30(8), 1402-1407.

Keszei, A. P., R. A. Goldbohm, L. J. Schouten, P. Jakszyn, & d. B. van (2013). Dietary N-nitroso compounds, endogenous nitrosation, and the risk of esophageal and gastric cancer subtypes in the Netherlands Cohort Study. *American Journal of Clinical Nutrition*, 97(1), 135-146.

Klaunig, J., & L. Kamendulis (2004). The role of oxidative stress in carcinogenesis. *Annual Review of Pharmacology and Toxicology*, 44 239-267.

Kuhnle, G. G. C., G. W. Story, T. Reda, A. R. Mani, K. P. Moore, J. C. Lunn, & S. A. Bingham (2007). Diet-induced endogenous formation of nitroso compounds in the GI tract. *Free Radical Biology and Medicine*, 43(7), 1040-1047.

Larsson, S. C., & A. Wolk (2012). Red and processed meat consumption and risk of pancreatic cancer: meta-analysis of prospective studies. *British journal of cancer*, 106, 603–607.

Larsson, S. C., L. Bergkvist, & A. Wolk (2006). Processed meat consumption, dietary nitrosamines and stomach cancer risk in a cohort of Swedish women. *International Journal of Cancer*, 119(4), 915-919.

Lehotay, S. J., K. A. Son, H. Kwon, U. Koesukwiwat, W. Fu, K. Mastovska, E. Hoh, & N. Leepipatpiboon (2010). Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables. *Journal of Chromatography A*, 1217(16), 2548-2560.

Leistner, L. (2000). Basic aspects of food preservation by hurdle technology. *International journal of food microbiology*, 55(1-3), 181-186.

Leth, T., S. Fagt, S. Nielsen, & R. Andersen (2008). Nitrite and nitrate content in meat products and estimated intake in Denmark from 1998 to 2006. *Food Additives & Contaminants: Part A*, 25(10), 1237-1245.

Li, L., J. Shao, X. Zhu, G. Zhou, & X. Xu (2013). Effect of plant polyphenols and ascorbic acid on lipid oxidation, residual nitrite and N-nitrosamines formation in dry-cured sausage. International *Journal of Food Science & Technology*, 48(6), 1157-1164.

Li, L., P. Wang, X. Xu, & G. Zhou (2012). Influence of Various Cooking Methods on the Concentrations of Volatile N-Nitrosamines and Biogenic Amines in Dry-Cured Sausages. *Journal of Food Science*, 77(5), C560-C565.

Lijinsky, W. (1987). Structure-activity relations in carcinogenesis by N-nitroso compounds. *Cancer and metastasis reviews*, 6(3), 301-356.

Liu, X., M. Miller, M. Joshi, D. Thomas, & J. Lancaster (1998). Accelerated reaction of nitric oxide with O-2 within the hydrophobic interior of biological membranes. *Proceedings of the National Academy of Sciences of the United States of America*, 95(5), 2175-2179.

McArdle, R., & Hamill, R. (2011). Utilisation of hydrocolloids in processed meat systems. In *Processed meats - Improving safety, nutrition and quality*, edited by Kerry, J.P & Kerry, J.F.., Woodhead Publishing, Cambridge, UK.

Massey, R. C., P. E. Key, R. A. Jones, & G. L. Logan (1991). - Volatile, non-volatile and total N-nitroso compounds in bacon. *Food Additives and Contaminants*, (5), 585.

Massey, R. C., C. Crews, M. J. Dennis, D. J. McWeeny, J. R. Startin, & M. E. Knowles (1985). Identification of a major new involatile N-nitroso compound in smoked bacon. *Analytica Chimica Acta*, 174(0), 327-330.

Masuda, M., H. F. Mower, B. Pignatelli, I. Celan, M. D. Friesen, H. Nishino, & H. Ohshima (2000). Formation of N-Nitrosamines and N-Nitramines by the Reaction of Secondary Amines with Peroxynitrite and Other Reactive Nitrogen Species: A Comparison with Nitrotyrosine Formation. *Chemical research in toxicology*, 13(4), 301-308.

Mol, H. G. J., P. Plaza-Bolaños, P. Zomer, T. C. de Rijk, A. A. M. Stolker, & P. P. J. Mulder (2008). Toward a Generic Extraction Method for Simultaneous Determination of Pesticides, Mycotoxins, Plant Toxins, and Veterinary Drugs in Feed and Food Matrixes. *Analytical Chemistry*, 80(24), 9450-9459.

Mottram, D. S., R. L. S. Patterson, R. A. Edwards, & T. A. Gough (1977). The preferential formation of volatile N-nitrosamines in the fat of fried bacon. *Journal of the science of food and agriculture,* 28(11), 1025-1029.

Mottram, D. S., R. L. S. Patterson, D. N. Rhodes, & T. A. Gough (1975). Influence of ascorbic acid and ph on the formation of n-nitrosodimethylamine in cured pork containing added dimethylamine. *Journal of the science of food and agriculture*, 26(1), 47-53.

Norat, T., S. Bingham, P. Ferrari, N. Slimani, M. Jenab, M. Mazuir, K. Overvad, A. Olsen, A. Tjønneland, F. Clavel, M. Boutron-Ruault, E. Kesse, H. Boeing, M. M. Bergmann, A. Nieters, J. Linseisen, A. Trichopoulou, D. Trichopoulos, Y. Tountas, F. Berrino, D. Palli, S. Panico, R. Tumino, P. Vineis, H. B. Bueno-de-Mesquita, P. H. M. Peeters, D. Engeset, E. Lund, G. Skeie, E. Ardanaz, C. González, C. Navarro, J. R. Quirós, M. Sanchez, G. Berglund, I. Mattisson, G. Hallmans, R. Palmqvist, N. E. Day, K. Khaw, T. J. Key, M. San Joaquin, B. Hémon, R. Saracci, R. Kaaks, & E. Riboli (2005). Meat, Fish, and Colorectal Cancer Risk: The European Prospective Investigation into Cancer and Nutrition. *Journal of the National Cancer Institute*, 97(12), 906-916.

Ozel, M. Z., F. Gogus, S. Yagci, J. F. Hamilton, & A. C. Lewis (2010). Determination of volatile nitrosamines in various meat products using comprehensive gas chromatography-nitrogen chemiluminescence detection. Food and Chemical Toxicology, 48(11), 3268-3273.

Pegg, R. B., & Shahidi, F. (2004). *Nitrite curing of meat: the N-nitrosamine problem and nitrite alternatives*. Food & Nutrition Press, INC. Online ISBN: 9780470385081

Pensabene, J., & W. Fiddler (1990). Determination of ten N-nitrosoamino acids in cured meat products. *Journal of the Association of Official Analytical Chemists*, 73(2), 226-230.

Pensabene, J., & W. Fiddler (1993). Effect of Carbohydrate Cryoprotecting Agents on the Formation of N-Nitrosodimethylamine in Surimi-Meat Frankfurters. *Journal of Food Safety*, 13(2), 125-132.

Pierre, F. H. F., O. C. B. Martin, R. L. Santarelli, S. Taché, N. Naud, F. Guéraud, M. Audebert, J. Dupuy, N. Meunier, D. Attaix, J. Vendeuvre, S. S. Mirvish, G. C. G. Kuhnle, N. Cano, & D. E. Corpet (2013). Calcium and α-tocopherol suppress cured-meat promotion of chemically induced colon carcinogenesis in rats and reduce associated biomarkers in human volunteers. *The American Journal of Clinical Nutrition*, 98(5), 1255-1262.

Pierson, M. D. (1982). Nitrite, nitrite alternatives, and the control of Clostridium botulinum in cured meats. *CRC critical reviews in food science and nutrition*, 17(2), 141-187.

Planas, C., Ó Palacios, F. Ventura, J. Rivera, & J. Caixach (2008). Analysis of nitrosamines in water by automated SPE and isotope dilution GC/HRMS: Occurrence in the different steps of a drinking water treatment plant, and in chlorinated samples from a reservoir and a sewage treatment plant effluent. *Talanta*, 76(4), 906-913.

Poulsen, M. E., M. Wenneker, J. Withagen, & H. B. Christensen (2012). Pesticide residues in individual versus composite samples of apples after fine or coarse spray quality application. *Crop Protection*, 35(0), 5-14.

Przybylski, C., & C. Segard (2009). Method for routine screening of pesticides and metabolites in meat based baby-food using extraction and gas chromatography-mass spectrometry. *Journal of Separation Science*, 32(11), 1858-1867.

Rasmussen, R. R., I. M. L. D. Storm, P. H. Rasmussen, J. Smedsgaard, & K. F. Nielsen (2010). Multi-mycotoxin analysis of maize silage by LC-MS/MS. *Analytical and Bioanalytical Chemistry*, 397(2), 765-776.

Rath, S., & Felix Guillermo, R. R. (2008). Nitrosamines, In *Handbook of Processed Meats and Poultry Analysis,* Edited by Leo M . L . Nollet and Fidel Toldr (pp. 687-705). CRC Press. ISBN: 978-1-4200-4531-4

Ripollés, C., E. Pitarch, J. V. Sancho, F. J. López, & F. Hernández (2011). Determination of eight nitrosamines in water at the ng/L levels by liquid chromatography coupled to atmospheric pressure chemical ionization tandem mass spectrometry. *Analytica Chimica Acta*, 702(1), 62-71.

Rogers, R.W.. Manufacturing of Reduced-Fat, Low-Fat, and Fat-Free emulsion Sausage, In *Meat science and applications.* Edited by Hui,Y.H.; Nip, W-K.; Rogers, R.W.; Young, O.A.. Marcel Dekker Inc., New York, US.

Rohrmann, S., K. Overvad, H. B. Bueno-de-Mesquita, M. U. Jakobsen, R. Egeberg, A. Tjønneland, L. Nailler, M. Boutron-Ruault, F. Clavel-Chapelon, V. Krogh, D. Palli, S. Panico, R. Tumino, F. Ricceri, M. M. Bergmann, H. Boeing, K. Li, R. Kaaks, K. Khaw, N. J. Wareham, F. L. Crowe, T. J. Key, A. Naska, A. Trichopoulou, D. Trichopoulos, M. Leenders, P. H. M. Peeters, D. Engeset, C. L. Parr, G. Skeie, P. Jakszyn, M. Sánchez, J. M. Huerta, M. L. Redondo, A. Barricarte, P. Amiano, I. Drake, E. Sonestedt, G. Hallmans, I. Johansson, V. Fedirko, I. Romieux, P. Ferrari, T. Norat, A. C. Vergnaud, E. Riboli, & J. Linseisen (2013). Meat consumption and mortality - results from the European Prospective Investigation into Cancer and Nutrition. *BMC Medicine*, 11(1).

Rywotycki, R. R., & Y. Ryszard (2002). The effect of selected functional additives and heat treatment on nitrosamine content in pasteurized pork ham. *Meat Science*, 60(4), 335-339.

Rywotycki, R. (2007). The effect of baking of various kinds of raw meat from different animal species and meat with functional additives on nitrosamine contamination level. *Food Chemistry*, 101(2), 540-548.

Rywotycki, R. (2003). Meat nitrosamine contamination level depending on animal breeding factors. *Meat Science*, 65(1), 669-676.

Santarelli, R. L., F. Pierre, & D. E. Corpet (2008). Processed Meat and Colorectal Cancer: A Review of Epidemiologic and Experimental Evidence. *Nutrition and cancer*, 60(2), 131-144.

Santarelli, R. L., J. Vendeuvre, N. Naud, S. Tache, F. Gueraud, M. Viau, C. Genot, D. E. Corpet, & F. H. F. Pierre (2010). Meat Processing and Colon Carcinogenesis: Cooked, Nitrite-Treated, and Oxidized High-Heme Cured Meat Promotes Mucin-Depleted Foci in Rats. *Cancer Prevention Research*, 3(7), 852-864.

Schothorst, R. C., & H. H. J. Somers (2005). Determination of N-nitrosodiethanolamine in cosmetic products by LC–MS–MS. *Analytical and Bioanalytical Chemistry*, 381(3), 681-685.

Sen, N. P., P. A. Baddoo, & S. W. Seaman (1986). N-Nitrosothiazolidine and N-Nitrosothiazolidine-4-Carboxylic Acid in Smoked Meats and Fish. *Journal of Food Science*, 51(3), 821-825.

Sen, N. P., & S. J. Kubacki (1987). Review of methodologies for the determination of nonvolatile N-nitroso compounds in foods. *Food additives and contaminants*, 4(4), 357-383.

Sen, N., P. Baddoo, & S. Seaman (1993). Studies on the Occurrence and Formation of 2-(Hydroxymethyl)-N-Nitrosothiazolidine-4-Carboxylic Acid (Hmntca) and 2-(Hydroxymethyl)-N-Nitrosothiazolidine (Hmnthz) in various Cured Smoked Meats, Fish and Cheese. Journal of the science of food and agriculture, 61(3), 353-356.

Sen, N. P., B. Donaldson, S. Seaman, J. R. Iyengar, & W. F. Miles (1976). Inhibition of nitrosamine formation in fried bacon by propyl gallate and L-ascorbyl palmitate. *Journal of Agricultural and Food Chemistry*, 24(2), 397-401.

Sen, N. P., S. W. Seaman, P. A. Baddoo, & D. Weber (1988). Further Studies on the Formation of Nitrosamines in Cured Pork Products Packaged in Elastic Rubber Nettings. *Journal of Food Science*, 53(3), 731-734.

Sen, N. P., S. Seaman, & W. F. Miles (1979). Volatile nitrosamines in various cured meat products: effect of cooking and recent trends. *Journal of Agricultural and Food Chemistry*, 27(6), 1354-1357.

Sewram, V., G. S. Shephard, W. F. O. Marasas, & de Castro, M. F. P. M. (2003). Improving Extraction of Fumonisin Mycotoxins from Brazilian Corn-Based Infant Foods. *Journal of food protection*, 66 854-859.

Simko, P. (2011). Heat and processing generated contaminants in processed meats. *In Processed meats - Improving safety, nutrition and quality*, edited by Kerry, J.P & Kerry, J.F. Woodhead Publishing, Cambridge, UK.

Skibsted, L. H. (2011). Nitric oxide and quality and safety of muscle based foods. *Nitric Oxide*, 24(4), 176-183.

Spiegelhalder, B., G. Eisenbrand, & R. Preussmann (1980). Occurence of volatile nitrosamines in food: A survey of the West German market. *IARC scientific publications*, No.31.

Startin, J. R. (1996). Individual non-volatile N-nitroso compounds in foods. *European Journal of Cancer Prevention*, 5 27-28.

Stuff, J. E., E. T. Goh, S. L. Barrera, M. L. Bondy, & M. R. Forman (2009). Construction of an N-nitroso database for assessing dietary intake. *Journal of Food Composition and Analysis*, 22(Supplement 1), S42-S47.

Sunen, E., B. Fernandez-Galian, & C. Aristimuno (2001). Antibacterial activity of smoke wood condensates against Aeromonas hydrophila, Yersinia enterocolitica andListeria monocytogenes at low temperature. *Food Microbiology*, 18(4), 387-393.

Sung, J. H., I. S. Kwak, S. K. Park, H. I. Kim, H. S. Lim, H. J. Park, & S. H. Kim (2010). Liquid chromatography-tandem mass spectrometry determination of N-nitrosamines released from rubber or elastomer teats and soothers. *Food Additives & Contaminants: Part A*, 27(12), 1745-1754.

Theiler, R. F., K. Sato, T. G. Aspelund, & A. F. Miller (1981). Model System Studies on N-Nitrosamine Formation in Cured Meats: The Effect of Curing Solution Ingredients. *Journal of Food Science*, 46(4), 996-998.

Theiler, R., K. Sato, T. Aspelund, & A. Miller (1984). Inhibition of N-Nitrosamine Formation in a Cured Ground Pork Belly Model System. *Journal of Food Science*, 49(2), 341-344.

Topuz, E., E. Aydin, & E. Pehlivanoglu-Mantas (2012). A Practical LC-MS/MS Method for the Detection of NDMA at Nanogram per Liter Concentrations in Multiple Water Matrices. *Water, Air, & Soil Pollution*, 223(9), 5793-5802.

Träger, A. W. B. (2013). Nitrosamines in nitrite preserved meat products. Master Thesis project report, National Food Insitute, Technical University of Denmark.

Tricker, A. R., & S. J. Kubacki (1992). - Review of the occurrence and formation of non-volatile N-nitroso compounds in foods. *Food Additives and Contaminants*, (1), 39.

Tricker, A. R., B. Pfundstein, E. Theobald, R. Preussmann, & B. Spiegelhalder (1991). Mean daily intake of volatile N-nitrosamines from foods and beverages in West Germany in 1989-1990. *Food and Chemical Toxicology*, 29(11), 729-732.

Tricker, A. R., & R. Preussmann (1991). Carcinogenic N-nitrosamines in the diet: occurrence, formation, mechanisms and carcinogenic potential. *Mutation Research/Genetic Toxicology*, 259(3-4), 277-289.

Tricker, A. R., M. J. Perkins, R. C. Massey, & D. J. McWeeny (1985). Some nitrosoamino acids in bacon adipose tissue and their contribution to the total N-nitroso compound concentration. *Zeitschrift für Lebensmittel-Untersuchung und Forschung*, 180(5), 379-383.

Wang, L., H. Hsia, & C. Wang (2006). Simultaneous Determination of Five Volatile and Non-Volatile N-Nitrosamines in Biological Fluids and Cosmetic Products by Liquid Chromatography with Photodiode Array Detection. *Journal of Liquid Chromatography & Related Technologies*, 29(12), 1737-1751.

Wong, H. L., S. M. Murphy, & S. S. Hecht (2005). Cytochrome P450 2A-catalyzed metabolic activation of structurally similar carcinogenic nitrosamines: N'-nitrosonornicotine enantiomers, N-nitrosopiperidine, and N-nitrosopyrrolidine. *Chemical research in toxicology*, 18, 61-69.

Wu, J., W. S. Rickert, A. Masters, & P. Joza (2012). Determination of N-nitrososarcosine in tobacco and smokeless tobacco products using isotope dilution liquid chromatography tandem mass spectrometry. *Analytical Methods*, 4(10), 3448-3452.

Xiong, W., H. Hou, X. Jiang, G. Tang, & Q. Hu (2010). Simultaneous determination of four tobacco-specific N-nitrosamines in mainstream smoke for Chinese Virginia cigarettes by liquid chromatography–tandem mass spectrometry and validation under ISO and "Canadian intense" machine smoking regimes. *Analytica Chimica Acta*, 674(1), 71-78.

Yurchenko, S., & U. Mölder (2007). The occurrence of volatile N-nitrosamines in Estonian meat products. *Food Chemistry*, 100(4), 1713-1721.

Yurchenko, S., & U. Mölder (2006). Volatile N-nitrosamines in various fish products. *Food Chemistry*, 96(2), 325-333.

Zeilmaker, M. J., M. I. Bakker, R. Schothorst, & W. Slob (2010). Risk Assessment of N-nitrosodimethylamine Formed Endogenously after Fish-with-Vegetable Meals. *Toxicological Sciences*, 116(1), 323-335.

Zhao, Y., S. L. Garrison, C. Gonzalez, W. D. Thweatt, & M. Marquez (2007). N-Nitrosation of amines by NO2 and NO: A theoretical study. *Journal of Physical Chemistry A*, 111(11), 2200-2205.

Zhao, Y., J. Boyd, S. E. Hrudey, & X. Li (2006). Characterization of New Nitrosamines in Drinking Water Using Liquid Chromatography Tandem Mass Spectrometry. *Environmental science & technology*, 40(24), 7636-7641.

Appendix A – Levels of NA in meat balls prepared with three different levels of nitrite

Table 1: Levels of NA found in meat balls prepared from minced pork meat with different levels of nitrite added (0, 150, 300 and 600 mg kg^{-1}) and stored at 10°C for 2 or 13 days. The meat balls were analysed in duplicate and the individual analytical results are presented (Träger, 2013).

	sodium nitrite added (mg kg^{-1})						
	0	150		300		600	
Stored for 2 days							
NHPRO		5.0	5.9	9.5	14.2	62.9	74.0
NPRO		17.4	17.7	49.9	40.4	190	192
NPIP						0.4	0.4
NTCA		22.1	20.3	23.8	25.7	22.8	26.1
NMTCA		11.6	11.9	16.0	17.0	13.5	16.2
Stored for 13 days							
NHPRO		4.5	4.9	5.1	8.1	21.5	30.9
NPRO		17.7	15.5	43.7	41.6	173	148
NPIP				0.2	0.3	0.9	1.1
NTCA		40.1	25.9	34.9	35.8	31.7	26.7
NMTCA						3.7	2.9

Appendix B – Experimental design, and Minitab figure for seven factor study on meat balls

Table 1: Experimental design of the 2-level 1/8 fraction factorial design (resolution IV) including seven factors. "–" indicate a low level and "+" indicate a high level of the respective factors. The design was generated using Minitab version 6.0. (Träger, 2013).

| Factors/Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
SodiumNitrite	-	+	-	+	+	+	-	+	-	-	-	-	-	+	+	+
SodiumChloride	-	+	-	-	-	-	+	+	+	+	-	-	+	-	+	+
SodiumErythorbate	-	+	+	-	+	+	-	-	-	+	+	-	+	-	+	-
SodiumTriPolyPhosphate	+	+	-	+	+	-	+	-	-	+	+	-	-	-	-	+
Dextrose	-	+	+	+	-	-	+	-	+	-	+	-	-	+	+	-
Liquid Smoke	+	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-
Storage Time	+	+	+	-	+	-	+	+	-	-	-	-	+	+	-	-

Main effects and interaction plots obtained by analysing the detected levels of NA in the 16 types of samples dictated by the factorial design above (Träger, 2013).
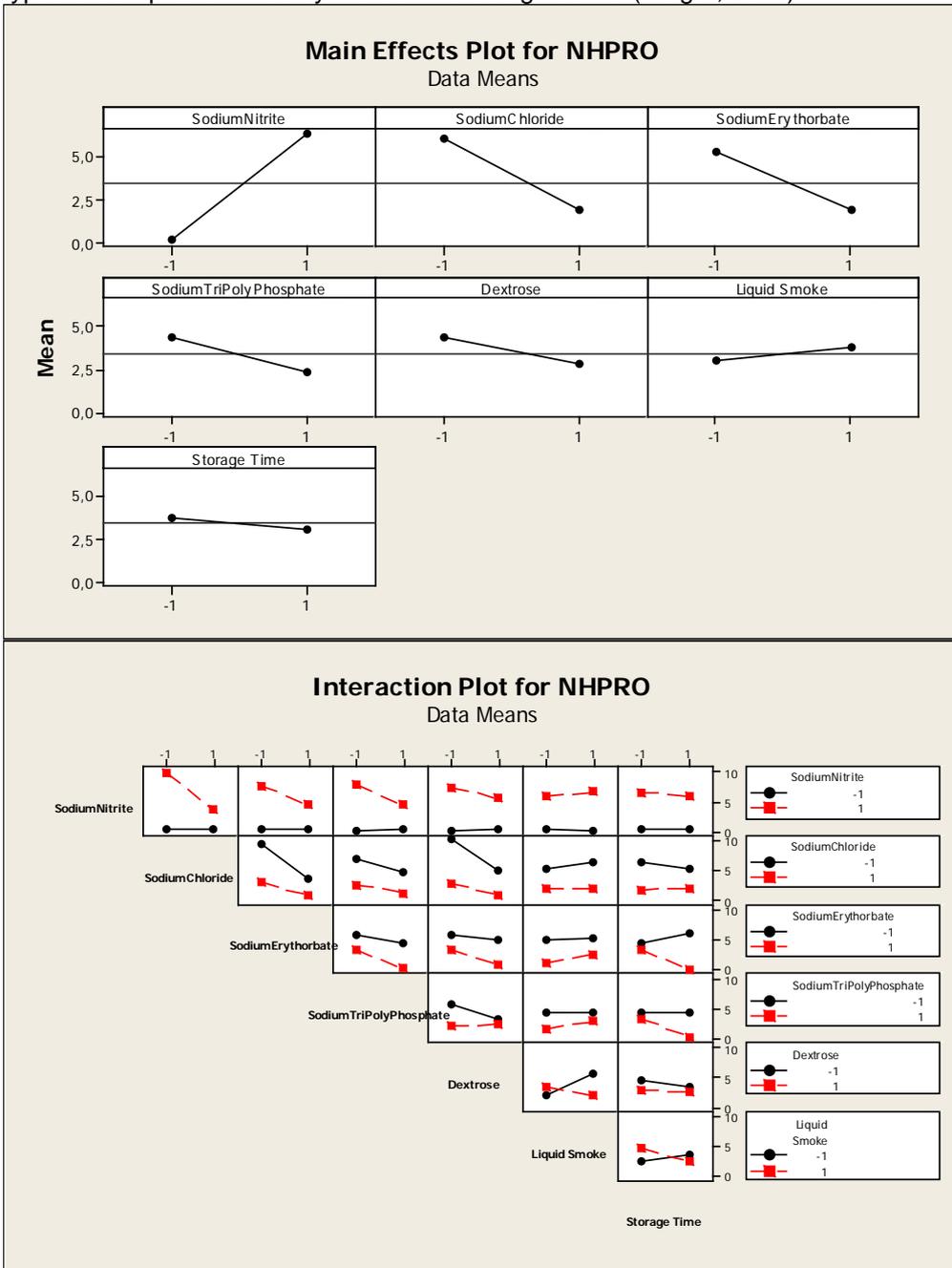
Main Effects Plot for NPRO
Data Means
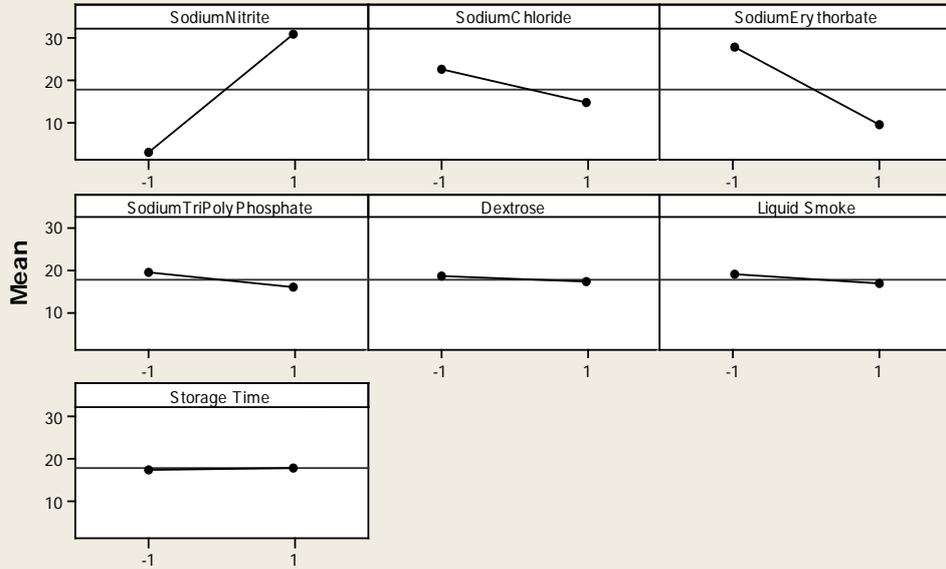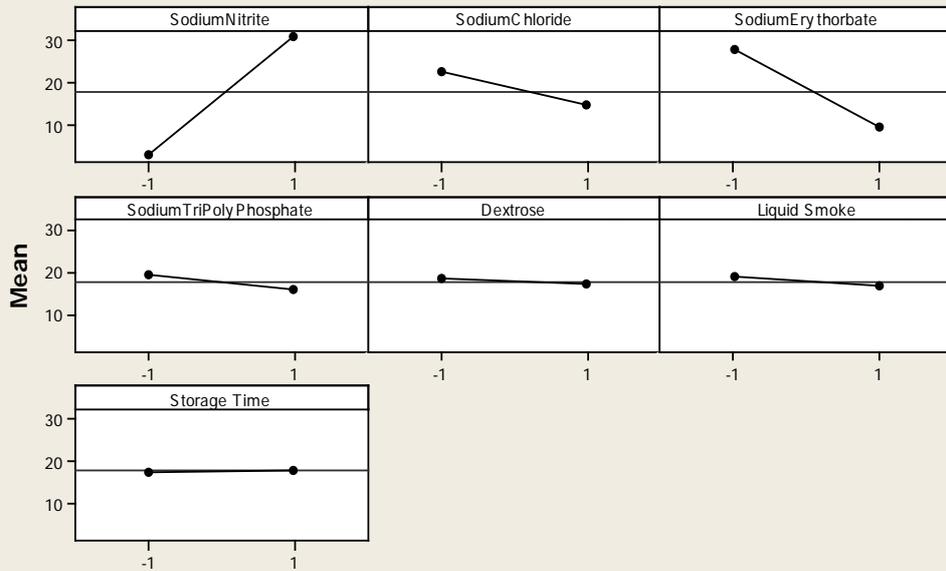
Main Effects Plot for NPRO
Data Means

Main Effects Plot for NTCA
Data Means
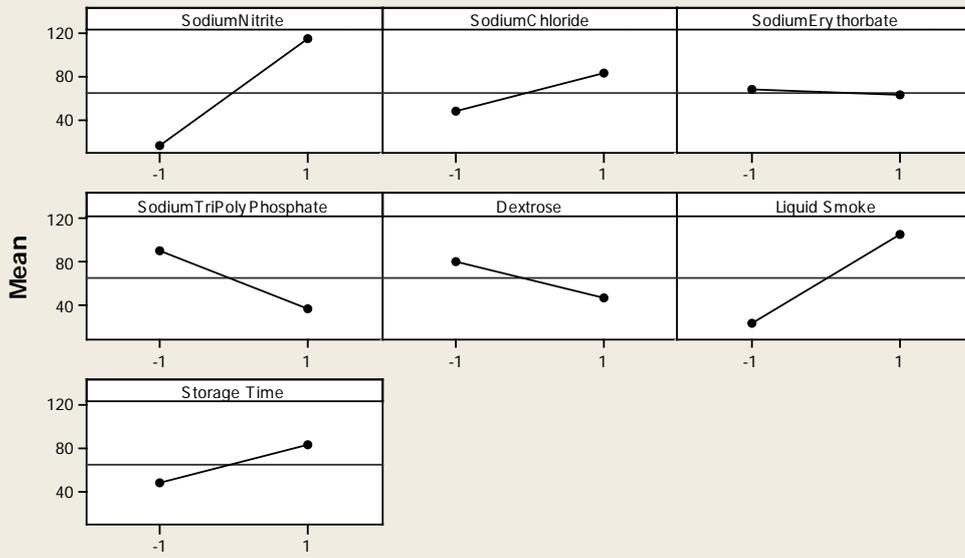
Interaction Plot for NTCA
Data Means
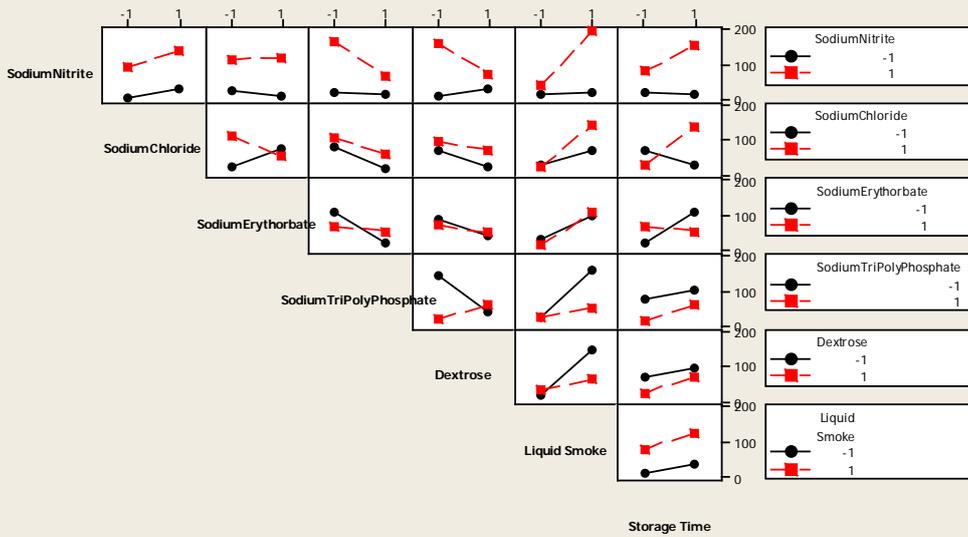
Main Effects Plot for NMTCA
Data Means
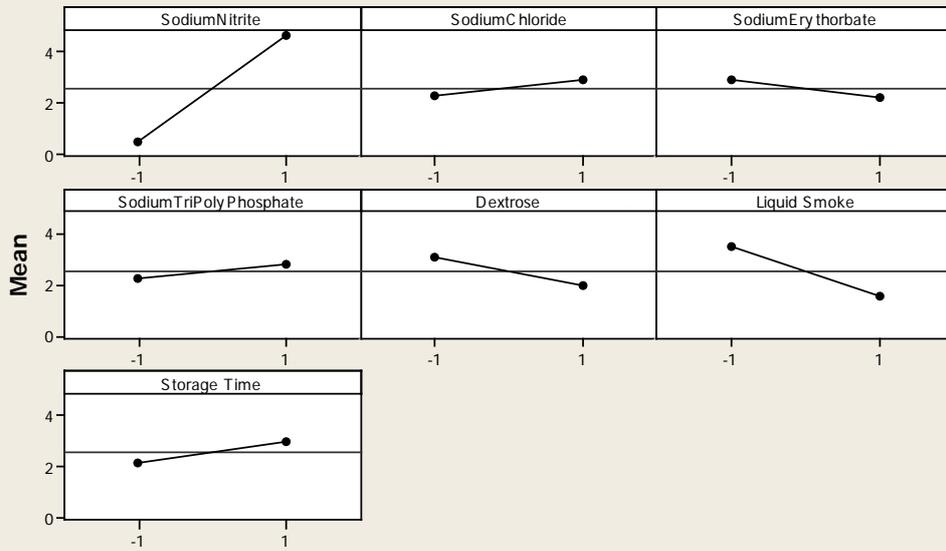
Interaction Plot for NMTCA
Data Means
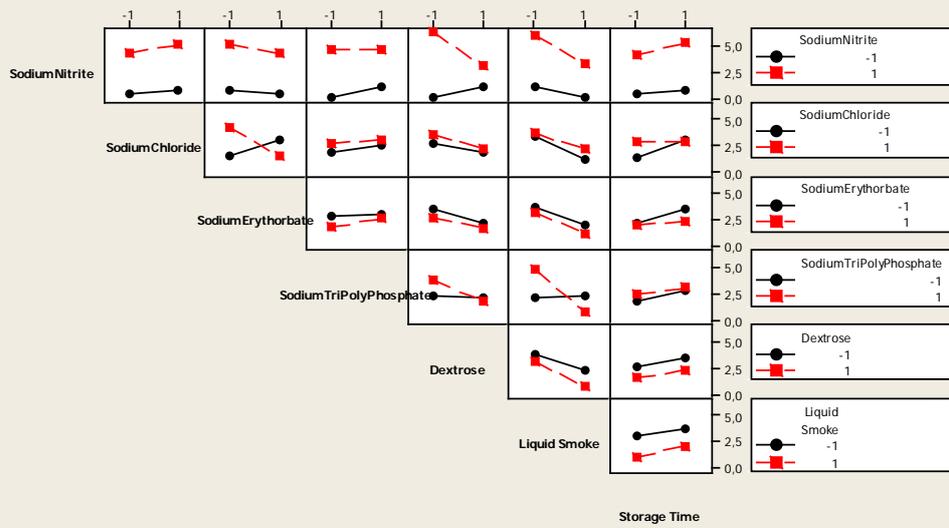
Appendix C - Experimental design, and Minitab figures for three factor study on meat balls

Table 1: Experimental design of the 2-level full factorial design including three factors. "–" indicate a low level and "+" indicate a high level of the respective factors. The design was generated using Minitab version 6.0. (Träger, 2013).

Factors/Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
SodiumNitrite	+	+	+	+	-	+	-	-	+	-	+	-	-	-	-	+
SodiumChloride	-	-	+	+	-	+	+	+	-	+	-	-	-	-	+	+
SodiumErythorbate	-	-	+	-	-	+	-	+	+	+	+	-	+	+	-	-

Main effects and interaction plots obtained by analysing the detected levels of NA in the 16 types of samples dictated by the factorial design above. An asterisk indicate that the main effect or the interaction was statistically significant (95% confidence level) (Träger, 2013).
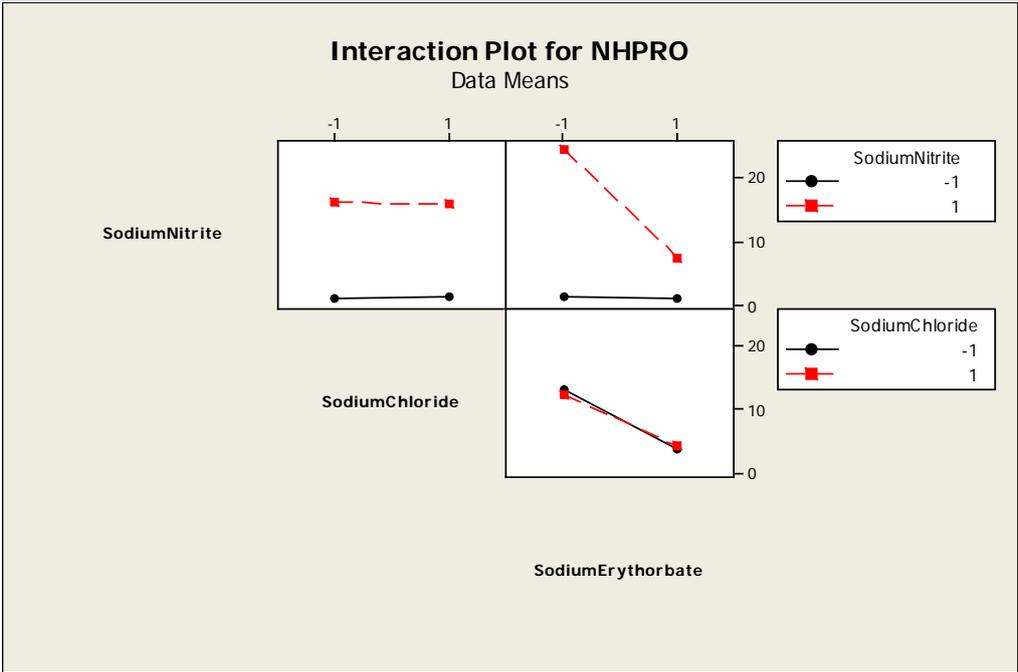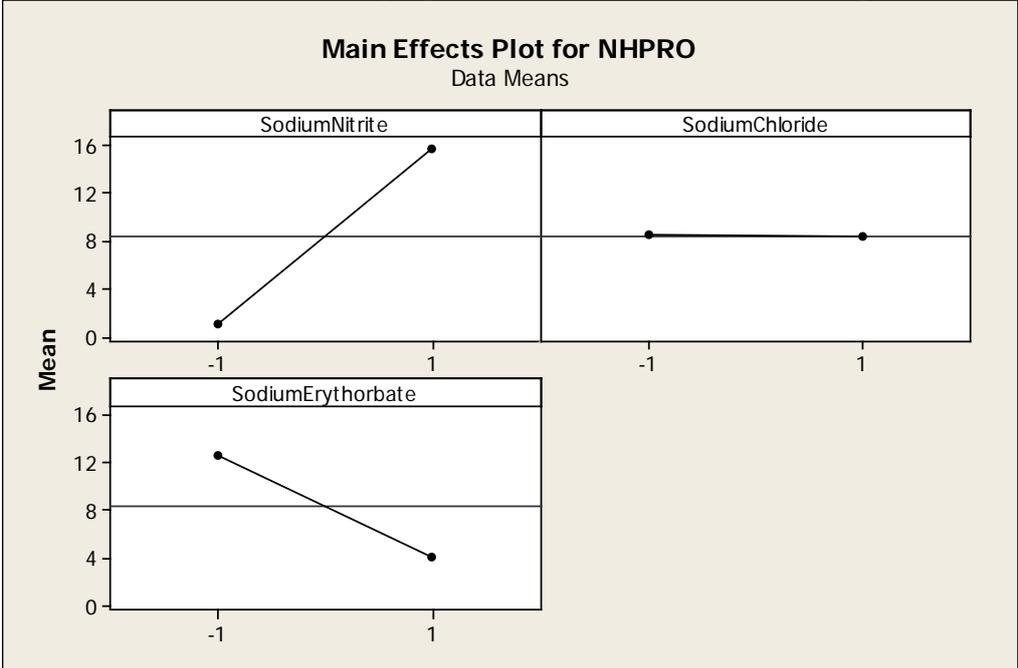
Main Effects Plot for NPRO
Data Means
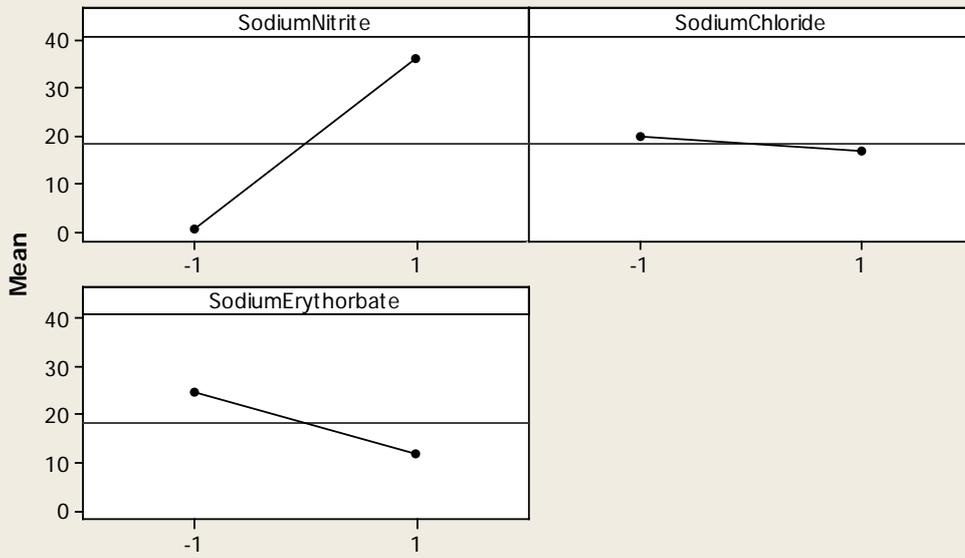
Interaction Plot for NPRO
Data Means
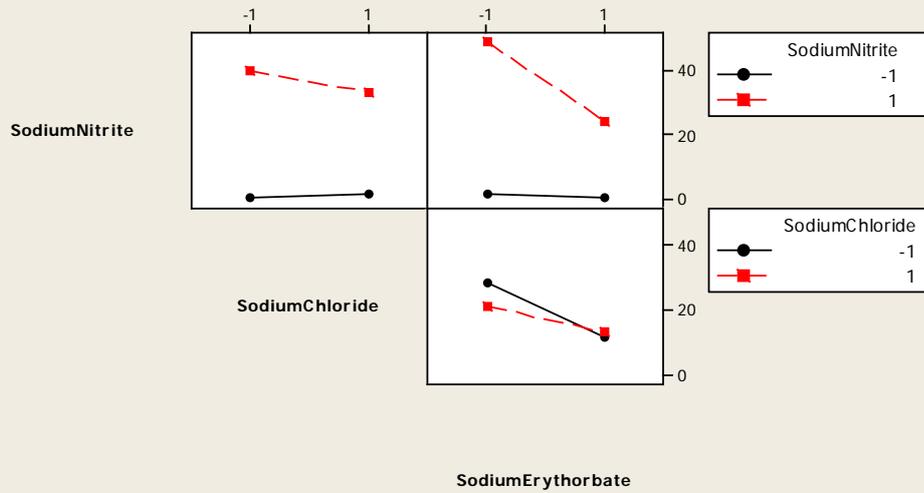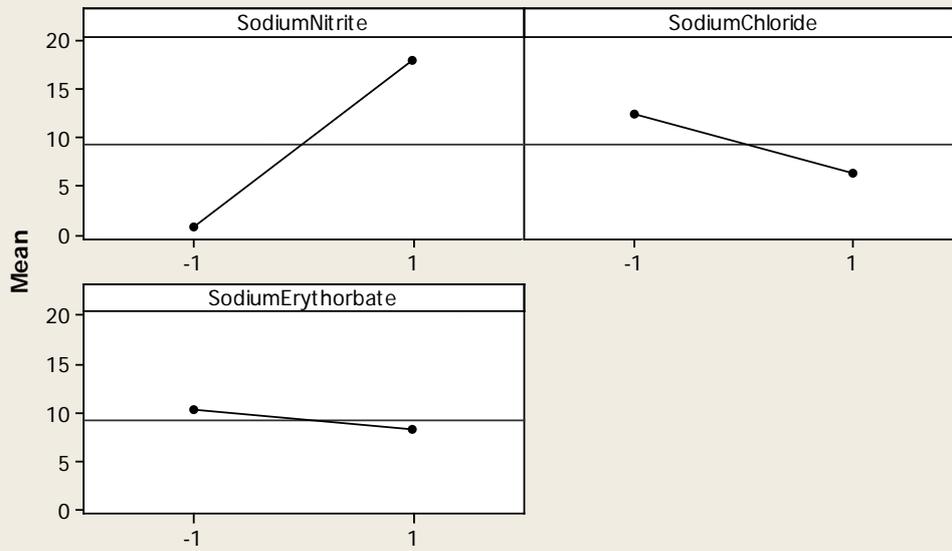
Main Effects Plot for NTCA
Data Means

Interaction Plot for NTCA
Data Means
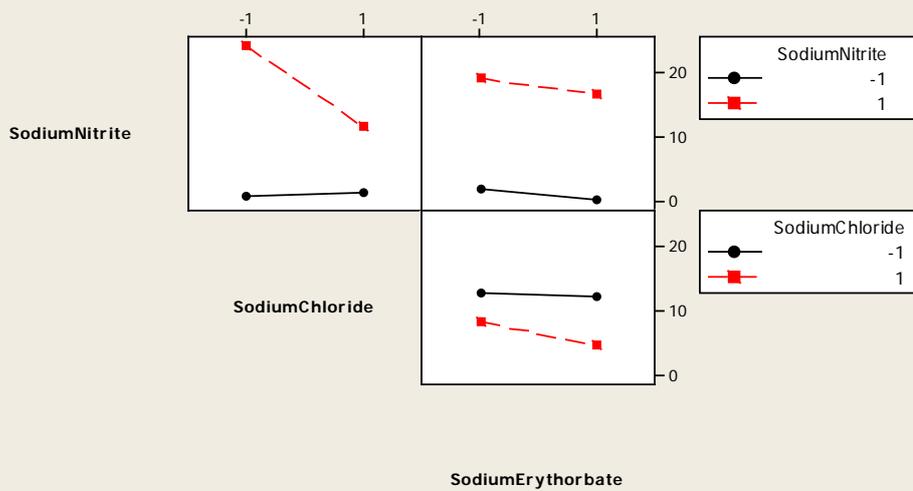
Main Effects Plot for NMTCA
Data Means
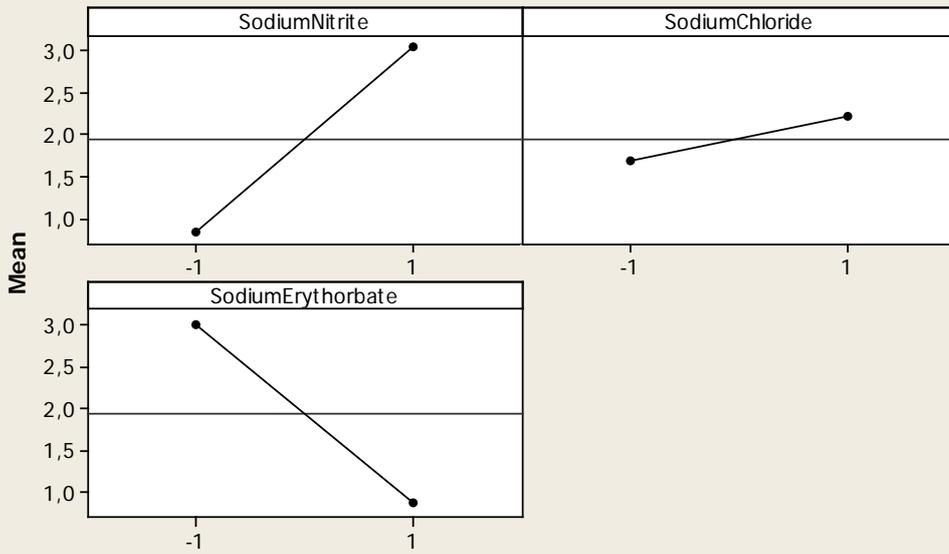
Interaction Plot for NMTCA
Data Means
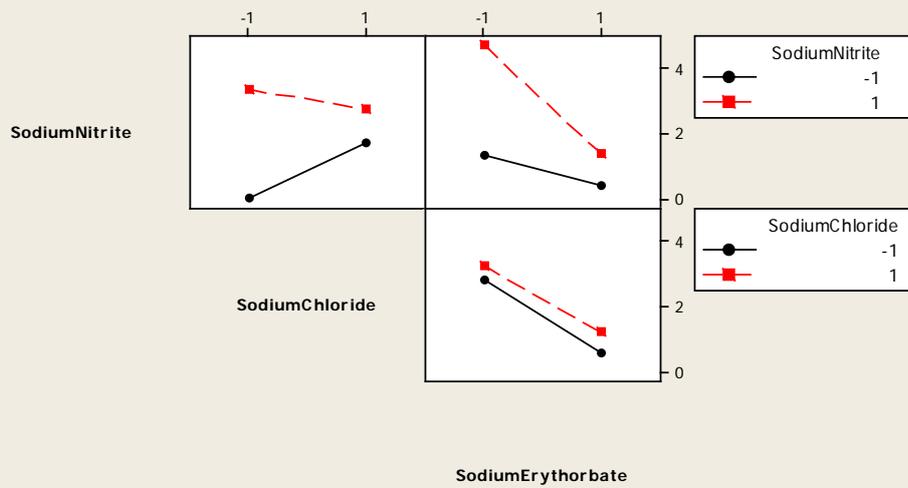
Appendix D - Supplementing figures to Paper III regarding NSAR, NDMA and NPYR

The results obtained for NSAR, NDMA and NPYR for factorial studies performed on sausages, described in Chapter 6.3 and 6.4 presented in the form of main effects plots, interaction plots and surface plots.
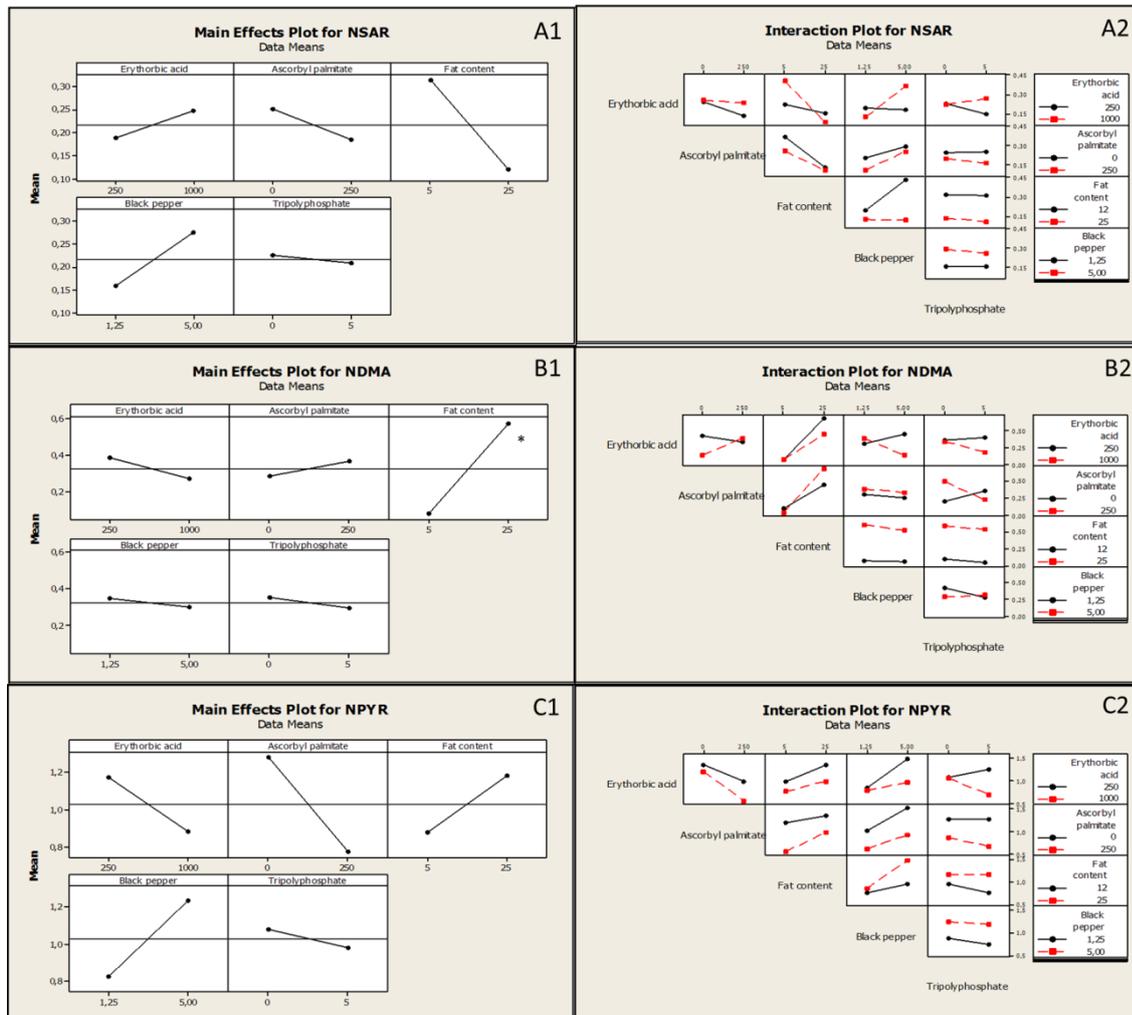
Figure 1: Main effects (A1-C1) and interactions (A2-C2) of erythorbic acid (250 or 1000 mg kg^{-1}), ascorbyl palmitate (0 or 250 mg kg^{-1}), fat content (~12 or ~25%) and black pepper (1.25 or 5 g kg^{-1}) on the levels of NSAR, NDMA and NPYR in cooked sausages prepared with 150 mg kg^{-1} sodium nitrite and stored for 24 hours at 5°C. Data analysis was performed using Minitab (version 6).
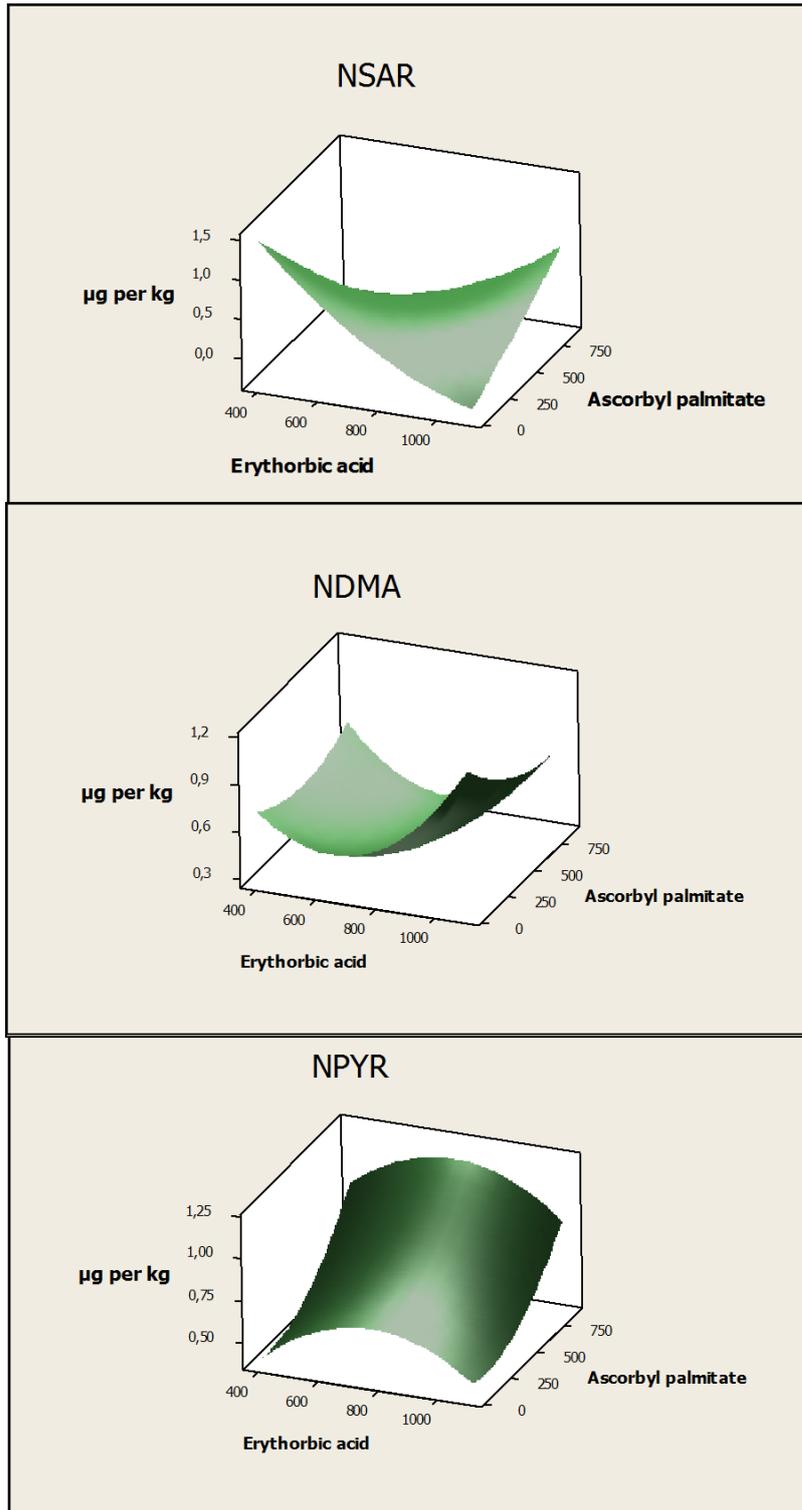
Figure 2: Response surface plots for the effect of erythorbic acid (396, 500, 750, 1000 and 1104 mg kg^{-1}) and ascorbyl palmitate (26, 150, 450, 750 and 874 mg kg^{-1}) on the levels of NSAR, NDMA and NPYR in cooked sausages prepared with sodium nitrite (150 mg kg^{-1}). Because of the low levels, close to the LOQ of the applied method, the effects are associated with relatively high uncertainty and the figures have therefore not been included in Paper III.
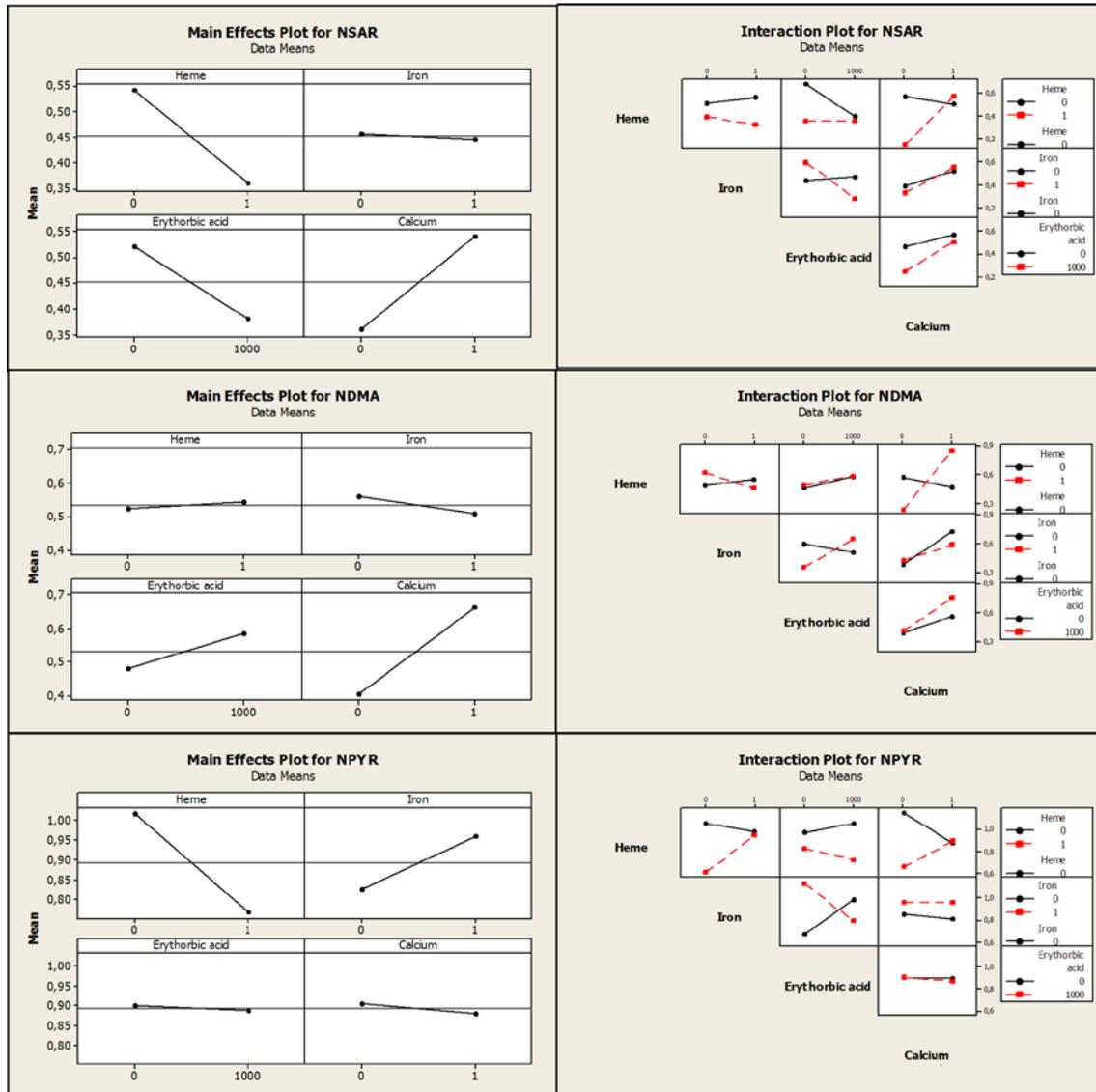
Figure 3: Main effects and interactions for the effect of heme as myoglobin (0 or 1.5 g kg^{-1}), iron as iron(III)sulphate hydrate (0 or 36 mg kg^{-1}), erythorbic acid (0 or 1000 mg kg^{-1}) and calcium as calcium carbonate (0 or 6 g kg^{-1}) on the levels of NSAR, NDMA and NPYR in cooked sausages prepared with 150 mg kg^{-1} sodium nitrite and stored for 24 hours at 5°C. Data analysis was performed using Minitab (version 6).

12. Papers I-III and Manuscript I

Paper I

Simultaneous determination of volatile and non-volatile nitrosamines in processed meat products by liquid chromatography tandem mass spectrometry using atmospheric pressure chemical ionisation and electrospray ionisation

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ABSTRACT

A sensitive, selective and generic method has been developed for the simultaneous determination of the contents ($\mu g\,kg^{-1}$ range) of both volatile nitrosamines (VNA) and non-volatile nitrosamines (NVNA) in processed meat products. The extraction procedure only requires basic laboratory equipment and a small volume of organic solvent. Separation and quantification were performed by the developed LC–(APCI/ESI)MS/MS method. The method was validated using spiked samples of three different processed meat products. Satisfactory recoveries (50–130%) and precisions (2–23%) were obtained for eight VNA and six NVNAs with LODs generally between 0.2 and $1\,\mu g\,kg^{-1}$, though for a few analyte/matrix combinations higher LODs were obtained (3 to $18\,\mu g\,kg^{-1}$). The validation results show that results obtained for one meat product is not always valid for other meat products. We were not able to obtain satisfactory results for N-nitrosohydroxyproline (NHPRO), N-nitrosodibenzylamine (NDBzA) and N-nitrosodiphenylamine (NDPhA). Application of the APCI interface improved the sensitivity of the method, because of less matrix interference, and gave the method a wider scope, as some NAs were ionisable only by APCI. However, it was only possible to ionize N-nitroso-thiazolidine-4-carboxylic acid (NTCA) and N-nitroso-2-methyl-thiazolidine-4-carboxylic acid (NMTCA) by ESI. The validated method was applied for the analysis of processed meat products and contents of N-nitrosodimethylamine (NDMA), N-nitrosopyrrolidine (NPYR), N-nitrosomethylaniline (NMA), N-nitrosoproline (NPRO), NTCA, and NMTCA were found in one or several nitrite cured meat products, whereas none were detected in non-nitrite cured bacon.

1. Introduction

Nitrite (E 249–E 250) has been used for preservation of meat products for decades and is still a widely used preservative for products as e.g. bacon, sausages and luncheon meats. Nitrite curing provides efficient inhibition of the growth of *Clostridium botulinum* [1], and it thereby lowers the risk of botulism, and in addition it results in the development of unique colour, flavours and aromas [2]. However, nitrite can react with secondary amines present in the meat to form N-nitrosamines (NAs), many of which are genotoxic [3]. Several studies and reviews (e.g. [4,5]) conclude that there is an association between dietary intake of processed meat and increased risk of cancer, especially gastric cancer. Recently a large study revealed an association between increased mortality

and a daily intake of processed meat above only 20 g [6]. Based on a review of all published cohort and case-control studies from 1985–2005, Jakszyn & Gonzalez 2006 [7] concluded that the available evidence supports a positive association between nitrite and nitrosamine intake and gastric cancer, as well as between the intake of meat including processed meat and gastric cancer and oesophageal cancer [7].

A literature study reveals that about 20 different NAs have been identified in processed meat products. In general the NAs are defined as either volatile NAs (VNA) or non-volatile NAs (NVNA) where the VNAs are applicable to extraction by steam distillation and/or to analysis by gas chromatography and the NVNA are not. The levels of NAs in nitrite preserved meat products vary greatly, from below detectability to levels in the order of thousand $\mu g\,kg^{-1}$, depending on the type of NA and the type of meat products. Of the most commonly found NAs in meat, N-nitrosodiethylamine (NDEA) has been evaluated as the most potent carcinogen, whereas N-nitrosodimethylamine

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(NDMA) has lower potency and N-nitrosopyrrolidine (NPYR) and N-nitrosopiperidine (NPIP) even lower [8]. N-nitrosoproline (NPRO) and N-nitrosohydroxyproline (NHPRO) have been shown to be noncarcinogenic [9]. Knowledge is however still needed regarding the genotoxicity and/or carcinogenicity of some NAs, such as N-nitroso-thiazolidine-4-carboxylic acid (NTCA) and N-nitroso-2-methyl-thiazolidine-4-carboxylic acid (NMTCA) [9]. In general the occurrence and toxicological profiles of the NVNA are less studied.

The VNAs, which are frequently found but in relatively low amounts of $\leq 5\,\mu g\,kg^{-1}$ [10–12], have been the subject of the majority of the available literature. Most of these studies were conducted in the 1970s and early 1980s. Furthermore, most of these early studies only investigated one to five NAs. NDMA and NPYR are the most frequently found VNA in nitrite/nitrate treated meat products. For example Spiegelhalder et al. [13] reported 32% and 11% of such products (n = 395) to contain detectable levels (>0.5 $\mu g\,kg^{-1}$) of NDMA and NPYR, respectively. NDMA and NPYR at levels above $5\,\mu g\,kg^{-1}$ occur only in 2% and 6% of the samples, respectively [13]. Other types of VNA were only detected occasionally. The analytical procedures for the analysis of these volatile compounds in foods were well established at that time. The methods employed in the majority of these earlier studies as well as many recent studies [14,15] used gas chromatography for separation and detection by thermal energy analyser (TEA) [16,17]. The TEA detector provided higher sensitivity compared to GC–MS systems available at that time. In comparison with today's MS systems the TEA detector and UV detector have relatively lower specificity and therefore also a higher risk of false positive findings. Furthermore, because the TEA detector has limited versatility and is of relatively high cost this detector is not available in most laboratories. In previous studies dichloromethane, has been widely used as extraction solvent and concentration procedure using a Kuderna-Danish apparatus for VNA analysis [14,18,19]. Dichloromethane in many cases is an efficient extraction solvent; however because of its possible carcinogenic effect it has not been used in the present work.

On the other hand, knowledge on the occurrence and nature of NVNA in food is limited. The reported NVNA are primarily N-nitrosamino acids [14], which occur in processed meat products at significantly higher levels than the VNA [10]. For example N-nitroso-2-hydroxymethyl-thiazolidine-4-carboxylic acid (NHMTCA), NTCA and NMTCA were found in smoked cured meats at concentrations up to 2100 $\mu g\,kg^{-1}$ [20], 1600 $\mu g\,kg^{-1}$ and 98 $\mu g\,kg^{-1}$ [21], respectively. Publications describing methods for the determination of N-nitrosamino acids in meat products are available [18,22,23]. These studies are based on LC–TEA and extraction procedure consuming large amounts of organic solvent. More recently publications on methods for determination of N-nitrosamino acids using LC–MS/MS are available, but in matrices as tobacco [24] and smokeless tobacco products [25].

Since the 1970s and 1980s, the technology used by the industry to process meat and the consumption patterns of processed meats has changed. For example, it has become common procedure during production of most nitrite cured meat to add ascorbate or erythorbate as an antioxidant; hygiene and maintenance of the cool chain has been improved; fat contents, sodium chloride and nitrite levels have been reduced for most products. All these factors can affect the NA levels and the levels of NAs in processed meat products may therefore have changed since the majority of the studies in this field were performed.

In order to perform a well-founded and updated assessment of the risk associated with the human exposure to NAs via the consumption of nitrite preserved meats, data on the occurrence and nature of NAs, including both VNA and NVNA, in a wide range of processed meat products available on the market, is needed. For that purpose an analytical method allowing simultaneous extraction and determination of both VNA and NVNA in meat products

is an important tool. A method based on LC will allow for the determination of the NVNA and highly polar NAs, i.e. NHPRO, N-nitrososarcosine (NSAR) and NDMA. Several recent publications describe separation and detection of several VNAs by LC–MS or LC–MS/MS [26–28]. Application of tandem mass spectrometry would further provide high sensitivity and specificity. A method for the simultaneous determination of both VNA and NVNA by LC–MS/MS has, to our knowledge, not been published so far.

The aim of the present study was to develop and validate a method for the simultaneous determination of NVNA and VNA in processed meat products. It was chosen to base the assay on high performance liquid chromatography combined with atmospheric pressure chemical ionisation (APCI) and/or electro spray ionisation (ESI) with detection by tandem mass spectrometry (MS/MS) since this would provide high sensitivity and specificity as well as have the possibility to include both groups of NAs in the same assay. The extraction procedure should be a common procedure, as simple as possible and preferably without the use of dichloromethane. The target NAs have previously been reported to occur in processed meat products and they were commercially available as pure standards (chemical structures of the target NAs are presented in Fig. 1). The method was applied to ten samples of processed meat products purchased at local supermarkets.

2. Experimental

2.1. Chemicals

Acetonitrile (extraction solvent) was of HPLC grade (Rathburn Chemicals Ltd, Walkerburn, Scotland). Formic acid (purity 98–100% for analysis), pipecolic acid (PIC) (purity 98%), and the methanol used as LC eluent (purity $\geq$99.9%, Fluka-Analytical) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). The pure standards of N-nitrososarcosine (NSAR), N-nitrosohydroxyproline (NHPRO), N-nitrosodibenzylamine (NDBzA), N-nitrosoproline (NPRO), N-nitrosomethylaniline (NMA), N-nitroso-2-methyl-thiazolidine 4-carboxylic acid (NMTCA) and N-nitroso-thiazolidine-4-carboxylic acid (NTCA) were purchased from Toronto research chemicals (Toronto, Canada), whereas the standards of N-nitrosodiethylamine (NDEA), N-nitrosodipropylamine (NDPA), N-nitrosomorpholine (NMOR), N-nitrosodimethylamine (NDMA) and N-nitrosodiphenylamine (NDPhA) were purchased from Sigma-Aldrich Co. N-nitrosomethylethylamine (NMEA), N-nitrosopyrrolidine (NPYR), N-nitrosodibutylamine (NDBA), N-nitrosopiperidine (NPIP) were purchased from Dr. Ehrenstorfer (Ausburg, Germany). The internal standards N-nitrosodimethylamine-d_6 (NDMA-d_6) and N-nitrosopyrrolidine-d_8 (NPYR-d_8) were purchased from Sigma-Aldrich Co. and CDN isotopes (Quebec, Canada), respectively. The purity of all the NA standards was $\geq$98% except for NMA which were of 95% purity.

Stock solutions in methanol of 1 mg mL^{-1} were prepared for each of the NAs. A mixture of all the NA standards (standard solution) was then prepared by volumetrically dilution of the stock solutions with acetonitrile to 10 $\mu g\,mL^{-1}$. The stock solutions were stored at $-60\,°C$ and the standard solution was stored at $-18\,°C$.

2.2. Extraction procedure

A fractional factorial design was set up to systematically evaluate the influence of different factors on the extraction efficiencies. A seven factor design was constructed using the software Minitab (Version 16.24). The seven factors were sample size, volume of extraction solvent, introducing a second extraction, acidifying the extraction solvent, clean-up by liquid/liquid extraction with hexane and dilution of the extract with water. The effect of the seven
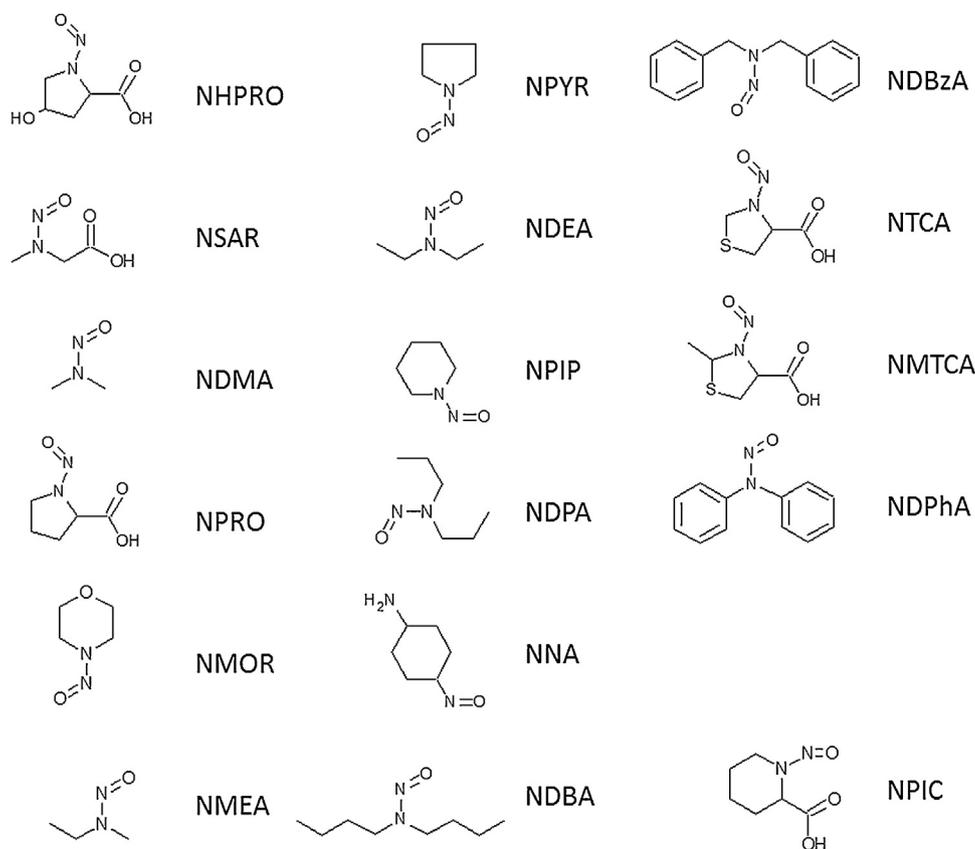
Fig. 1. Structures of the 16 N-nitrosamines relevant for the target NAs and NPIC. NHPRO (N-nitrosohydroxyproline), NSAR (N-nitrososarcosine), NDMA (N-nitrosodimethylamine), NPRO (N-nitrosoproline), NMOR (N-nitrosomorpholine), NMEA (N-nitrosomethylethylamine), NPYR (N-nitrosopyrrolidine), NDEA (N-nitrosodiethylamine), NPIP (N-nitrosopiperidine), NDPA (N-nitrosodi-n-propylamine), NMA (N-nitrosomethylaniline), NDBA (N-nitrosodibutylamine), NDBzA (N-nitrosodibenzylamine), NTCA (N-Nitroso-thiazolidine-4-carboxylic acid), NMTCA (N-Nitroso-2-methyl-thiazolidine 4-carboxylic acid), NDPhA (N-nitrosodiphenylamine) and NPIC (N-nitrosopipecolic acid).

factors on the recovery of the NAs were studied by extracting spiked samples of an organically produced chicken based luncheon meat sausage (9% fat) purchased from the local supermarket. The chicken sausage had been produced without the addition of nitrite, nitrate or vegetable concentrates and no NAs were therefore expected to be present. Eight samples were spiked with a mixture of the NAs resulting in a spike level of 50 μg kg⁻¹ sample for each of them and extracting by different procedures combining high or low/no treatment of each of the seven factor For the final optimized extraction procedure 2.5 g of homogenized processed meat product was weighed into a 50 mL polypropylene (PP) tube (Sarstedt, Nümbrecht, Germany). NPYR-d_8, NDMA-d_6 (both 9 μg kg⁻¹ sample) and pipe colic acid (PIC) (1 mg kg⁻¹ sample) in acetonitrile were added to each sample. If nitrosation processes occurred during the extraction procedure this would be indicated by the occurrence of N-nitrosopipecolic acid (NPIC) in the extract. QC samples were further spiked with 1, 3 or 30 μg kg⁻¹. After addition of 7.5 mL cold acetonitrile with 1% formic acid, the tubes were briefly vigorously shaken by hand and then for 10 min on a rotor table (Reac 2, Buch and Holm, Herlev, Denmark). The extraction was further facilitated by having a ceramic homogenizer (Agilent Technologies, Santa Clara, CA, USA) in each tube. After centrifugation, (Megafuge 3 OR, Heraeus Sepatech Gmbh, Osterode, Germany) for 10 min at 4500 g, 15 mL of the clear supernatant was transferred to a 15 mL PP tube and stored at −60 °C for minimum 15 min. The extracts were allowed to thaw and when still cold, centrifuged for 10 min at 4500 g. By this freezing out step insoluble co-extractives (e.g. lipids) precipitated and could thereby be separated from the extract. 5 mL of the acetonitrile phase was transferred to a Hamilton glass tube

(VWR-Bie & Berntsen, Herlev, Denmark) and evaporated under a stream of nitrogen (at 30 °C ± 0.5) (Supertherm, Mikrolab Aarhus A/S, Højbjerg, Denmark) to a volume of approx. 0.25 mL. The volume was adjusted to 1.0 mL by addition of Milli-Q water. An aliquot was transferred to 0.45 μm filter vial (PP filters, Whatman, Buckinghamshire, UK) and diluted 1:1 with Milli-Q. Calibration solutions were also prepared in filter vials and consisted of one part standard mixture diluted with either water, to obtain the appropriate calibration concentrations (0.334–100 ng mL⁻¹), or an appropriate meat sample extract to obtain matrix matched calibrations. NDMA-d_6 and NPYR-d_8 in acetonitrile were added as internal standards (ISTDs) to each calibration vials resulting in a final concentration of 6 ng mL⁻¹. These ISTDs were primarily used for assigning the peaks of NDMA and NPYR, but also for correction of the NDMA and NPYR recoveries when validating on kassler. The sample and calibration solutions were filtered and transferred to auto sampler vials with deactivated glass inserts (Agilent Technologies, Santa Clara, CA, USA), for analysis by LC–MS/MS first with an APCI and then an ESI interphase.

When the meat samples contained less than 70% water, some of the sample was replaced by Milli-Q water to obtain a water content of 70%. The water contents of the samples were estimated using data reported in the Danish food data bank (www.foodcomp.dk) or determined experimentally by drying of 5 g samples (in duplicate) under vacuum at 70 °C for at least 16 h or until the gravimetric determination did not change. No less than 1.8 g of sample was however extracted, thus e.g. salami samples which typically contain 30–35% water were adjusted to about 50% water.

2.3. Chromatographic separation and detection

The separation was performed by LC on an Agilent 1200 Series HPLC (Agilent Technologies, Santa Clara, CA, US) with a Poroshell Phenyl-Hexyl column 150 × 2.1 mm, 3 μm column (Agilent Technologies). The final mobile phase consisted of water (Eluent A) and methanol (Eluent B) both with 0.1% formic acid for both APCI and ESI. The gradient programme for APCI was as follows: the initial percentage of B was 5%, increasing to 10% over 3.5 min, then to 20% over 2 min, 80% over 4.5 min and finally to 90% over 1 min and held for 5 min. Between each run the column was equilibrated for 6 min with 2% B. The latter step was included, because it was found, that the amount of organic modifier in the first part of the eluent programme hereby could be increased, resulting in increased ionisation efficiency and still maintaining adequate retention of the highly polar NAs (NHPRO, NSAR and NDMA). The eluent programme for the analysis using ESI was as follows: the initial percentage of B was 30% B, which was increased to 50% over 3 min and then to 90% B over 2 min and held for 5 min. Between runs the column was equilibrated for 5 min with 30% B. The injection volume for both the APCI and ESI analysis was 3.5 μL.

MS/MS detection was performed on an Agilent 6460 Series Triple Quadrupole (Agilent Technologies) equipped with either an APCI or a Jet Stream ESI source. Collision gas was nitrogen. Detection was performed in Multiple Reaction Monitoring (MRM) mode with unit resolution of 0.7 atomic mass for both quadrupole 1 and 3. The MRM reactions were divided into four groups/segments and the dwell time was optimised to obtain at least 20 data-points across each peak (Fig. 2). MS parameters including choice of ionisation type, precursor ions, product ions, fragmentor and collision energies as well as source setting for the analytes included in the method are presented in Table 1. The transition giving the most intense instrument response was employed for quantification and the second most sensitive for qualification.

Mass Hunter workstation software (version B.01.04, Agilent Technologies) was used for instrument control, data acquisition and processing of data. Identification of the analytes in spiked matrices and real samples was based on the comparison of their retention times and quantifier (Q)/qualifier (q) ion ratios with those observed for matrix matched standard solutions.

2.4. Validation

Validation of the method was performed in accordance with ISO 5725-2 [29] to demonstrate specificity, precision, accuracy and lower limit of detection (LOD). Mean recovery, relative standard deviation for repeatability (RSD$_r$) and LOD were calculated for each of the 16 NAs (incl. NPIC). LOD was calculated as three times the standard deviation at the lowest accepted spike level no reference material was available, instead the accuracy of the method was determined using three different types of recovery test samples, i.e. kassler (~3% fat), salami (~24% fat) and bacon (15–30% fat) all purchased from local supermarkets. Each type of test samples were spiked with 1, 3 or 30 μg kg^{-1} of the selected NAs, with three to four replicates at each level.

3. Results and discussion

The aim was to develop a common method for the determination of both VNA and NVNA in processed meat products. Thus the employed extraction method should be able to extract the target NAs that represent a wide range in polarity from processed meat products with varying contents of fat, water, salt and spices etc. On the other hand the amount of co-extracted matrix should be as low as possible in order to prevent interference in the analytical response. LC can provide separation of a wide range of analytes if the right analytical column and mobile phases are selected but matrix induced ion suppression in the LC–MS/MS analysis should also be considered when choosing the optimum LC–column. Detection and quantification by tandem MS can provide high selectivity
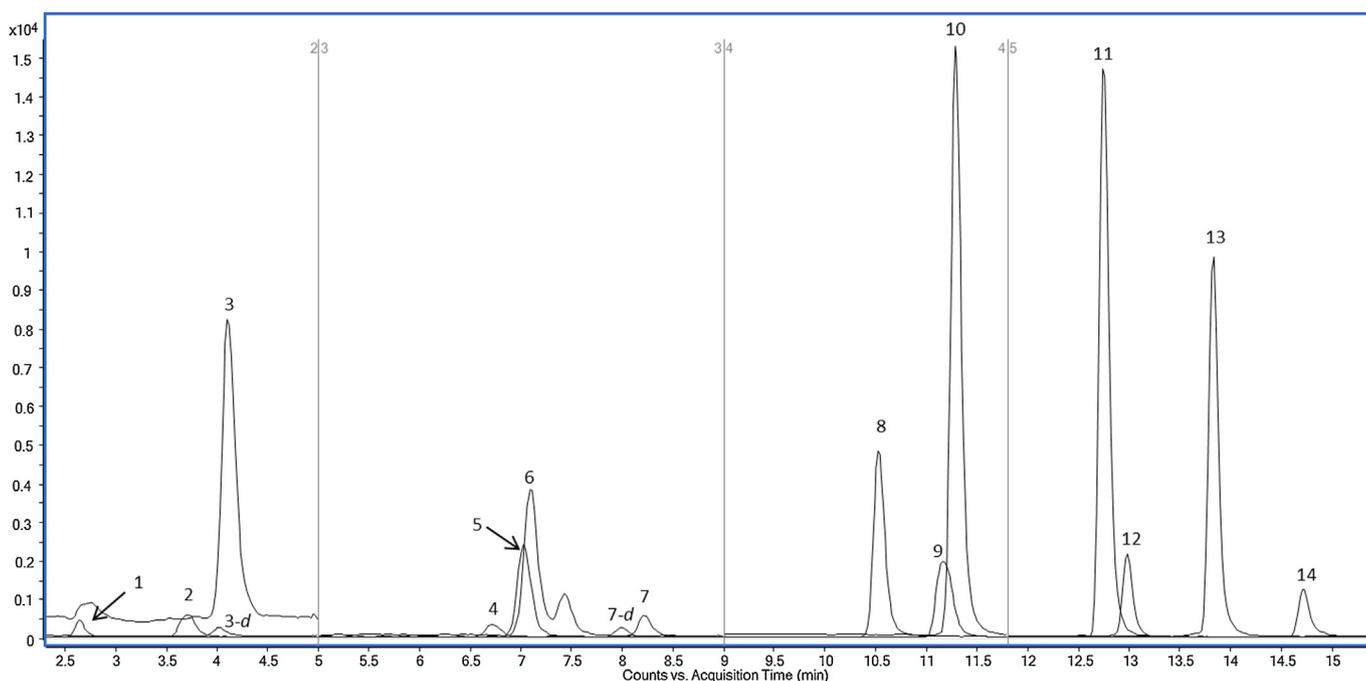
Fig. 2. HPLC(APCI)MS/MS chromatogram of standard mixture solution of 100 ng mL^{-1} of the VNA and NVNA: (1) N-nitrosohydroxyproline (NHPRO), (2) N-nitrososarcosine (NSAR), (3) N-nitrosodimethylamine (NDMA), (3d) NDMA-d_6, (4) N-nitrosoproline (NPRO), (5) N-nitrosomorpholine (NMOR), (6) N-nitrosomethylethylamine (NMEA), (7) N-nitrosopyrrolidine (NPYR), (7d) NPYR-d_8, (8) N-nitrosodiethylamine (NDEA), (9) N-nitrosopipecolic acid (NPIC), (10) N-nitrosopiperidine (NPIP), (11) N-nitrosodi-n-propylamine (NDPA), (12) N-nitrosomethylaniline (NMA), (13) N-nitrosodibutylamine (NDBA) and (14) N-nitrosodibenzylamine (NDBzA).

Table 1
APCI and ESI MS/MS optimised conditions for the NAs.

Compound name	Abbrev.	Nominal mass	Ionisation	Segment1	RT	Quantification m/z	Coll. energy	Qualification m/z	Coll. energy
N-nitrosohydroxyproline*	NHPRO	160.05	APCI (+)	2	2.6	161→68	20	161→86	10
N-nitrososarcosine*	NSAR	118.04	APCI (+)	2	3.6	119→44.1	5	119→61	15
N-nitrosodimethylamine-d_6 (ISTD)	NDMA-d_6	80.09	APCI (+)	2	3.9	81.1→46.1	2	81.1→81.1	0
N-nitrosodimethylamine	NDMA	74.05	APCI (+)	2	4	75 .1→75.1	0	75.1→43	10
N-nitrosoproline*	NPRO	144.05	APCI (+)	3	6.6	145→70	15	145→68	15
N-nitrosomorpholine	NMOR	116.06	APCI (+)	3	6.8	117.1→86	10	117.1→73	10
N-nitrosomethylethylamine	NMEA	88.06	APCI (+)	3	6.9	89→61	5	89→43	15
N-nitrosopyrrolidine-d_8 (ISTD)	NPYR-d_8	108.06	APCI (+)	3	7.9	109.1→62	10	109.1→64.1	10
N-nitrosopyrrolidine	NPYR	100.06	APCI (+)	3	8.1	101.1→41	15	101.1→59	10
N-nitrosodiethylamine	NDEA	102.08	APCI (+)	4	10.5	103→75	5	103→29	10
N-nitroso pipecolicacid (control for artefact NAs formation)	NPIC	158.16	APCI (+)	4	11.1	159→83.8	10	None	N.app.
N-nitrosopiperidine	NPIP	114.08	APCI (+)	4	11.2	115→41	20	115→69	10
N-nitrosodipropylamine	NDPA	130.11	APCI (+)	5	12.7	131→ 43	10	131→89	5
N-nitrosomethylaniline*	NMA	136.06	APCI (+)	5	13	137→66	15	137→107	5
N-nitrosodibutylamine	NDBA	158.14	APCI (+)	5	13.8	159.1→57	10	159.1→103	5
N-nitrosodibenzylamine	NDBzA	226.11	APCI (+)	5	14.7	227→91	10	227→181	2
N-nitroso-thiazolidine-4-carboxylic acid*	NTCA	162.01	ESI (−)	2	3.8	161→71	2	161→117	2
N-nitroso-2-methyl-thiazolidine-4-carboxylic acid*	NMTCA	176.03	ESI (−)	3	5.5	175→71.1	3	None	N.app.
N-nitrosodiphenylamine*	NDPhA	198.08	ESI (+)	4	8.9	199→169	5	199→66	20

N.app.: Not applicable; Compounds marked with a * are NVNA; (1) Operating conditions for APCI segment 2/3/4/5 were as follows: gas temperature 325/325/300/275 °C; APCI heater 300/300/250/250, gas flow 7/6/6/6 L min^{-1}, nebulizer 20/20/20/15 psi. For all four segments the capillary voltage was 4000, corona needle voltage 5 and delta EMV 800. Operating conditions for ESI segment 2/3/4 were as follows: gas temperature 325/325/275 °C, delta EMV 0/0/800. For all three segments the gas flow was 7 L min^{-1}, nebulizer 40 psi, sheath gas temperature 300 °C, sheath gas flow 11 L min^{-1}, nozzle voltage 500 V, capillary voltage 3000 V.

as well as high sensitivity. These considerations were the point of departure for the development of the method.

3.1. Chromatographic and detection parameters

3.1.1. Separation

The best separation of the NAs was obtained using a Poroshell Phenyl-Hexyl column and a gradient elution programme as described. Several reversed phase columns (Genesis, PFP, Kinetex, Kinetex XB-C18, Acclaim PA2, Zorbax eclipse Plus C8, Poroshell Phenyl Hexyl) were tested for their ability to separate the NAs. NHPRO, NSAR, NDMA, NPRO and NMOR were found difficult to retain and therefore also to separate from each other. The Zorbax eclipse Plus C8 (Agilent Technologies, Santa Clara, CA, US) column performed similar to the Poroshell Phenyl Hexyl column, with a bit more retention of the mentioned analytes. However, the Poroshell column provided generally more narrow peaks and the peaks of NHPRO and NSAR, occurring as both R- and S-form, did not split, as was the case on the other types of columns including the Zorbax eclipse Plus C8 column.

Gradient elution starting with a low content of organic modifier was necessary in order to prevent the highly polar NAs (NHPRO, NSAR and NMDA) from eluting together with the void volume. Both acetonitrile and methanol was tested as organic modifiers. The highly polar NAs with carboxylic acid groups, NHPRO, NSAR and NPRO, were better retained with methanol than with acetonitrile. Ripolles et al. [28] found that acetonitrile resulted in higher signal intensities in NA analysis than methanol, but only a slight difference was observed in the present study. Furthermore, APCI is applicable with a mobile phase of up to 100% methanol but only with up to about 80% acetonitrile. Thus, methanol was chosen.

Lowering the pH of the mobile phase, by adding 0.1% formic acid, increased the degree of protonation and thereby also the retention of the N-nitrosamino acids, NHPRO, NSAR and NPRO slightly, but it also increased the signal intensity of the majority of the NAs. Increasing the acid concentration up to 1% formic acid did not further improve the retention or the signal intensities.

Having the analytes entering the ion source with a low amount of methanol (2%) was found to reduce the ionisation efficiency. The ionisation efficiency was higher if the content of methanol in the eluent applied in the beginning of the eluent programme was increased by from 2% to 5%; however this also reduced the retention time of the early eluting analytes. If the column was equilibrated, between each run for at least 5 min, with only 2% methanol and shifting to 5% methanol upon injection of the sample, it was however possible to almost maintain the retention and at the same time have a more efficient ionisation.

Regarding the optimization of the injection volume, the sensitivity was increased up to a volume of 3.5 μL matrix matched standard solution. If the injection volume was increased beyond 3.5 μL peak broadening, reduced retention times and higher noise levels were observed. Hence the capacity of the column was exceeded.

The developed chromatographic method allows for the separation of seven NVNA and nine VNA relevant for nitrite preserved meat products as well as NPIC. An LC(APCI)MS/MS chromatogram of thirteen of the target NAs (calibration standard of 100 ng mL^{-1}) is presented in Fig. 2. Not all the peaks were base line separated. However, when detection is performed in MRM mode quantification is still possible since the specific transitions are targeted. It is preferable to have the analytes separated in groups so that different time segments can be defined in the MS/MS method without having signals overlapping the defined segments. Because NDPhA, NTCA and NMTCA were not ionisable by APCI (as described in the following section) these were included in an LC(ESI)MS/MS method and were easily separated.

3.1.2. Detection

Ionisation by both ESI and APCI in both positive and negative mode was tested for all compounds. Thirteen out of sixteen NAs included in this study were found to ionise by APCI in positive mode. NDPhA, NTCA, and NMTCA could only be ionised by ESI, the two latter only in negative mode. NPRO, NMA, NHPRO, NMOR, NDBA, NPIP and NPYR were ionisable by both APCI (positive) and ESI (positive) with a higher signal response by ESI when analysing pure standards.

However, when analysing matrix matched calibration standards the differences in sensitivity was negligible, indicating less matrix induced ion suppression in the APCI interface and/or less formation of adducts. For that reason only NTCA, NMTCA and NDPhA were analysed using ESI. The ionisations applied appear from Table 1.

The reduced matrix induced ion suppression, observed when using APCI compared to ESI may be explained by the fact that the ionisation processes on APCI occurs in the gas phase. The analyte molecules are separated more in space from the matrix molecules when in the gas phase and thereby less affected by them. APCI has also been shown to be useful for the ionisation of several VNA [28], which traditionally have been analysed by GC. Production of $[M+H]^+$ was found to be the primary product ions, which is also in agreement with what was observed by Ripollés et al. [28] for ionisation of VNA by APCI.

Two product ions of the $[M+H]^+$ ions were determined for each of the NAs included in the method except for NDMA, NPIC and NMTCA. For NDMA, it was only possible to determine one set of precursor and product ion, though the product ion was of low mass, making it unspecific. NDMA was therefore quantified by collecting the molecular ion in the first quadrupole, having the collision energy at null in the second, and collecting the molecular ion again in the third quadrupole. This approach allowed for analysis of NDMA in the MRM acquisition mode. Only one product ion could be determined for NMTCA by ESI in negative mode, however since no interfering peaks were observed in any of the matrices analysed the specificity was found satisfactory. No interfering peaks were observed for NPIC for any of the analysed matrices.

3.2. Extraction

In order to extract both the polar and the relatively non-polar NAs by the same extraction procedure, a new method needed to be developed. Acetonitrile was chosen as the extraction solvent because it has been proven as a generic solvent for several purposes in our laboratory [30,31]. Acetonitrile is also able to extract a wide range of analytes and co-extracts less of the interfering matrix components, including fat/lipids, than if extracting with e.g. methanol, acetone [32] or ethyl acetate [33].

Based on the experience obtained in our laboratory and from published literature on generic extraction methods it was found important to evaluate the influence of the following factors on the efficiency with which the NAs were extracted from spiked samples ($50 \mu g\,kg^{-1}$) of chicken sausage: (1) sample size (2.5 or 5 g); (2) volume of extraction solvent (7.5 or 15 mL); (3) performance of an additional extraction after water addition (2.5 mL water added and a second extraction performed) or not; (4) use of neutral or acidic acetonitrile (1% formic acid) as extraction solvent; (5) extracting for 10 or 60 min; (6) clean-up by liquid/liquid extraction with hexane or not and (7) dilution of the extract 1:1 with water or not. A fractional factorial design (data not shown) was applied to these seven factors and it was found that: (1/2) The relation between sample and acetonitrile should be around 1:3 in order to have efficient protein precipitation; (3) The extraction efficiency of the highly polar NAs was not increased by introducing a second extraction after addition of water; (4) Addition of formic acid to the extraction solvent increased the extraction efficiency especially for the N-nitrosamino acids (NHPRO, NSAR). The improved extraction efficiency by addition of formic acid to the extraction solvent is in accordance with results obtained by Mol et al. [32], who found that the extraction efficiency of both acidic and basic analytes were improved. The same group suggested, that lowering the pH resulted in less binding of acidic analytes being protonated and basic analytes being either neutral or protonated (cationic) and thereby less prone to bind to amino and deprotonated acidic functionalities on the matrix particles, respectively [32]. Acidified extraction solvent

has also been shown to be necessary for efficient extraction of the mycotoxin fumonisin, which is a compound with carboxylic acid groups, as the N-nitrosoamino acids [34]. Further, (5) an extraction time of 10 min was as efficient as 60 min and (6) washing with heptanes had no significant effect on the signal intensities. (7) Dilution of the final extracts 1:1 with water significantly improved the peak shapes of the highly polar NAs, i.e. NHPRO, NSAR and NPRO, which eluted very early on several of the tested columns. The peak shapes of especially NHPRO and NSAR improved when having the sample in a weaker solvent than acetonitrile. It was therefore tested if the acetonitrile could be removed from the crude extract by evaporation leaving a small aqueous remedy, without losing the volatile NAs. No significant loss due to the vaporization step was observed, however the solubility of the most non-polar NAs (e.g. NDPhA) may be compromised. The recoveries remained at acceptable levels and within the range of 70–120% for most analytes.

Freezing of the acetonitrile extracts resulted in precipitation of low soluble constituents, as e.g. lipids. Removal of these constituents decreased the amount of precipitates formed when the acetonitrile phase was evaporated. However precipitates were still observed when the acetonitrile phase was evaporated. When the aqueous remedy was filtrated (filter vial 0.45 μm) generally a clear extract was obtained. PIC was added to the spiked test samples and this did not result in the occurrence of NPIC in the analysed extracts. The absence of NPIC indicates that no significant NA production had occurred during the extraction Because only basic analytical equipment, single use extraction tubes, few steps and small amount of organic solvent were used the developed extraction method is evaluated as fast, simple, coast effective and environmentally sound.

3.3. Validation

The results of the validation, including recoveries, RSD_r and LOD are presented in Table 2. Calibration curves were obtained by least-square regression of the peak areas versus analyte concentration using minimum five concentration levels, ranging from 0.33 to $100 \,ng\,mL^{-1}$ corresponding to $0.2–68 \,\mu g\,kg^{-1}$ sample, in duplicate. Linearity between response and analyte concentrations was observed for most NA and test sample combinations. However, in most cases a quadratic curve gave a slightly better correlation for the APCI analysis. Calibration solutions were prepared every fortnight by dilution of a $10 \,\mu g\,mL^{-1}$ standard mixture with water. Bracketing calibration was performed in each analytical batch. Regressions were performed with $1/x$ weighting. The correlation coefficients were ≥0.95. No NA contamination and/or interfering compounds originating from solvents and materials were observed in extracts of reagent blanks. Recoveries, after subtraction of any content in the blank samples, were in general found acceptable, i.e. 50–120%, 60–110% and 70–120% at the spike levels of 1, 3 and $30 \,\mu g\,kg^{-1}$, respectively [35]. The RSD_r were in general also at an acceptable level, i.e. ≤23% [35]. However, low recoveries were obtained for NHPRO (21–54%) at the two highest spike levels and for NDBzA (20–70%) at all three spike levels, though especially for salami. The low recoveries may be explained by a reduced solubility. The solvent used may be too apolar for efficient extraction of NHPRO and too polar for efficient extraction of NDBzA. Recoveries for NDMA were low (∼50%) at all spike levels, which is demonstrated by the recoveries obtained for bacon and salami (Table 2). However, satisfactory recoveries were obtained if the internal standard, NDMA-d_6, was used for correction. This is demonstrated by the recoveries of 75–96% for the two highest spike levels for kassler (Table 2). NDMA-d_6 was only used for the correction of the recovery of NDMA from kassler. The recovery of NPYR from kassler was also corrected though using NPYR-d_8. This

Table 2

Validation results including recoveries, relative repeatability (RSDr%), limit of detection (LOD), and limit of quantification (LOQ) obtained for spiked samples of kassler (pork, smoked and boiled), bacon (un-fried) and salami.

| Meat products | Kassler, pork, smoked, boiled | | | | | | | | Bacon, un-fried | | | | | | | | Salami | | | | | | | |
| Spike level µg kg⁻¹ | 1 | | 3 | | 30 | | LOD | LOQ | 1 | | 3 | | 30 | | LOD | LOQ | 1 | | 3 | | 30 | | LOD | LOQ |
Recoveries (Rec. %), repeatability (RSDr %), LOD and LOQ	Rec. %	RSDr, %	Rec. %	RSDr, %	Rec, %	RSDr, %			Rec. %	RSDr, %	Rec. %	RSDr, %	Rec. %	RSDr, %			Rec. %	RSDr, %	Rec. %	RSDr, %	Rec. %	RSDr, %		
N-nitrosohydroxyproline (NHPRO)	86	6	45	13	26	3	N.app	N.app	70	20	43	18	35	9	N.app	N.app	61	43	54	7	21	23	N.app	N.app
N-nitrososarcosine (NSAR)	41	31	58	7	58	6	0.4	0.8	89	23	53	27	71	8	0.6	1.2	36	78	42	15	67	10	0.6	1.1
N-nitrosodimethylamine (NDMA)	58*	15	96*	4	75*	1	0.3	0.5	135	8	64	42	54	7	3.3	6.7	119	24	59	7	67	17	0.9	1.7
N-nitrosoproline (NPRO)	93	12	66	8	64	7	0.3	0.7	94	17	61	9	70	6	0.5	0.9	85	10	68	12	66	14	0.2	0.5
N-nitrosomorpholine (NMOR)	99	8	81	10	99	6	0.2	0.5	100	6	89	6	77	6	0.2	0.3	83	21	87	18	94	9	0.5	1.0
N-nitrosomethylethylamine (NMEA)	59	12	56	10	62	3	0.2	0.4	100	2	75	13	60	7	0.05	0.1	108	35	84	11	72	16	0.8	1.7
N-nitrosopyrrolidine (NPYR)	82*	23	95*	10	75*	3	0.9	1.8	166	22	67	9	75	10	0.5	1.0	103	38	125	30	127	15	17.1	34.2
N-nitrosodiethylamine (NDEA)	43	31	40	20	46	10	0.7	1.4	67	14	70	14	62	10	0.3	0.6	99	8	87	16	74	16	0.2	0.5
N-nitrosopipecolic acid (NPIC)	99	14	91	17	84	7	0.4	0.8	72	7	71	10	102	6	0.1	0.3	84	36	96	16	85	12	1.3	2.7
N-nitrosopiperidine (NPIP)	82	13	86	11	82	6	0.3	0.6	97	9	85	8	86	3	0.3	0.5	98	11	99	6	97	11	0.3	0.6
N-nitrosodi-n-propylamine (NDPA)	58	27	53	21	51	2	0.5	0.9	68	23	71	6	73	12	0.5	0.9	84	12	87	9	76	20	0.3	0.6
N-nitrosomethylaniline (NMA)	81	13	80	9	79	5	0.3	0.6	91	16	78	20	72	7	0.4	0.9	78	22	78	8	75	23	0.5	1.0
N-nitrosodibutylamine (NDBA)	30	26	63	29	63	6	1.7	3.3	129	19	89	22	57	23	0.7	1.4	71	2	30	20	35	59	0.0	0.1
N-nitrosodibenzylamine (NDBzA)	57	22	45	23	38	15	N.app	N.app	74	10	33	9	34	22	N.app	N.app	19	30	28	39	38	133	N.app	N.app
N-nitrosothiazolidine-4-carboxylic acid (NTCA)	–	–	130	18	56	10	2.1	4.2	169	146	76	34	76	7	4.9	9.7	740	52	453	9	79	26	18.4	36.8
N-nitroso-2-methyl-thiazolidine-4-carboxylic acid (NMTCA)	33	85	86	7	59	3	0.6	1.2	67	25	61	15	87	2	0.8	1.7	224	26	92	43	88	12	9.4	18.7

N.app.: means not applicable, no satisfactory validation results was obtained; *: Recoveries corrected. NDMA-d_6 and NPYR-d_8 was used for correction of NDMA and NPYR recoveries, respectively.

correction also resulted in recoveries between 75 and 95%. Also Eerola et al. [26] reported the recovery of NDMA to be about50%. Their method involved extraction with dichloromethane, several clean-up steps and analysis by LC(APCI)-MS. Thus, even with more elaborate extraction methods low recoveries were observed. Low recoveries and low precision were observed for NDBzA when extracted from salami. Low recoveries of NDBzA were also observed for kassler and bacon; however the precision was acceptable for these two matrices. The low recoveries of NDMA observed in the present work, as well as by Eerola et al. [26], may occur because of evaporation of this small analyte during the concentration step.

The developed method has satisfactory precision (RSD ≤ 23%) for at least one of the tested spike levels for all three matrices (kassler, bacon, salami), except for NDPhA and NDBzA. Lower precision was observed for NDBzA in salami (≥30%), however clear peaks were observed at all three spike levels. The low precision perhaps arose because of some interfering matrix.

The instrumental response of NDPhA also varied greatly, either because of great variation in extraction efficiencies but more likely because of incompatibility with the developed MS method. NDPhA is thermally instable [36], and the low and unstable responses could be the result of degradation caused by the relatively high temperature in the source (275–300 °C). Instability of NDPhA has been reported by others [36]; however Ripolles et al. [28] did not observe degradation in the APCI interface.

LODs for kassler and bacon ranged from 0.05 to 4.9 μg kg^{-1} and for salami from 0.2 to 18.4 μg kg^{-1}. Relatively high LODs were obtained for NTCA (18.4 μg kg^{-1}) and NMTCA (9.4 μg kg^{-1}) in salami, because of content, of up to 69 and 18 μg kg^{-1}, respectively, in the unspiked salami samples. The two lowest spike levels only accounted for a small percentage of the original NTCA and NMTCA contents and this resulted in high uncertainty on the calculated recoveries. An LOD of 17 μg kg^{-1} was obtained for NPYR in salami because of high level of matrix induced noise in the region where this compound elutes. Thus it was not possible to obtain equally low LODs for the individual analytes for all three types of matrices. Furthermore, the validation show that results obtained for one matrix is not always possible to extrapolate to all other e.g. processed meat matrixes.

Massey et al. [18] employed two different extraction procedures for the extraction of NDMA, NPYR (steam distillation and dichloromethane extraction) NSAR, HNPRO, NTCA and NMTCA (repeated ethyl acetate extractions and SPE clean-up) from bacon and analysis by GC and LC with TEA detection. They obtained LODs of 2 μg kg^{-1} for NDMA and NPYR, 5 μg kg^{-1} for NHPRO and NMTCA, 7 μg kg^{-1} for NTCA and 10 μg kg^{-1} for NSAR [18]. Drabik-Markiewicz et al. [37] analysed for VNA (NDMA, NDEA, NDBA, NPIP and NPYR) in meat products with a method involving vacuum distillation and repeated extractions with dichloromethane and analysis by GC–TEA. They obtained LODs of 0.2–0.8 μg kg^{-1}. Thus, the new method described in the present study can despite its simplicity fully compete with the sensitivities obtained by other methods.

Some but generally minor matrix effects were observed for the different analyte/matrix combinations, when comparing the instrumental responses obtained by analysing an aqueous standard solution with that obtained by analysing the same standard solution matched 1:1 with meat extract. However, the analytical response obtained for standard solution matched with extract of kassler, bacon or salami in percent of that obtained for aqueous standard solutions all induced significantly higher signals for NHPRO (2–4.5 times higher, Fig. 3). Slight suppression of the NSAR and NDBzA was induced by the kassler extracts (Fig. 3A). The bacon extracts also induced slight suppression of the NSAR response and a slightly higher response for NMA and NDBA (Fig. 3B). Suppression of
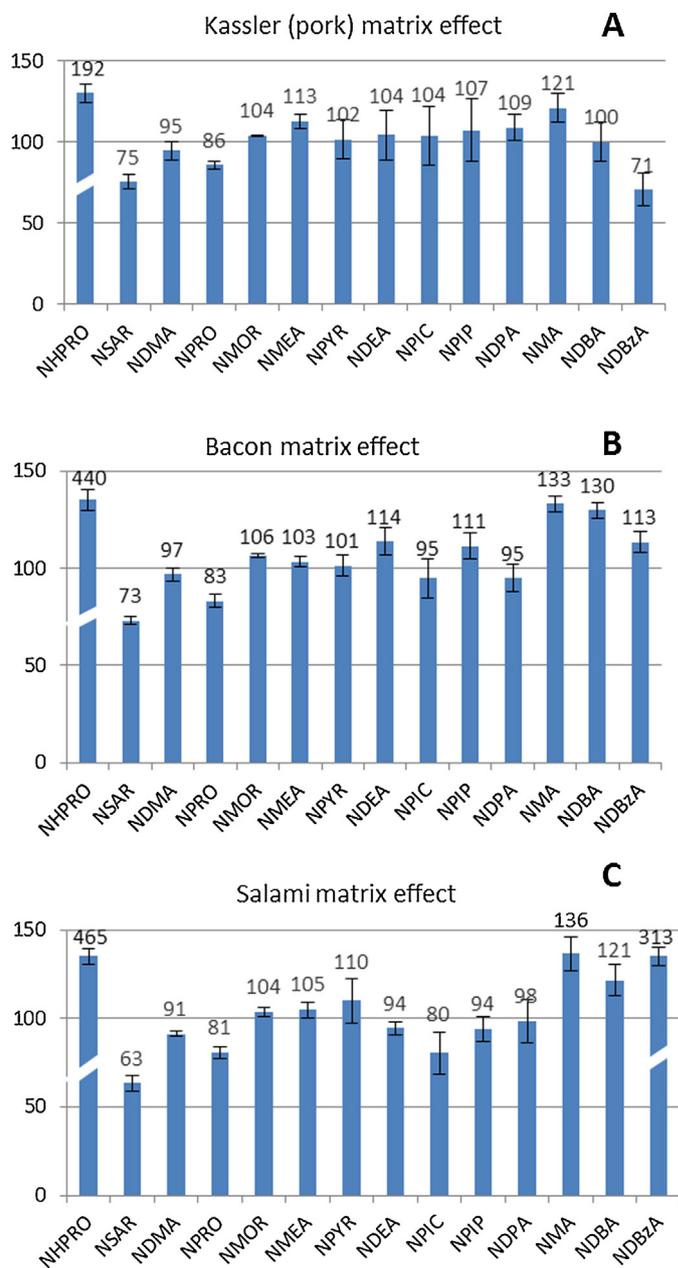

Fig. 3. The response of the individual NAs observed when analyzing standard solution of 100 ng mL^{-1} matched 1:1 with either kassler (A), bacon (B) or salami (C) extracts in percentage of response observed by the analysis of same standard solution matched 1:1 with Milli-Q water. NHPRO (N-nitrosohydroxyproline), NSAR (N-nitrososarcosine), NDMA (N-nitrosodimethylamine), NPRO (N-nitrosoproline), NMOR (N-nitrosomorpholine), NMEA (N-nitrosomethylethylamine), NPYR (N-nitrosopyrrolidine), NDEA (N-nitrosodiethylamine), NPIP (N-nitrosopiperidine), NDPA (N-nitrosodi-n-propylamine), NMA (N-nitrosomethylaniline), NDBA (N-nitrosodibutylamine), NDBzA (N-nitrosodibenzylamine) and NPIC (N-nitrosopipecolic acid).

the NSAR response was also induced by the salami extracts whereas slightly higher response was observe for NMA and much higher for NDBzA (Fig. 3C). These increased responses observed could not be explained by contents in the matrix. The matrix effects on NTCA and NMTCA analysed by LC(ESI neg.)MS/MS were not specifically examined because several analytical runs using different matrixes had demonstrated that the responses for unmatrix matched and matrix matched calibration solutions were the same, if the latter was corrected for the contents in the matrix used.

Table 3
Contents of NAs (μg kg^{-1}) in processed meat products purchased from the local supermarkets determined by LC–(APCI/ESI)MS/MS.

Sample	NHPRO	NSAR	NMDA	NPRO	NMOR	NMEA	NPYR	NDEA	NPIP	DNPA	NMA	NDBA	NDBzA	NTCA	NMTCA
Ham, dry cured smoked	9.4	–	–	5.52	–	–	1.0	–	–	–	–	–	–	171	22
Pork filet, smoked	–	–	1.3	–	–	–	1.0	–	–	–	–	–	–	28	–
Ham medallion, smoked	2.0	–	2.1	–	–	–	1.3	–	–	–	–	–	–	448	28
Pork, flank spiced, cooked	3.8	–	2.0	–	–	–	–	–	–	–	–	–	–	–	3.2
Bacon	–	–	1.8	–	–	–	0.4	–	–	–	–	–	–	41	–
Bacon, chili, without nitrite	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Salami[a]	–	–	–	–	–	–	–	–	–	–	–	–	–	76	7.5[b]
Turkey salami	3.9	–	–	3.1	–	–	–	–	–	–	0.6	–	–	111	39
Salami, black pepper	–	–	–	–	–	–	1.4	–	–	–	–	–	–	191	19
Pepperoni	–	–	–	7.5	–	–	–	–	–	–	–	–	–	217	31

(–) indicate not detected.

[a] Results stated are averages of double determinations.

[b] The result is below the LOD for NMTCA obtained for salami. The variation of the two double determinations (7.82 and 7.07 μg kg^{-1}) were however low and the result therefore evaluated as valid.

3.4. Application to nitrite preserved meat products

The developed analytical method was applied to ten samples of processed meat products purchased from the local supermarkets, i.e. (1) Ham, dry cured smoked, (2) pork filet, (3) ham medallion, smoked, (4) pork, flank, spiced, cooked (5) bacon, (6) bacon prepared without nitrite, (7) salami, (8) turkey salami, (9) salami with black pepper, and (10) pepperoni. The samples represent several types of processed meat products, either nitrite cured (sample 1–5, 7–10) or non-nitrite cured (sample 6), heat treated (sample 4) or not heat treated (sample 2 and 3), smoked (sample 1–3, 5, 6–10) or not smoked (sample 4), fermented (sample 7–10) or not fermented (sample 1–6) (Table 3).

Quality control samples, prepared by spiking samples of the kassler also used for the validation, with 3 and 30 μg kg^{-1}, were included in each sequence to monitor the recoveries and general performance of the method. It was chosen to base the quantification on standards in solvent, because the matrix effects observed for the compounds successfully validated were evaluated as minor. Based on the matrix effects observed for the three representative matrices, kassler, bacon and salami (Fig. 3) and the validation results (Table 2) the occurrences of NHPRO and NDBzA are therefore presented only as qualitative results.

NTCA was the NA detected in most samples (8 out of 10) and at highest levels (28–217 μg kg^{-1}) (Table 3). NTCA was though not detected in the non-smoked pork, flank, spiced and cooked or in the bacon prepared without nitrite. This result is in accordance with the findings by others [21], that the NTCA precursor originates from the smoke and is not formed in the absence of nitrite. NMTCA was detected in seven of the ten samples, at levels ranging from 3 to 39 μg kg^{-1}. NPRO was detected in three of the samples at levels ranging from 3 to 7 μg kg^{-1}. The hydroxylated counterpart, NHPRO, was detected in four of the samples with contents ranging from ~2 to ~9 μg kg^{-1}. These results are generally in accordance with the findings by others, e.g. reviewed by Tricker & Kubacki [21]. NMA was detected at low level (0.6 μg kg^{-1}) in turkey based salami. NMA has been reported to occur in rubber products [38,39], and is generally not found in processed meat products. The NMA detected in the turkey based salami may therefore result from contamination from e.g. rubber casing or netting used during production or storage, though the exact production conditions for this product are not known to the authors.

Low contents (≤ 2 μg kg^{-1}) of NDMA and NPYR were detected in four and five of the ten samples, respectively. It was expected in the present study that the levels of NDMA, NDEA, NPIP and NPYR in the samples would be low or even absent. This was expected because the levels of NDMA and NPYR have been reported by others to occur in many cured meat products at levels from <1 to about 5 μg kg^{-1} [11,40]. Furthermore, Drabik-Markiewicz et al. [37] found, that if

the levels of nitrite added to bacon during production were less than 120 mg kg^{-1} the levels of NDMA, NDEA, NPIP and NPYR remained below LOQ (0.8, 0.9, 2.5 and 1.6 μg kg^{-1}, respectively). The maximum permitted amount of sodium nitrite that can be added to most meat products in Denmark is 60 mg kg^{-1}, whereas it is 150 mg kg^{-1} for most products in the other EU member states, for that reason generally lower levels of NAs were expected in nitrite preserved meat products on the Danish market than in the same products on the European market.

4. Conclusion

A fast, simple and environmentally rational method based on acetonitrile extraction and LC–(APCI/ESI)MS/MS was developed for simultaneous determination of VNA and NVNA in processed meat products. The method was successfully validated for 13 out of 16 NAs. The LODs for the validated NAs are between 0.2 and 1 μg kg^{-1}, with only few exceptions. Some variability was observed in the LODs obtained, depending on the type of meat product used. This indicates that validation results obtained using one meat product not necessarily can be extrapolated to all meat products. The method could not be validated for NHPRO, NDBzA and NDPhA. The method is proved suitable for the determination of NAs in processed meat products, represented by products purchased from the Danish market and may therefore be a useful tool for an updated risk assessment of the dietary intake of NAs from processed meat products including both the VNA and the NVNA.

References

[1] A.M. Gibson, T.A. Roberts, A. Robinson, J. Food Technol. 19 (1984) 29.
[2] K. Honikel, Meat Sci. 78 (2008) 68.
[3] IARC, N-Nitroso Comp. 17 (1978), last accessed December 2013, http://monographs.iarc.fr.
[4] G.G.C. Kuhnle, S.A. Bingham, J.A. Milner, D.F. Romagnolo, in: J.A. Milner, D.F. Ramagnolo (Eds.), Nutrition and Health: Bioactive Compounds and Cancer, 2010, p. 195.
[5] T. Norat, E. Riboli, Nutr. Rev. 59 (2001).
[6] S. Rohrmann, K. Overvad, H.B. Bueno-de-Mesquita, M.U. Jakobsen, R. Egeberg, A. Tjønneland, L. Nailler, M. Boutron-Ruault, F. Clavel-Chapelon, V. Krogh, D. Palli, S. Panico, R. Tumino, F. Ricceri, M.M. Bergmann, H. Boeing, K. Li, R. Kaaks, K. Khaw, N.J. Wareham, F.L. Crowe, T.J. Key, A. Naska, A. Trichopoulou, D. Trichopoulos, M. Leenders, P.H.M. Peeters, D. Engeset, C.L. Parr, G. Skeie, P. Jakszyn, M. Sánchez, J.M. Huerta, M.L. Redondo, A. Barricarte, P. Amiano, I. Drake, E. Sonestedt, G. Hallmans, I. Johansson, V. Fedirko, I. Romieux, P. Ferrari, T. Norat, A.C. Vergnaud, E. Riboli, J. Linseisen, BMC Med. 11 (2013) 63.

[7] P. Jakszyn, C.A. Gonzalez, World J. Gastroenterol. 12 (2006) 4296.
[8] M. Jägerstad, K. Skog, Mutat. Res. 574 (2005) 156.
[9] A.R. Tricker, R. Preussmann, Mutat. Res. Gen. Toxicol. Environ. 259 (1991) 277.
[10] M.J. Hill, R.C. Massey, D.E.G. Shuker, S. Leach, A.R. Tricker, R. Preussmann, J.R. Rowland, 169, New York/Ellis Horwood. ISBN 0-89573-605-0 (1988).
[11] N. Campillo, P. Viñas, N. Martínez-Castillo, M. Hernández-Córdoba, J. Chromatogr. A 1218 (2011) 1815.
[12] S. Yurchenko, U. Mölder, Food Chem. 100 (2007) 1713.
[13] B. Spiegelhalder, G. Eisenbrand, R. Preussmann, E.A. Walker, L. Griciute, M. Castegnaro, M. Boerzsoenyi, Sci. Pub. 31 (1980).
[14] C. Crews, Qual. Assur. Saf. Crop 2 (2010) 2.
[15] S. Rath, R.R. Felix Guillermo, in: L. Nollet, F. Toldra (Eds.), Handbook of Processed Meats and Poultry Analysis, CRC Press, 2008, p. 687.
[16] G. Drabik-Markiewicz, B. Dejaegher, E. De Mey, T. Kowalska, H. Paelinck, Y. Vander Heyden, Food Chem. 126 (2011) 1539.
[17] B.P. Dunn, H.F. Stich, Food and Chem. Toxicology 22 (1984) 609.
[18] R.C. Massey, P.E. Key, R.A. Jones, G.L. Logan, Food Addit. Contam. (1991) 585.
[19] N.P. Sen, S. Seaman, W.F. Miles, J. Agric. Food Chem. 27 (1979) 1354.
[20] R.C. Massey, C. Crews, M.J. Dennis, D.J. McWeeny, J.R. Startin, M.E. Knowles, Anal. Chim. Acta 174 (1985) 327.
[21] A.R. Tricker, S.J. Kubacki, Food Addit. Contam. (1992) 39.
[22] N.P. Sen, S.J. Kubacki, Food Addit. Contam. 4 (1987) 357.
[23] A.R. Tricker, M.J. Perkins, R.C. Massey, D.J. McWeeny, Z. Lebensm. Unters. Forsch. 180 (1985) 379.
[24] J. Wu, W.S. Rickert, A. Masters, P. Joza, Anal. Methods 4 (2012) 3448.
[25] S. Essen, H. Kimpton, C. Wright, K. McAdam, Poster Presented at the CORESTA Smoke Science and Product Technology Joint Study Groups Meeting, Graz, Austria, 2011, Last accessed December 2013, http://www.batscience.com/groupms/sites/BAT_7AWFH3.nsf/vwPagesWebLive/ABC2A912C9C3C84EC1257922003E936D/$FILE/CORESTA%20Poster%20Graz%20STPOST20%20CW.pdf?openelement.
[26] S. Eerola, I. Otegui, L. Saari, A. Rizzo, Food Addit. Contam. 15 (1998) 270.
[27] M. Krauss, J. Hollender, Anal. Chem. 80 (2008) 834.
[28] C. Ripollés, E. Pitarch, J.V. Sancho, F.J. López, F. Hernández, Anal. Chim. Acta 702 (2011) 62.
[29] ISO, ISO 5725–2 (1994), Last accessed December 2013, http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_tc_browse.htm?commid=49808.
[30] M.E. Poulsen, M. Wenneker, J. Withagen, H.B. Christensen, Crop Prot. 35 (2012) 5.
[31] R.R. Rasmussen, I.M.L.D. Storm, P.H. Rasmussen, J. Smedsgaard, K.F. Nielsen, Anal. Bioanal. Chem. 397 (2010) 765.
[32] H.G.J. Mol, P. Plaza-Bolaños, P. Zomer, T.C. de Rijk, A.A.M. Stolker, P.P.J. Mulder, Anal. Chem. 80 (2008) 9450.
[33] S.J. Lehotay, K.A. Son, H. Kwon, U. Koesukwiwat, W. Fu, K. Mastovska, E. Hoh, N. Leepipatpiboon, J. Chromatogr. A 1217 (2010) 2548.
[34] V. Sewram, G.S. Shephard, W.F.O. Marasas, M.F.P.M. de Castro, J. Food Prot. 66 (2003) 854.
[35] NMKL, Nordic Committee on Food Analysis No 4, third ed., 2009, Last accessed December 2013, http://www.nmkl.org/.
[36] Y. Zhao, J. Boyd, S.E. Hrudey, X. Li, Environ. Sci. Technol. 40 (2006) 7636.
[37] G. Drabik-Markiewicz, K. Van den Maagdenberg, E. De Mey, S. Deprez, T. Kowalska, H. Paelinck, Meat Sci. 81 (2009) 479.
[38] V. Anna, S. Rimma, O. Lev, G. Jenny, Anal. Chim. Acta 685 (2011) 162.
[39] L.C.JJr. Goss, S. Monthey, H. Issel, Rubber Chem. Technol. 79 (2006) 541.
[40] J.E. Stuff, E.T. Goh, S.L. Barrera, M.L. Bondy, M.R. Forman, J. Food Comp. Anal. 22 (2009) 42.

Paper II

Occurrence of volatile and non-volatile *N*-nitrosamines in processed meat products and the role of heat treatment

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ABSTRACT

Most of the available data on the occurrence of *N*-nitrosamines (NA) in processed meat products have been generated in the 1980s and 1990s and especially data on the occurrence of non-volatile NA (NVNA) are scarce. Therefore we have studied the levels of volatile nitrosamines (VNA) and NVNA in processed meat products on the Danish market ($N = 70$) and for comparison also products on the Belgian market ($N = 20$). The effect of heat treatment on the NA levels, in selected samples, was also studied, in order to enable an evaluation of how preparation before consumption affects the levels of NA. For the Danish products the mean levels of the VNA were generally low (≤ 0.8 µg kg^{-1}), whereas the mean levels of the NVNA were considerably higher (≤ 118 µg kg^{-1}). Slightly higher mean levels were indicated for the Belgian products (i.e. VNA ≤ 1.5 µg kg^{-1} and NVNA ≤ 270 µg kg^{-1}). The sums of VNA were higher than 10 µg kg^{-1} for one Danish sample and two Belgian samples. Levels of up to 2000 and 4000 µg kg^{-1} of *N*-nitroso-thiazolidine-4-carboxylic acid (NTCA) an NVNA occurred in the Danish and the Belgian samples, respectively. The majority of the Danish processed meat products contain NVNA but also VNA occur. The levels of NA are comparable with those reported in previous and recent studies; however the frequency in which they are found may be lower and thereby also the mean contents. The levels of *N*-nitrosopiperidine (NPIP) increased by frying and baking, whereas varying impacts were observed for *N*-nitrosoproline (NPRO), *N*-nitrosodimethylamine (NDMA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosodiethylamine (NDEA) and *N*-nitrosomethylaniline (NMA) depending on the type of product and/or the heat treatment. The levels of the NVNA, NTCA and *N*-nitroso-2-methyl-thiazolidine 4-carboxylic acid (NMTCA) decreased after frying and baking.

1. Introduction

Several publications report a link between meat intake and colorectal cancer risk. Based on an evaluation of the publications in this area, Kuhnle, Bingham, Milner, and Romagnolo (2010, pp. 195–212) conclude that there is a significant, though modest increase in the risk of developing colorectal cancer for consumers of red meat (mainly beef) and processed meat (mainly pork) (Kuhnle et al., 2010). The risk of cancer was estimated to increase by 29% with a daily consumption of 100 g red meat and by 21% with a daily consumption of 50 g of processed meat. The scientifically based evidence for an association between meat intake and increased risk of colorectal cancer led in 2007 the World Cancer Research Fund to include the phrase: "Limit intake of red meat and avoid processed meat" as one of 10 universal guidelines for healthy nutrition

(Demeyer, Honikel, & De Smet, 2008). In 2013 the Danish Veterinary and Food Administration changed their recommendations for a healthy diet to include the recommendation to limit the consumption of red and processed meat.

Thus, a greater risk is associated with the intake of processed meat, which in general is signified by the use of nitrite (E 249–E 250) or nitrate (E 251–252) for preservation. The mechanisms linking the consumption of red meat and processed meat to cancer are still unclear. *N*-nitrosamines (NA) are produced in nitrite/nitrate preserved meat products. The NA is a large group of compounds of which many are carcinogenic (IARC, 1998). Thus the occurrence of NA in processed meats may play a significant role in why the observed adverse health effects are more pronounced for processed meat than for meat in general.

Since the consumption of nitrite preserved meat products is associated not only with an increased exposure to nitrite but also to the carcinogenic NA, the Danish authorities have been seeking to minimize the use of nitrite, without compromising the microbiological safety of the products. Denmark therefore considered it

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necessary to maintain national provisions for the use of nitrite for meat preservation, allowing the addition of maximum 60 mg kg^{-1} to most meat products intended for the Danish market. Though to some products as flank pork (spiced, boiled) which is spiced with parsley and different types of non-Danish traditional products as Wiltshire bacon it is allowed to add 100 mg kg^{-1} and 150 mg kg^{-1}, respectively. The common EU legislation (Directive 2006/52/EC) allows an addition of 150 mg kg^{-1} meat. Several studies have indicated that there is a positive correlation, though not necessarily linear, between the amount of nitrite added and the amount of NA formed (e.g. (Drabik-Markiewicz et al., 2009; Robach, Owens, Paquette, Sofos, & Busta, 1980; Yurchenko & Mölder, 2007)). Thus the levels of NA are expected to be low in products on the Danish market and lower than in products on the common EU market. However, no data is available in support of this assumption.

The majority of the available studies on NA formation and occurrence only include the so-called volatile *N*-nitrosamines (VNA) and rarely the so-called non-volatile *N*-nitrosamines (NVNA). Many of the VNA are known to be carcinogenic and the majority of the remainder is assumed to be so, whereas only a few of the NVNA are assumed to be carcinogenic. Even though most of the NVNA are non-carcinogenic or less potent carcinogens, their presence in processed meat products may still be of significance, when assessing the human health risks associated with the use of nitrite/nitrate. This may be the case if weakly carcinogenic NVNA occur frequently and in large amounts and/or if non-carcinogenic NVNA can be precursors of the carcinogenic NA.

Studies indicate that the levels of NA in processed meat may increase during further processing e.g. frying, baking or other heat treatments (Drabik-Markiewicz et al., 2009; Drabik-Markiewicz et al., 2011; Rywotycki, 2007; Yurchenko & Mölder, 2006, 2007). It is suggested that decarboxylation of NVNA as e.g. *N*-nitrososarcosine (NSAR) and *N*-nitrosoproline (NPRO) may be the cause of the heat induced increase in the levels VNA, i.e. *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR), respectively. In order to perform a well-founded evaluation of the levels of NA in the products as consumed, data on the role of heat treatment on the levels of both VNA and NVNA is needed.

We therefore studied the occurrence of NA in meat products available on the Danish market. Samples from the Belgian market were also analysed for comparison. Besides that, the samples were analysed for residual levels of nitrite or nitrate in order to study a possible correlation between residual levels of nitrite or nitrate and the levels of NA. The levels of VNA and NVNA were examined in six selected products before and after heat treatment. A method we recently developed and validated allowed for the simultaneous quantification of several VNA and NVNA in the meat products (Herrmann, Duedahl-Olesen, & Granby, 2014).

2. Methods

2.1. Samples

2.1.1. Samples from the Danish market

A total of 70 samples were taken in connection with the present study. Forty-nine of the samples were taken by authorized personnel at importers, wholesalers and production sites and comprised of several types of products (Table 1). This survey sampling was undertaken by the Danish Veterinary and Food Administration during fall 2012 ($N = 49$). Furthermore twenty-one samples were purchased from local supermarkets during May to September 2013 ($N = 21$). These samples were mainly products of which the Danish population have a high consumption, i.e. salami, sausages, ham and bacon. Of the samples taken on the Danish market, 41 were produced in Denmark while the remaining were produced in Germany ($N = 13$), Poland ($N = 3$), Sweden ($N = 4$), Spain ($N = 2$), Slovenia ($N = 1$), and for six samples the origin had not been noted. Regardless of the place of production the products available on the Danish market should comply with the more strict national provisions with regard to the use of nitrite and nitrate.

2.1.2. Samples from the Belgian market

Samples from other EU member states were represented by 20 samples purchased in local supermarkets in Belgium in October 2013 ($N = 20$). Twelve of these products were produced in Belgium, three in Germany, three in Spain and two in France.

Table 1

Mean levels of VNA (uncoloured columns) and NVNA (shaded columns) (µg kg^{-1}) in processed meat products on the Danish or Belgian market. The mean of all quantifiable contents, number of samples with contents and maximum levels detected are presented for each compound and commodity group combination.

Compound name	N[3]	N-nitrosodi-methylamine NDMA Mean / det. / Max	N-nitroso-morpholine NMOR Mean / det. / Max	N-nitroso-methylethylamine NMEA Mean / det. / Max	N-nitroso-pyrrolidine NPYR Mean / det. / Max	N-nitroso-diethylamine NDEA Mean / det. / Max	N-nitroso-piperidine NPIP Mean / det. / Max	N-nitroso-sarcosine NSAR Mean / det. / Max	N-nitroso-proline NPRO Mean / det. / Max	N-nitroso-methylaniline NMA Mean / det. / Max	N-nitroso-thiazolidine-4-carboxylic acid NTCA Mean / det. / Max	N-nitroso-2-methyla-thiazolidine-4-carboxylic acid NMTCA Mean / det. / Max
Results for Danish samples												
Bacon	6	1.2 / 4 / 2.0		0.5 / 1 / 0.5	1.4 / 3 / 2.1		0.07 / 1 / 0.07		2.2 / 2 / 3.1	0.4 / 2 / 0.6	134 / 6 / 535	6.1 / 5 / 23
Kassler (pork saddle, smoked and boiled)	8	0.8 / 3 / 1.1	0.05 / 1 / 0.05	0.1 / 1 / 0.1	0.4 / 2 / 0.5		0.2 / 2 / 0.2		0.1 / 2 / 0.2		12 / 8 / 27	1.4 / 6 / 2.0
Chicken/Turkey (luncheon meat)	2				0.4 / 1 / 0.4				1.8 / 2 / 2.0	0.4 / 1 / 0.4	79 / 2 / 150	21 / 1 / 21
Meat sausage (luncheon meat, pork)	1			0.3 / 1 / 0.3							6 / 1 / 6.3	3.3 / 1 / 3.3
Medister sausage (pork, Danish speciality)	1								0.6 / 1 / 0.6		18 / 1 / 18	
Sausages	9	1.0 / 3 / 1.6			1.4 / 5 / 2.2	0.1 / 2 / 0.2		2.0 / 1 / 2.0	4.4 / 4 / 11	1.1 / 1 / 1.1	33 / 9 / 99	3.2 / 6 / 7.6
Flank pork (spiced, boiled)	6	0.8 / 3 / 0.9	0.08 / 2 / 0.1	0.3 / 1 / 0.3	0.4 / 1 / 0.4				0.9 / 5 / 2.0	0.3 / 2 / 0.3	9 / 5 / 25	4.4 / 5 / 12
Ham[1]	8	2.0 / 3 / 3.0	0.03 / 1 / 0.03	0.2 / 4 / 0.4	1.2 / 7 / 2.3	0.2 / 1 / 0.2	0.04 / 1 / 0.04	0.4 / 1 / 0.4	4.0 / 7 / 10	0.3 / 1 / 0.3	325 / 8 / 2034	11 / 8 / 23
Salami[2]	24	1.6 / 15 / 4.0	0.5 / 1 / 0.5		2.1 / 10 / 3.4	0.3 / 6 / 0.3	0.1 / 5 / 0.2	1.0 / 1 / 1.0	4.6 / 11 / 24	0.5 / 6 / 0.9	117 / 24 / 347	10 / 24 / 39
Filet, smoked	5	1.1 / 4 / 1.8		0.2 / 1 / 0.2	4.0 / 4 / 13	0.3 / 2 / 0.3			17 / 3 / 30	0.4 / 2 / 0.5	292 / 5 / 723	14 / 5 / 34
Mean content (positive results, all products)[4]		1.3	0.1	0.3	2	0.3	0.1	20	4.3	0.47	120	8.1
Mean content (all results, all products)[4]	70	0.7	0.01	0.04	0.8	0.04	0.02	1.5	2.3		118	7.3
Results for Belgian samples												
Bacon	4	1.6 / 4 / 2.6			2.2 / 2 / 2.2		0.2 / 1 / 0.2	1.0 / 2 / 1.0	0.4 / 4 / 0.6	0.5 / 1 / 0.5	36 / 4 / 89	3.2 / 4 / 7
Ham[1]	7	1.5 / 4 / 2.2		0.4 / 1 / 0.4	1.5 / 5 / 2.0		0.07 / 1 / 0.07		2.5 / 3 / 6	0.2 / 2 / 0.3	6 / 5 / 13	6 / 5 / 15
Salami[2]	9	2.6 / 5 / 7.2	0.5 / 1 / 0.5		2.7 / 7 / 6.9		0.3 / 7 / 0.6		15.9 / 9 / 75	0.3 / 3 / 0.4	581 / 9 / 4030	13 / 8 / 18
Mean content (positive results, all products)[4]		1.9	0.5	0.4	2.2	0	0.3	1.0	9.5	0.3	300	8.9
Mean content (all results, all products)[4]	20	1.3	0.02	0.05	1.5	0	0.1	0.1	7.6	0.09	270	7.6

1: Includes smoked and boiled as well as dried cured ham; 2: Includes salami, chorizo and pepperoni; 3: Number of samples analysed. 4: The means are based on all positive findings also those below LOQ if the retention times and ion ratios matched those of the standard solutions or quality control samples. Non-detects were not included when calculating the means for the individual product groups nor when calculation the mean of "positive results, all products". When calculating the means of "all results, all products" non-detects were set to zero.

2.1.3. Samples for heat treatment studies

The samples used to study the impact of heat treatment on the levels of NA were purchased at Danish local supermarkets or Danish local butcher stores. Six different products were included; brine cured bacon ("bacon 1"); needle-pumped cured bacon ("bacon 2"); ham boiled and smoked ("ham 1"); serrano ham dry cured ("ham 2"); pepperoni; and chorizo.

Enough sample of each type was purchased so that it could be divided into six portions. Three of these portions were subjected to heat treatment before analysis whereas the other three were analysed directly. Each of the six subsamples were analysed in duplicate. The two bacon types were fried on a pan until brown and crispy, which required 9 min of frying. The two types of ham, pepperoni and chorizo were baked in an oven preheated to about 250 °C until light brown. "Ham 1", "ham 2", pepperoni and the chorizo required baking for 9, 7.5, 9 and 5 min in order to appear light brown. Pan frying was chosen for the treatment of bacon because most bacon consumed in Denmark is consumed as fried crispy bacon and baking in oven was chosen for ham, pepperoni and chorizo since these products may be likely choices for pizza toppings. The fat melting off during the heat treatment was included in the analytical sample since it was considered to represent a worst case scenario.

2.2. 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 ≥99.9%, Fluka-Analytical) were purchased from Sigma–Aldrich Co. (St Louis, MO, USA). The pure standards of *N*-nitrososarcosine (NSAR), *N*-nitrosohydroxyproline (NHPRO), *N*-nitrosodibenzylamine (NDBzA), *N*-nitrosoproline (NPRO), *N*-nitrosomethylaniline (NMA), *N*-nitroso-2-methyl-thiazolidine 4-carboxylic acid (NMTCA) and *N*-nitroso-thiazolidine-4-carboxylic acid (NTCA) were purchased from Toronto Research Chemicals (Toronto, Canada), whereas the standards *N*-nitrosodiethylamine (NDEA), *N*-nitrosodipropylamine (NDPA), *N*-nitrosomorpholine (NMOR) and *N*-nitrosodimethylamine (NDMA) were purchased from Sigma–Aldrich Co. *N*-nitrosomethylethylamine (NMEA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosodibutylamine (NDBA), *N*-nitrosopiperidine (NPIP) were purchased from Dr. Ehrenstorfer (Ausburg, Germany). The internal standards *N*-nitrosodimethylamine-d_6 (NDMA-d_6) and *N*-nitrosopyrrolidine-d_8 (NPYR-d_8) were purchased from Sigma–Aldrich Co. and CDN Isotopes (Quebec, Canada), respectively. The purity of all of the NA standards was ≥98% except for NMA which were of 95% purity. Sodium nitrite, Sodium nitrate and Sodium hydroxide was purchased from Sigma–Aldrich Co. The SPE cartridges (C-18 Chromabond, EC 500 mg/6 ml) were purchased from Macherey–Nagel (Nagel GmbH & Co, Germany).

2.3. Analysis of VNA and NVNA

The contents of eight VNA and five NVNA in the samples were determined according to a method recently developed and validated at our laboratory (Herrmann et al., 2014). The method is described in brief below.

2.3.1. Extraction method

2.5 g of the homogenized sample was after the addition of internal standards (ISTD) (NPYR-d_8 and NDMA-d_6) extracted for 10 min with 7.5 ml 1% formic acid in acetonitrile. After centrifugation the clear supernatant was removed and frozen. The extract was thawed until it became fluent and then centrifuged. 5 ml of the

acetonitrile phase was evaporated under a gentle stream of nitrogen to a volume of approximately 0.25 ml which was adjusted to 1.0 ml with Milli-Q water resulting in the final extract. An aliquot was mixed 1:1 with Milli-Q water, filtered and analysed.

2.3.2. LC–MS/MS method

Separation was performed on an Agilent 1200 Series HPLC (Agilent Technologies, Santa Clara, CA, US) with a Poroshell PhenylHexyl 150 × 2.1 mm, 3 μm column (Agilent Technologies). A mobile phase gradient programme was applied, using 0.1% formic acid in water and methanol, respectively. The injection volume for both the APCI and ESI analysis was 3.5 μL.

MS/MS detection was performed on an Agilent 6460 Series Triple Quadrupole (Agilent Technologies) equipped with either an APCI or a Jet Stream ESI source. Collision gas was nitrogen. Detection was performed in Multiple Reaction Monitoring (MRM) mode. MassHunter Workstation software (version B.01.04, Agilent Technologies) was used for instrument control, data acquisition and for processing of the qualitative and quantitative data.

The quantitative and qualitative analyses were performed by external calibration (0.334–1000 ng ml^{-1}) and compared with the retention times and quantifier ion/qualifier ion ratios obtained by analysing NA standard solutions and spiked QC samples.

The LODs obtained with the described method were generally <1 μg kg^{-1}, though with some exceptions. LOD for NDBA and NTCA in kassler were 1.7 and 2.1 μg kg^{-1}, respectively; LOD for NDMA and NTCA in bacon were 3.3 and 4.9 μg kg^{-1}, respectively; and LOD for NPYR, NTCA and NMTCA in salami were 17.1, 18.4 and 9.4 μg kg^{-1}, respectively. Relatively high LODs were obtained for NTCA and NMTCA, because the "natural" content in the unspiked salami samples were relatively high, i.e. 69 and 18 μg kg^{-1}, respectively. The high LOD for NPYR salami resulted from a too high variation of the results (relative standard deviation of 38%) though the recovery was around 100% even at a spike level of 1 μg/kg. The validation results are presented in detail in Herrmann et al. (2014). Quality Control (QC) samples of kassler spiked at two levels and in duplicates were included in each analytical run. Each positive finding of an NA content in samples was in the present study verified by comparing it with the retention times and quantifier/qualifier ion ratios observed for the external standards, the spiked QC samples, and for NDMA and NPYR also the internal standard.

2.4. Analysis of nitrite and nitrate

The samples from the Danish market were analysed for nitrite and nitrate at the regional laboratory of the Danish Veterinary and Food Administration (Villadsen, Jakobsen, & Eriksen, 2012) by a Flow Injection Analysis (FIA) method based on Leth, Fagt, Nielsen, and Andersen (2008) as accredited analysis. The samples taken from the Belgian market were analysed at our laboratory using the method described below.

2.4.1. Extraction method

A 5 g homogenized meat sample was added 50 ml water and extracted for 15 min by ultrasonication (Branson 5510, VWR) followed by centrifuging for 10 min at 3600 rpm (Sigma 3–18K, Buch & Holm, Denmark). A 2 ml aliquot of the extract was cleaned up on an SPE cartridge (Telos, C18 (EC), Mikrolab Aarhus A/S, Denmark) and into an injection vial.

2.4.2. LC–UV method

The detection of sodium nitrite and sodium nitrate was performed using ion chromatography coupled to UV detection at 225 nm (HPLC and Diode Array Detector, series1100, Agilent Technologies) with separation on a Dionex IonPac AS11 RFIC

4×250 mm column and a Dionex IonPac AG11 4×50 mm guard column. The LODs for sodium nitrate and sodium nitrite using this method are 1 mg kg^{-1}. The method was developed and validated at our laboratory. The performance of the method was further evaluated by analysing reference material from FAPAS proficiency test material 1592 meat, 2013. The obtained result for sodium nitrate was 150 mg kg^{-1} (assigned value 143 mg kg^{-1}) and for sodium nitrite it was 189 mg kg^{-1} (assigned value 178 mg kg^{-1}).

3. Results and discussion

3.1. NA analysis

3.1.1. Meat products on the Danish market

The samples contained relatively low mean levels of the individual VNA ≤ 1 µg kg^{-1} (Table 1). The most frequently found VNA were NDMA and NPYR. The levels of NDMA ranged from non-detectable to 4 µg kg^{-1}. The levels of NPYR ranged from non-detectable to 13 µg kg^{-1}.

NDMA was detected in >50% of the samples of bacon, salami and smoked filet. NPYR was detected in $\geq 50\%$ of the bacon, sausages, ham and smoked filet samples. In general products which were boiled during processing, e.g. flank pork, kassler, seem less likely to contain detectable levels of VNA.

General levels of VNA reported in the literature are also low, i.e. ≤ 5 µg kg^{-1} (Campillo, Viñas, Martínez-Castillo, & Hernández-Córdoba, 2011; Hill et al., 1988, p. 169; Yurchenko & Mölder, 2007). NDMA and NPYR are the most frequently found VNA in nitrite/nitrate treated meat products. For example Spielgelhalder, Eisenbrand, and Preussmann (1980) reported 32% and 11% of such products ($N = 395$) to contain detectable levels (>0.5 µg kg^{-1}) of NDMA and NPYR, respectively. Levels above 5 µg kg^{-1} of NDMA and NPYR occurred only in 2% and 6% of the samples, respectively (Spielgelhalder et al., 1980). Other types of VNA were only detected occasionally. In the present study no levels of NDMA above 5 µg kg^{-1} occurred in the Danish samples but in one of the twenty Belgian samples (5%). NPYR occurred at concentrations above 5 µg kg^{-1} in one Danish (1%) and one Belgian sample (5%). Thus, the levels and frequencies of findings in the present study are comparable to those found by others. If looking at more recent studies low levels of VNA were also found (Campillo et al., 2011; Ozel, Gogus, Yagci, Hamilton, & Lewis, 2010). Though the average levels of NDMA (2.8 and 0.3 µg kg^{-1}), NPYR (1.4 and 0.9 µg kg^{-1}), NPIP (1.1 and 0.9 µg kg^{-1}) and NDEA (0.7 and 0.3 µg kg^{-1}) by Campillo et al. (2011) and Ozel et al. (2010) in 21 and 22 samples of processed meat products from the Spanish and the Turkish market, respectively, are higher than those found in the present study. The individual levels, detected by these two groups, were low, however the frequency with which they were detected was higher and the mean levels were therefore also higher.

NDBA was not detected in any samples and therefore not included in Table 1. NDPA was detected in a few samples at very low levels (≤ 0.2 µg kg^{-1}). NDPA was previously detected in meat products such as sausages and salami (Ozel et al., 2010) and also in cheese (Dellisanti, Cerutti, & Airoldi, 1996). However, considering that the levels we found were very low and below the LOD of 0.3 µg kg^{-1} the results are not presented.

Several NVNA were also detected in the Danish samples. NSAR was only detected in a few samples and mostly at low levels, except for one smoked filet sample containing 82 µg kg^{-1}. This smoked filet sample also contained the highest level of NPRO (30 µg kg^{-1}) and NPYR. NTCA was detected and quantified in all but one sample, i.e. a sample of flank pork. The highest content of NTCA (2000 µg kg^{-1}) was found in a sample of ham. NMTCA was detectable and quantifiable in all but seven samples, though at

significantly lower levels than NTCA. NTCA also occur in unsmoked but nitrite preserved meat products, though at low levels (≤ 18 µg kg^{-1}), i.e. in meat sausage (luncheon meat) and boiled ham. The highest level of NMTCA (39 µg kg^{-1}) occurred in a sample of salami.

The results of NTCA are in accordance with the reported results by Massey, Key, Jones, and Logan (1991) who found that NTCA (and N-nitrosothiazolidine) occurred in all nitrite preserved smoked bacon (Massey et al., 1991). The present results support this observation and further indicate that, NTCA is also unambiguously occurring in other types of smoked meat products. Levels of NTCA and NMTCA up to 1600 µg kg^{-1} and 98 µg kg^{-1}, respectively, in nitrite preserved and smoked meat products have been reported (Tricker & Kubacki, 1992). These levels of NTCA and NMTCA are comparable to the levels found in the present study. The high levels of NTCA and NMTCA are found in smoked products supporting that precursors for NTCA and NMTCA occur in the smoke (Massey et al., 1991) and/or that the formation of these two NA are stimulated by the increase in temperature during the smoking process. The toxicological significance of the detected levels of NTCA and NMTCA cannot be evaluated because the available data is insufficient for a toxicological evaluation of these two NA. They are expected to be non-carcinogenic but this still needs to be verified.

3.1.2. Meat products on the Belgium market

The Belgian samples include bacon, ham and salami samples. Among the Danish samples 54% were represented by these three types of products. Assuming a positive correlation between the amount of added nitrite and the formation of NA, lower levels of NA are expected in products on the Danish market than on the Belgian market. This is expected because the more strict Danish national provisions allow for an addition of lower levels of nitrite/nitrate than the common EU legislation.

Generally the levels of VNA and NVNA found in the Belgian samples were similar to the levels found in the Danish samples. However, if looking at the mean contents of NA in the Belgian and in the Danish samples (Table 1) it seems that some means of NA are higher for the Belgian samples and others are higher for the Danish samples. The contents of NTCA and NMTCA may be more linked with the smoke processes used than the amount of nitrite added. For the remaining NA the means of six NA (NSAR, NDMA, NPRO, NMOR, NPYR and NPIP) are higher for the Belgian samples whereas only the levels of two NA (NDEA and NMEA) are higher for the Danish samples. Though, if applying a student t-test (one tailed, type 2, $P \geq 0.95$ or $P \geq 0.99$) to the data it is only the NPIP contents that are significantly higher in the Belgian samples. Thus, the present results weakly indicate that the levels of NA occurring in meat products produced according to the common EU regulation may be higher than those occurring in processed meat produced in accordance with the provisional Danish regulation. If a final conclusion is to be drawn in regard to whether the addition of e.g. 150 mg kg^{-1} nitrite will result in higher average NA levels than if 60 mg kg^{-1} nitrite was added, further studies are needed. E.g. a survey of the NA content in samples, for which the precise terms of production including the amount of nitrite added, are known.

No maximum limits have been established by EU for NA in processed meat products, though, a limit of 10 µg kg^{-1} total volatile N-nitrosamines has been set for cured meat products in the United States (Crews, 2010). For the samples on the Danish market the sum of VNA were all ≤ 4.9 µg kg^{-1}, except for one sample of smoked filet for which the sum amounted to 15.0 µg kg^{-1}. For the samples on the Belgian market the sum of VNA were all ≤ 3.6 µg kg^{-1}, except for two samples of chorizo. The sums of VNA for these two chorizo samples amounted to 11.4 and 10.2 µg/kg. Thus only three samples

amongst both Danish and Belgian samples are not in compliance with the maximum limit of 10 µg kg^{-1} set in the US.

Recently a desk study was undertaken in EU in order to monitor the implementation of Directive 2006/52/EC in the EU Member States (WGA 13/02/07 draft report on the use of nitrites in MS rev 1, not made available because it is a draft) with regard to the use of nitrites by the industry. The report concludes that the amount of nitrite typically applied to meat products are generally lower than what is allowed to be added, however also generally higher than the levels allowed in Denmark. The amounts of nitrite added to meat products in Denmark were also typically lower than what is allowed in accordance with the more strict national provisions. This report therefore support that commonly more nitrite is added to meat products intended for the common European market than to meat products intended for the Danish market. Besides the amount of nitrite/nitrate added to the meat during production, a large number of factors can however have an impact on the levels of NA formed as well as the residual levels of nitrite/nitrate. These factors include composition of the feed given to the animals before slaughter, quality of the raw meat used (e.g. level of stress, microbial activity), use of additional additives (antioxidants, phosphates, sugars, salt), use of spices (e.g. paprika, black pepper) and starter cultures, temperature applied during drying and smoking processes, storage conditions etc. All of these factors may influence the levels of NA formed and may be as significant as or even more significant than the amount of nitrite added and may therefore mask any correlation between added amount of nitrite and degree of NA formation. Furthermore no information was available on the amounts of nitrite/nitrate added to the individual samples of the present survey.

3.2. Nitrite/nitrate analysis

3.2.1. Meat products on the Danish market

All samples were analysed by the LC–UV method and the samples taken by the Danish Veterinary and Food Administration during fall 2012 were also analysed by the FIA based method shortly after sampling. Significantly higher nitrite levels were found when the sample were analysed briefly after sampling than when they had been stored at −18 to −20 °C for several month. Thus the nitrite was not stable under these storage conditions. It was therefore decided to use the analytical results obtained by the FIA based method for the samples taken by Danish Veterinary and Food Administration and the analytical results obtained by the LC–UV method for the remaining samples. Good correlation was found between the nitrate contents determined using the two methods. Thus the nitrate contents were stable during storage at −18 to −20 °C.

The nitrite (NaNO$_2$) levels ranged from <3 mg kg^{-1} to 36 mg kg^{-1} with a mean content of 6.0 mg kg^{-1}. The nitrate (NaNO$_3$) levels ranged from <5 mg kg^{-1} to 124 mg kg^{-1} with a mean value of 27.7 mg kg^{-1}. Good correlation was found between the nitrate contents in the samples analysed by both the LC–UV method and the FIA based method, indicating that nitrate is stable during storage at −18 to −20 °C. When calculating the mean values the <LOD values were set to 0.5 times the LODs (i.e. 0.5*3 mg kg^{-1} for NaNO$_2$ and 0.5*5 mg kg^{-1} for NaNO$_3$).

The five highest levels of nitrate were found in a sample of ham (129 mg kg^{-1}), flank pork (124 mg kg^{-1}), and three salami samples (71–120 mg kg^{-1}). The five highest levels of nitrite were found in two samples of kassler (36 and 19 mg kg^{-1}), a sample of meat sausage (luncheon meat) (28 mg kg^{-1}) and in two dinner sausages (26 and 20 mg kg^{-1}). In general, salamis contained low levels of nitrite (≤3 mg kg^{-1}) and accounted for the majority of the samples with a high content of nitrate (15–120 mg kg^{-1}).

No correlation between the detected residual levels of nitrite and/or nitrate and the levels of the individual levels of the detected NA could be demonstrated.

3.2.2. Meat products on the Belgian market

The nitrite (NaNO$_2$) levels in the Belgian samples ranged from 0.3 mg kg^{-1} to 25 mg kg^{-1} with a mean content of 4.0 mg kg^{-1}. The nitrate (NaNO$_3$) levels ranged from 1.5 mg kg^{-1} to 178 mg kg^{-1} with a mean value of 32.9 mg kg^{-1}. No significant difference in the nitrite and nitrate levels found in the Danish and the Belgian samples could be determined when applying the student t-test (one tailed, type 2, $P \geq 0.95$).

The fact that no differences, in the levels of nitrite or nitrate, could be detected, between the samples taken from the Danish market and the Belgian market, is in good agreement with the progress in the legislation. Initially the use of nitrite and nitrate was regulated by Directive 95/2/EC laying down maximum residual levels of nitrites and nitrates as well as 'indicative ingoing amounts'. However, since poor correlation has been demonstrated between added amounts and the resulting residual amounts, the legislation was altered by the implementation of Directive 2006/52/EC which defines maximum amounts for E 249 potassium nitrite and E 250 sodium nitrite that may be added during manufacturing.

3.3. Heat treatment

A heat treatment study was conducted to mimic the preparation which may take place in the home or in e.g. the fast food industry. The levels of VNA may increase as a result of heat treatment. Especially the levels of NDMA and NPYR have been shown to be affected by e.g. frying (Yurchenko & Mölder, 2007). Some of the minor effects reported may be the result of concentration of the product when water evaporates from the product during the cooking process. The heat treatment may also speed up processes in the meat including nitrosation or result in release of nitrogen oxide or other nitrosating species bound to lipids and hereby giving rise to the production of NA (Hotchkiss, Vecchio, & Ross, 1985). The levels of VNA and NVNA measured in the non-heat treated and heat treated samples in the present study are presented in Fig. 1. The levels presented for the heat treated samples are calculated as µg kg^{-1} non-heat treated meat products. Thus the results for the heat treated samples are corrected for the concentration occurring as a result of water evaporating. The weight losses ranged from 17 to 55%, lowest for the chorizo and highest for "bacon 2".

Each bar in Fig. 1 represents the average level found in three individually heated samples analysed in duplicate. A significant difference in the levels of NA in heat treated products compared to non-heat treated products were found for those data marked with one or two asterisks indicating significance at the 95% or the 99% confidence level, respectively.

It was only for NPIP, NTCA and NMTCA that the same effect of heat treatment was observed for all products. The levels of NPIP increased for all products with a factor of three to five, though only significantly for "bacon 2", "ham 2" and pepperoni. The levels of NTCA were significantly lower after the heat treatment, except for "ham 1". All levels of NMTCA also decreased following heat treatment and significantly for the two types of bacon, "ham 2" and pepperoni. The results obtained for NHPRO indicate that also the levels of this NVNA decrease when the products are submitted to heat treatment (data not presented since only indicative because of analytical uncertainties).

The levels of NPRO, NDMA, NPYR, NDEA and NMA are all found to either increase or decrease as a result of heat treatment, depending on the type of products and/or the type of heat
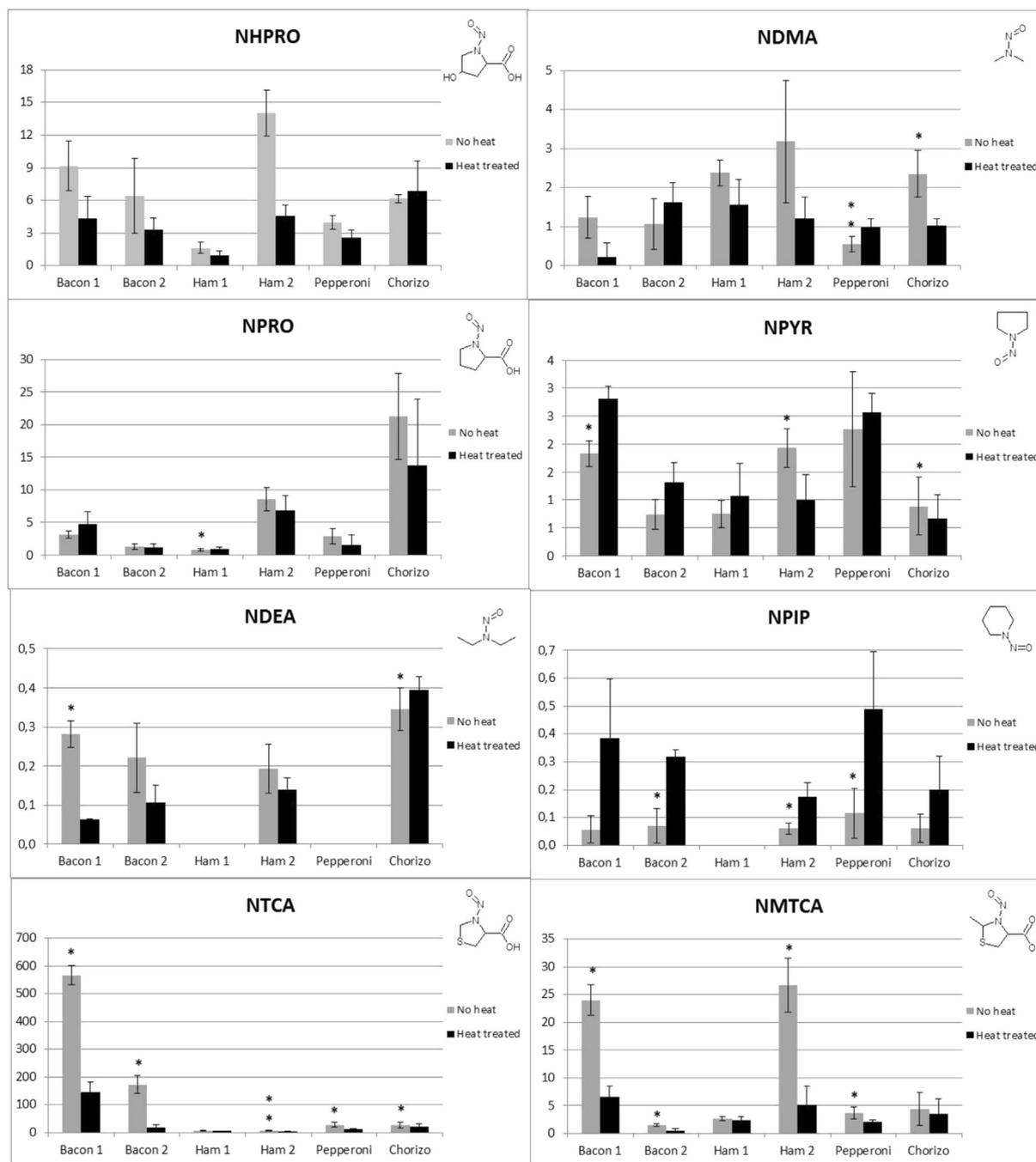
Fig. 1. The levels of NAs found in six different products after and before heat treatment, either by frying on a pan (Bacon 1 and 2) or backing in an oven preheated to 250 °C (Ham 1 and 2 and pepperoni and chorizo). Each bar represents three samples of heat treated or not heat treated respectively. If the bars are indicated with one or two asterisk (*) the difference in the NA level between the non-heat treated and the heat treated product was found significant at a 5% or 1% level (i.e. a test value for a type 1 one tailed student t-test of ≤0.05 or ≤0.01).

treatment. The present results do not support a relationship between the levels of NPRO and NPYR, in a way that a decrease in the NPRO levels is linked to an increase in the NPYR level, as would be expected if NPRO was decarboxylated to NPYR (Fig. 2), as suggested by e.g. Tricker and Kubacki (Tricker & Kubacki, 1992). NSAR was not detected in the products employed in this setup, thus changes in the NDMA contents as a result of NSAR decarboxylation are also considered as minor. However as will be discussed below the production of NPYR and NDMA may have occurred during the heat treatment even though a decrease was observed.
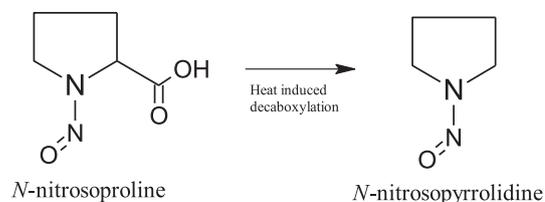
Fig. 2. Structure of N-nitrosoproline (NPRO) and its decarboxylated counterpart N-nitrosopyrrolidine (NPYR).

Some publications are available in which the NA levels are determined in the same batch of sample before and after heat treatment (Drabik-Markiewicz et al., 2009; Drabik-Markiewicz et al., 2011; Li, Wang, Xu, & Zhou, 2012; Rywotycki, 2007; Yurchenko & Mölder, 2006, 2007). The levels of VNA generally increased by heat treatment in these studies. Yurchenko and Mölder e.g. found that pan frying of nitrite preserved mutton resulted in a four times increase of NDMA (0.5–2.0 µg kg^{-1}) and up to 8 times increase of NPYR (from 1.9 to 15 µg kg^{-1}) (Yurchenko & Mölder, 2007). Li et al. (2012) on the other hand found that pan frying of nitrite preserved sausages only resulted in a 1.3 times increase in the NPYR level (Li et al., 2012). The variations in the results may be caused by a variation in the temperatures obtained in the products. It has been shown that maximum levels of NDMA and NPYR occur if products are heated to approximately 200 °C and if the temperature is further increased, slightly lower levels are found (Drabik-Markiewicz et al., 2009; Drabik-Markiewicz et al., 2011). This drop in the levels is expected to occur because of evaporation. The boiling points of NDMA, NDEA, NPYR and NPIP is 154, 176, 214 and 217 °C (at 721–760 mmHg), respectively. Thus it is likely that evaporation of especially NDMA and perhaps NDEA have occurred in the setup applied in the present study, and thereby compensated for any heat induced formation taking place (e.g. for pepperoni). The centrum temperature of the products for which a decrease in the NPYR level was observed in the present study ("ham 2" and chorizo) may have been higher than the boiling point of NPYR (214 °C) resulting in a positive net loss.

NMA was detected in the "bacon 1" and "bacon 2" samples and at a very low level in the heat treated "ham 1". The same level of NMA was found for the "bacon 1" sample after heat treatment, whereas a large reduction was observed for the "bacon 2" sample. Thus no general trend was found.

4. Conclusion

VNA and NVNA occur in nitrite/nitrate preserved meat products on the Danish market and the Belgium market. The mean levels of the VNA were generally low, whereas the mean levels of the NVNA were considerably higher. Although lower levels of nitrite/nitrate are allowed to be added during the manufacturing of products for the Danish market than if it is for the common European market, the majority of the Danish processed meat products still contain NA. No clear difference between the levels of NA in the products from the Danish and the Belgian market was found. The levels of NA found in the present study are comparable with those reported in previous and recent studies; however the frequency with which they are found may be lower and thereby also the mean contents. A clear positive effect of heat treatment on the level of NPIP was demonstrated, whereas varying impacts were observed for NPRO, NDMA, NPYR, NDEA and NMA depending on type of product and/or heat treatment. A clear heat induced decrease in the levels of NTCA and NMTCA was demonstrated.

5. Future

The expected differences between the average levels of NA in processed meat products from the Danish market and the Belgian market was not observed in the present study. Further work is needed in order to elucidate how different nitrite additions in the production of a meat products influence the formation of both VNA and NVNA, e.g. in the form a small scale production of a representative meat product allowing for the addition of different level of nitrite having all other factors equal.

References

Campillo, N., Viñas, P., Martínez-Castillo, N., & Hernández-Córdoba, M. (2011). Determination of volatile nitrosamines in meat products by microwave-assisted extraction and dispersive liquid–liquid microextraction coupled to gas chromatography–mass spectrometry. *Journal of Chromatography A, 1218*(14), 1815–1821.

Crews, C. (2010). The determination of N-nitrosamines in food. *Quality Assurance and Safety of Crops & Foods, 2*(1), 2–12.

Dellisanti, A., Cerutti, G., & Airoldi, L. (1996). Volatile N-nitrosamines in selected Italian cheeses. *Bulletin of Environmental Contamination and Toxicology, 57*(1), 16–21.

Demeyer, D., Honikel, K., & De Smet, S. (2008). The World Cancer Research Fund report 2007: a challenge for the meat processing industry. *Meat Science, 80*(4), 953–959.

Drabik-Markiewicz, G., Dejaegher, B., De Mey, E., Kowalska, T., Paelinck, H., & Vander Heyden, Y. (2011). Influence of putrescine, cadaverine, spermidine or spermine on the formation of N-nitrosamine in heated cured pork meat. *Food Chemistry, 126*(4), 1539–1545.

Drabik-Markiewicz, G., Van den Maagdenberg, K., De Mey, E., Deprez, S., Kowalska, T., & Paelinck, H. (2009). Role of proline and hydroxyproline in N-nitrosamine formation during heating in cured meat. *Meat Science, 81*(3), 479–486.

Herrmann, S. S., Duedahl-Olesen, L., & Granby, K. (2014). Simultaneous determination of volatile and non-volatile nitrosamines in processed meat products by liquid chromatography tandem mass spectrometry using atmospheric pressure chemical ionisation and electrospray ionisation. *Journal of Chromatography A, 1330*(0), 20–29.

Hill, M. J., Massey, R. C., Shuker, D. E. G., Leach, S., Tricker, A. R., Preussmann, R., et al. (1988). *Nitrosamines. Toxicology and microbiology.* ISBN 0-89573-605-0.

Hotchkiss, J. H., Vecchio, A. J., & Ross, H. D. (1985). N-Nitrosamine formation in fried-out bacon fat: evidence for nitrosation by lipid-bound nitrite. *Journal of Agricultural and Food Chemistry, 33*(1), 5–8.

IARC. (1998). IARC monographs on the evaluation of carcinogenic risks to humans. Some N-Nitroso compounds. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 17*.

Kuhnle, G. C., Bingham, S. A., Milner, J. A., & Romagnolo, D. F. (2010). *Meats, protein and cancer.*

Leth, T., Fagt, S., Nielsen, S., & Andersen, R. (2008). Nitrite and nitrate content in meat products and estimated intake in Denmark from 1998 to 2006. *Food Additives & Contaminants: Part A, 25*(10), 1237–1245.

Li, L., Wang, P., Xu, X., & Zhou, G. (2012). Influence of various cooking methods on the concentrations of volatile N-nitrosamines and Biogenic Amines in dry-cured sausages. *Journal of Food Science, 77*(5), C560–C565.

Massey, R. C., Key, P. E., Jones, R. A., & Logan, G. L. (1991). Volatile, non-volatile and total N-nitroso compounds in bacon. *Food Additives and Contaminants*, (5), 585.

Ozel, M. Z., Gogus, F., Yagci, S., Hamilton, J. F., & Lewis, A. C. (2010). Determination of volatile nitrosamines in various meat products using comprehensive gas chromatography-nitrogen chemiluminescence detection. *Food and Chemical Toxicology, 48*(11), 3268–3273.

Robach, M. C., Owens, J. L., Paquette, M. W., Sofos, J. N., & Busta, F. F. (1980). Effects of various concentrations of sodium nitrite and potassium sorbate on nitrosamine formation in commercially prepared bacon. *Journal of Food Science, 45*(5), 1280–1284.

Rywotycki, R. (2007). The effect of baking of various kinds of raw meat from different animal species and meat with functional additives on nitrosamine contamination level. *Food Chemistry, 101*(2), 540–548.

Spielgelhalder, B., Eisenbrand, G., & Preussmann, R. (1980). *Occurrence of volatile nitrosamines in food: A survey of the West German market.* IARC scientific publications. No.31.

Tricker, A. R., & Kubacki, S. J. (1992). Review of the occurrence and formation of non-volatile N-nitroso compounds in foods. *Food Additives and Contaminants*, (1), 39.

Villadsen, J., Jakobsen, M., & Eriksen, S. M. (2012). *Delprojekt 2-Nitrit og nitrat overvägning i kød- og fiskeprodukter.* http://www.foedevarestyrelsen.dk.

Yurchenko, S., & Mölder, U. (2006). Volatile N-nitrosamines in various fish products. *Food Chemistry, 96*(2), 325–333.

Yurchenko, S., & Mölder, U. (2007). The occurrence of volatile N-nitrosamines in Estonian meat products. *Food Chemistry, 100*(4), 1713–1721.

Paper III

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

2　**sausages**

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6　## Abstract

7　Literature on formation and mitigation of *N*-nitrosamine (NA) and especially non-volatile NA (NVNA) in

8　meat products is scarce and the present study is therefore a relevant contribution to the field. We found

9　positive correlation between the levels of *N*-nitrosopiperidine (NPIP), *N*-nitrosohydroxyproline (NHPRO),

10　*N*-nitrosoproline (NPRO), *N*-nitrosothiazolidine-4-carboxylic acid (NTCA) and *N*-nitroso-2-methyl-

11　thiazolidine-4-carboxylic acid (NMTCA) and the amount of nitrite added to cooked pork sausages. The

12　levels studied were 0, 60, 100, 150, 250 and 350 mg kg^{-1}. The levels of *N*-nitrosodimethylamine (NDMA)

13　and *N*-nitrosopyrrolidine (NPYR) remained at or below limit of quantification. Erythorbic acid inhibited the

14　formation of NHPRO, NPRO, NPIP and NTCA. This inhibition was for NTCA and NMTCA counteracted

15　by addition of free iron. Ascorbyl palmitate had less inhibitory effect than erythorbic acid and a combination

16　of the two provided no further protection. Increasing the black pepper content increased the levels of NPIP

17　and NMTCA. Only slight effects of increased fat content and addition of tripolyphosphate were observed.

18　**Keywords:** haem; iron; erythorbic acid; ascorbyl palmitate; factorial design

19　**Chemical compounds studied in this article:** *N*-nitrosothiazolidine-4-carboxylic acid

20　(NTCA)(PubChem CID:104890); *N*-nitroso-2-methyl-thiazolidine-4-carboxylic acid

21　(NMTCA)(PubChem CID:115101); *N*-nitrosoproline (NPRO)(PubChem CID:24141); *N*-

22　nitrosohydroxyproline (NHPRO)(PubChem CID:61873); *N*-nitrosopiperidine (NPIP)(PubChem CID:7526);

23　*N*-nitrososarcosine (NSAR)(PubChem CID:25811); *N*-nitrosodimethylamine (NDMA)

24   (PubChem CID:6124); *N*-nitrosopyrrolidine (NPYR)(PubChem CID:13591); Erythorbic acid

25   (PubChem CID:54675810); 6-O-palmitoyl-L-ascorbic acid (Ascorbyl palmitate) (PubChem CID:54680660)

Introduction

27   Sodium nitrite (nitrite) has for decades been widely used for preservation of meat products and is an efficient

28   inhibitor of the growth of *Clostridium botulinum* and thereby decreases the risk of this organism producing

29   toxins and heat-resistant spores. Nitrite also provides the processed meat with its characteristic red colour;

30   flavours and aromas known from products such as bacon and it inhibits lipid oxidation processes (Skibsted,

31   2011). However, *N*-nitrosamines (NA) may be formed during production and storage of nitrite preserved

32   meat products. The group of NA include both the so called volatile NA (VNA) and the non-volatile NA

33   (NVNA). The levels of these compounds in nitrite preserved meat products vary greatly, from below

34   detectability (<1 µg kg^{-1}) to levels in the order of thousands µg kg^{-1}, depending on the type of NA. In

35   particular the NVNA are found in high amounts (Hill *et al*., 1988). The NA is a large group of compounds of

36   which the majority is carcinogenic (IARC, 1978). The VNA are generally potent carcinogens (e.g. *N*-

37   nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR)) whereas the NVNA are weak

38   carcinogens (*N*-nitrososarcosine (NSAR)), or assumed to be non-carcinogenic (e.g. *N*-nitrosoproline

39   (NPRO), *N*-nitrosohydroxyproline (NHPRO), *N*-nitroso-thiazolidine-4-carboxylic acid (NTCA) and *N*-

40   nitroso-2-methyl-thiazolidine 4-carboxylic acid (NMTCA)). However, the assumption that NVNA as NPRO,

41   NHPRO, NTCA and NMTCA are non-carcinogenic, needs to be verified by actual toxicological *in vivo*

42   studies. Theoretically there is a risk of these compounds being decarboxylated into their carcinogenic

43   counterparts (NPYR, NHPYR, NThZ and NMThZ) either during heat treatment or by microbial activity in

44   the large intestine. As long as it cannot be verified whether these presumable non-carcinogenic NA,

45   contribute to the adverse health effects observed by consumption of processed meat or not, their formation

46   should be prevented as much as possible.

47   Studies have indicated that there is a positive though not necessarily linear correlation, between the amount

48   of nitrite added and the amount of NA formed (Drabik-Markiewicz, Van den Maagdenberg, De Mey,

49   Deprez, Kowalska & Paelinck, 2009, Drabik-Markiewicz, Dejaegher, De Mey, Kowalska, Paelinck &

50   Vander Heyden, 2011, Yurchenko & Mölder, 2007). These studies also indicate that the effects observed on

51   the NA levels by changes in the amount of nitrite added during preparation, i.e. the ingoing amount of nitrite,

52   may be different for the different NA and/or for the different test systems/meat products. Furthermore, the

53   majority of the available publications only deal with the VNA, i.e. typically NDMA, *N*-nitrosodiethylamine

54   (NDEA), NPYR and *N*-nitrosopiperidine (NPIP). Thus, data on the possible relationship between ingoing

55   amount of nitrite and the extent of NA formation in a meat product for both VNA and NVNA are scarce or

56   non-existing.

57

58   Besides the ingoing amount of nitrite a wide range of factors may potentially affect the formation of NA.

59   These factors are related to meat quality, fat content, processing, maturation and handling at home. Factors

60   related to processing include additives, heat applied during drying or smoking, precursors (added via wood

61   smoke, spices or other ingredients), storage/maturation conditions and packaging. Processing factors can

62   easily be controlled and their role in NA formation have been widely studied (e.g. (Hill *et al*., 1988, Li,

63   Wang, Xu & Zhou, 2012, Li, Shao, Zhu, Zhou & Xu, 2013, Sebranek & Fox, 1985). Though these studies

64   only deal with the VNA (NDMA, NPYR and in a few cases NDEA), whereas studies including the NVNA

65   are scarce (Janzowski, Eisenbrand & Preussmann, 1978).

66

67   Antioxidants are widely used as additives in meat processing because they increase the storage stability.

68   There is a large amount of literature on the effects of antioxidants on lipid oxidation processes, whereas

69   literature on the effect on the NA formation in meat products is limited (Li, Wang, Xu & Zhou, 2012, Li,

70   Shao, Zhu, Zhou & Xu, 2013, Mottram, Patterson, Rhodes & Gough, 1975, Rywotycki & Ryszard, 2002,

71   Sen, Donaldson, Seaman, Iyengar & Miles, 1976). These studies on the effect of adding antioxidants to meat

72   also only deal with NDMA, NPYR and NDEA and to our knowledge only one study tests the effect of

73   adding different levels of antioxidant (Mottram, Patterson, Rhodes & Gough, 1975). Thus data on the effect

74   of adding different levels of ascorbate/ascorbic acid/erythorbic acid (i.e. varies forms of vitamin C) on the

75   NA formation is needed in order to provide advice on the levels to be added during production and

76   preferably regarding both VNA and NVNA.

77

78   The different forms of vitamin C are polar antioxidants and because both oxygen and nitrogen oxide

79   produced by reduction of nitrite are more soluble in lipid (Combet *et al*., 2007) it has been suggested that the

80   levels of nitrosating species produced in the lipid phase can be higher than in the aqueous lean phase of the

81   meat. Nitrosating species liberated from the lipid phase has been suggested as the reason for the increase in

82   NPYR during frying of bacon (Sen, Donaldson, Seaman, Iyengar & Miles, 1976). Thus a combination of a

83   polar (e.g. erythorbic acid) and a non-polar antioxidant (e.g. ascorbyl palmitate) might inhibit the formation

84   of NA more efficiently than one antioxidant. Polyphosphates are often used additives in meat processing

85   because they increase the water holding capacity of the meat as well as stabilize the emulsion created in e.g.

86   sausages (Bianchi, 1971). When the lipid and the lean phase are emulsified it might facilitate exchange of

87   molecules between the two phases and thereby also exchange of nitrosating species and antioxidants.

88

89   Black pepper is used in the preparation of many types of sausages including cooked sausages and salamis

90   and NPIP is often detected in these types of products (De Mey *et al*., 2014). Black pepper contains piperidine

91   and *N*-nitrosopiperidin (Mey *et al*., 2014, Tricker, Pfundstein, Theobald, Preussmann & Spiegelhalder, 1991)

92   however it has to our knowledge not been confirmed by controlled studies that the addition of black pepper

93   to meat products results in occurrence of NPIP. NDMA, NPIP and NPYR may be produced when spices are

94   mixed with nitrite, thus spices may be a source of NA precursors (Sen, Donaldson, Charbonneau & Miles,

95   1974).

96

97   Haem has been suggested to play an essential role in the endogenous formation of NA following

98   consumption of red and processed meat (Lunn *et al*., 2007, Pierre *et al*., 2013, Santarelli *et al*., 2010) and

99   haem (Cross, Pollock & Bingham, 2003) are associated with increased endogenous formation of nitroso

100   compounds in both rodents and humans. Thus haem may play a role in the endogenous formation of nitroso

101   compounds in general and therefore of NA as well. The haem iron may be released from myoglobin or haem

102 as it passes through the digestive tract and it can therefore not be ruled out whether free iron plays a role in

103 the nitroso compound formation. Free iron induces lipid oxidation processes in meat which is inhibited by

104 the presence of antioxidants as is the NA formation, thus there may be some link between lipid oxidation

105 processes and NA formation. The role of haem/myoglobin and free iron in NA formation in meat has to our

106 knowledge not been studied.

107 *Aim*

108 To elucidate some of the areas mentioned above for which sufficient data is lacking we sat up the following

109 aims for the present study: 1) To examine the effect of ingoing amount of nitrite on the amount of NA

110 formed during the production of a cooked sausages; 2) To examine whether the formation of NA in the

111 nitrite cured cooked sausages were affected by the presence of a water soluble (erythorbic acid) and a fat

112 soluble antioxidant (ascorbyl palmitate), fat content, tripolyphosphate or black pepper; 3) To examine which

113 concentrations of the two mentioned antioxidants that resulted in the most efficient inhibition of the

114 formation of NA and if a combination of the two provides a higher degree of protection against NA

115 formation than just one of them; 4) To examine whether haem or free iron play a role in the formation of NA

116 in cooked sausages as has been suggested for the endogenous NA formation. In order to examine the effect

117 of each individual factor, possible interactions between them and to work with at realistic number of

118 experimental setups, factorial designs were employed.

119 # Experimental

120 ## Chemicals

121 Acetonitrile (extraction solvent) was of HPLC grade (Rathburn Chemicals Ltd, Walkerburn, Scotland).

122 Formic acid (purity 98-100% for analysis), pipecolic acid (purity 98%), and the methanol for LC eluent

123 (purity ≥99.9%, Fluka-Analytical) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

124 Erythorbic acid, ascorbyl palmitate, tripolyphosphate, iron(III)sulphate hydrate and myoglobin from equine

125 heart were also purchased from Sigma-Aldrich Co. The pure standards of *N*-nitrososarcosine (NSAR), *N*-

126 nitrosohydroxyproline (NHPRO), *N*-nitrosodibenzylamine (NDBzA), *N*-nitrosoproline (NPRO), *N*-

127 nitrosomethylaniline (NMA), *N*-nitroso-2-methyl-thiazolidine 4-carboxylic acid (NMTCA) and *N*-nitroso-

128 thiazolidine-4-carboxylic acid (NTCA) were purchased from Toronto Research Chemicals (Toronto,

129 Canada), whereas the standards *N*-nitrosodiethylamine (NDEA), *N*-nitrosodipropylamine (NDPA), *N*-

130 nitrosomorpholine (NMOR) and *N*-nitrosodimethylamine (NDMA) were purchased from Sigma-Aldrich Co.

131 *N*-nitrosomethylethylamine (NMEA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosodibutylamine (NDBA), *N*-

132 nitrosopiperidine (NPIP) were purchased from Dr. Ehrenstorfer (Ausburg, Germany). The internal standards

133 *N*-nitrosodimethylamine-d_6 (NDMA-d_6) and *N*-nitrosopyrrolidine-d_8 (NPYR-d_8) were purchased from

134 Sigma-Aldrich Co. and CDN Isotopes (Quebec, Canada), respectively. The purity of all of the NA standards

135 was ≥98% except for NMA which was of 95% purity. Sodium nitrite (Fluka-Analytical) was purchased from

136 Sigma-Aldrich Co..

137 *Cooked pork sausages (representative meat product)*

138 Cooked pork sausages were chosen as representative meat product, because sausages account for a major

139 part of the total consumption of processed meat products by the Danish as well as other European

140 populations (Linseisen *et al*., 2002). By choosing a minced meat product the ingredients are also more easily

141 distributed and any relevant equilibria are reached faster.

142 Trimmed fresh pork loin with a fat content of approximately 12% (www.foodcomp.dk) was minced

143 (Kenwood, MG470, Elgiganten, Glostrup Denmark) and all ingredients common for all samples in the setup

144 were added during mixing (Bear Varimixer, AR5A, A/S Wodschow & Co, Broendby, Denmark). The

145 sausage meat was prepared from tap water (26%), minced meat (67%), potato flour (4%), ground black

146 pepper (*Piper nigrum*, Santa Maria A/S, Broendby, Denmark)(0.125 or 0.5%), sodium chloride (2%),

147 paprika (0.5%), nitrite (0-350 mg kg^{-1} depending on the setup). Aliquots of the thoroughly mixed sausage

148 meat were transferred to a mini chopper (Phillips hand blender with chopper, HR1372, Punkt1, Roedovre,

149 Denmark) and further mixed with the ingredients/factors to be tested and chopped until they were evenly

150 distributed. The sausage meat that needed no further additions was chopped in the same way. Then the meat

151 was filled into sheep casings using either a sausage stuffer for larger portions or a single use plastic piping

152   bag equipped with a plastic stuffing horn for smaller portions. The sausages were hanged on an oven grate

153   and dried for 50 min at 70°C in an oven for the larger portions (UNOX, XVC705, Vigodrzere-Podova, Italy)

154   or a drying cabinet for smaller portions (Memmert drying cabinet, U40, Schwabach, Germany).

155   All sausages in all four setups were stored at -60°C after the end of preparation and any other

156   storage/treatments and until analysis.

157   *Experimental design and statistical analysis*

158   Four experimental setups were performed using cooked pork sausages as a model to meet the aims specified

159   for the present study i.e. **1)** A setup where the NA levels in cooked sausages prepared with six different

160   levels of nitrite was determined. **2)** A five factor 2-level factorial experiment to study the role of erythorbic

161   acid, ascorbyl palmitate, fat content, tripolyphosphate and black pepper on the NA formation. **3)** A full

162   central composite experiment to study the effect of thirteen different combinations of five levels of

163   erythorbic acid and five levels of ascorbyl palmitate on the NA formation. **4)** To study the role of haem and

164   iron in the NA formation a four factor 2-level factorial experiment was carried out including also erythorbic

165   acid and calcium as factors. Whether there was a significant difference in the NA levels in sausages prepared

166   with the high or the low level of each of the factors in the two 2-level factorial experiments was tested by

167   Student's *t*-test at 95% confidence levels. The statistical software Minitab (version 16) was used for setting

168   up the design and for the analysis of the results.

169   **Role of the ingoing amount of nitrite, drying and storage time (first setup)**

170   The role of different levels of in going amount of nitrite in the NA formation in sausages, the influence of

171   storage time after preparation and the effect of frying the sausages was studied. First sausages were prepared

172   in accordance with the recipe described above only varying the amount of in going nitrite. Six levels of

173   nitrite was employed i.e. 0, 60, 100, 150, 250 and 350 mg kg^{-1}. Twenty-four sausages, of approximately 25-

174   35 grams each, were prepared for each level of nitrite, i.e. a total of 144 sausages. The 24 sausages prepared

175   for each of the six nitrite levels were divided into four sub-groups. Sub-group 1 was packed immediately

176   after preparation (t_0) and put into the freezer after approximately four hours. Sub-group 2 was packed

177 immediately after the drying process (t_1) and frozen after about two hours. Sub-group 3 and 4 were packed

178 immediately after the drying process and stored at 5°C for 24 hours (t_2) and then frozen. Sub-group 4 was

179 used for studying the effect of pan frying. These sausages were fried for 10 minutes one group at a time

180 using the same frying pan. During these 10 minutes the centrum temperature of the sausage reached 100°C.

181 The weight loss was registered allowing for calculation of the NA content per kg^{-1} of the sausages not fried.

182 **Role of erythorbic acid, ascorbyl palmitate, fat content, black pepper and tripolyphosphate**

183 **(second setup)**

184 This second setup, a 2-level fractional factorial design (resolution V), was set up to study the effect of

185 erythorbic acid (250 or 1000 mg kg^{-1}), ascorbyl palmitate (0 or 250 mg kg^{-1}), increased fat content (12 or

186 25%), black pepper (1.25 or 5 g kg^{-1}) and tripolyphosphate (0 or 5 g kg^{-1}) on the formation of NA in cooked

187 sausages prepared with 150 mg kg^{-1} sodium nitrite. The design included the preparation of sausages with 16

188 different combinations of the five factors. A minimum of six sausages, each of about 15 gram, were

189 prepared for each of the 16 preparations. The sausages were packed in sealed plastic bags with minimum

190 three in each. One bag of each of the 16 preparations was stored either for 24 hours or 5 days at 5°C before

191 freezing.

192 **Mitigation by erythorbic acid and/or ascorbyl palmitate (third setup)**

193 The third setup, a full central composite experimental design, included 13 combinations of five different

194 concentrations of erythorbic acid (396, 500, 750, 1000 and 1104 mg kg^{-1}) and ascorbyl palmitate (26, 150,

195 450, 750 and 874 mg kg^{-1}) in cooked sausages prepared with 150 mg kg^{-1} sodium nitrite. These 13

196 combinations included four samples representing a "cube" portion, two axial or "star" points, a center point

197 and four replicates. Four sausages, each of approximately 15 grams, were prepared for each of the 13

198 combination of the two antioxidants.

199 **Role of haem and iron (fourth setup)**

200 The role of haem iron, in the form of myoglobin from equine heart, and free iron, in the form of

201 iron(III)sulphate hydrate, in the formation of NA in cooked sausages prepared with 150 mg kg^{-1} sodium

202 nitrite was studied. Calcium, in the form of calcium carbonate, and erythorbic acid was also included as a

203 factor in this factorial design because these two factors may counteract a possible effect of haem and iron,

204 respectively. By including erythorbic acid in this setup it was also possible to test the effect of this factor

205 again. The full 2-level factorial design setup required preparation of sausages from sausage meat prepared

206 with 16 different combinations of the four factors, i.e. added myoglobin (0 or 1.5 g kg^{-1}), iron(III)sulphate

207 hydrate (0 or 36 mg kg^{-1}), erythorbic acid (0 or 1000 mg kg^{-1}) and/or calcium carbonate (0 or 6 g kg^{-1}). Four

208 sausages, of approximately 15 gram each, were prepared for each of the 16 preparations.

N-nitrosamine analysis

210 The contents of eight VNA and five NVNA in the samples were determined according to a method recently

211 developed and validated at our laboratory (Herrmann, Duedahl-Olesen & Granby, 2014). In the following the

212 method will only be described in brief.

Extraction method

214 2.5 g of homogenized sample with internal standard (ISTD) added (NPYR-d_8 and NDMA-d_6) is extracted

215 with 7.5 ml 1% formic acid in acetonitrile. After centrifugation the supernatant was removed and frozen. The

216 thawed extract was centrifuged (4500 *g*). 5 ml of the acetonitrile phase was evaporated under a stream of

217 nitrogen to a volume of ~0.25 ml and then adjusted to 1.0 ml with Milli-Q water. After diluting 1:1 with

218 Milli-Q water the extract was filtered and analysed.

LC-MS/MS method

220 The final extracts were analysed by LC(APCI/ESI)-MS/MS as described in Herrmann *et al.* (2014). The

221 analytical platform consisted of an Agilent 1200 Series HPLC (Agilent Technologies, Santa Clara, CA, US)

222 coupled with an Agilent 6460 Series Triple Quadrupole (Agilent Technologies) equipped with either an

223 APCI or a Jet Stream ESI source. The analytical column was a Poroshell PhenylHexyl column 150x2.1mm,

224 3 µm column (Agilent Technologies). A mobile phase gradient programme was applied, using 0.1% formic

225 acid in water and methanol, respectively. The injection volume was 3.5 µL.

226   Quantitative and qualitative analysis were performed by external calibration (0.334 to 1000 ng ml^{-1}) and

227   compared with the retention times and quantifier ion/qualifier ion ratios obtained by analysing NA standard

228   solution and/or spiked QC samples (Herrmann, Duedahl-Olesen & Granby, 2014).

Results and discussion

Role of the ingoing amount of nitrite, drying and time (first setup)

231   By increasing the ingoing amount of nitrite (0, 60, 100, 150, 250, 350 mg kg^{-1}) the levels of NHPRO, NPRO,

232   NTCA, NMTCA (Figure 1A), NSAR and NPIP (Figure 1C) increased in the sausages. A steep increase in

233   the level of NMTCA was observed by adding 60 mg kg^{-1}. Higher levels of nitrite only increased the NMTCA

234   levels slightly, indicating that other factors than nitrite is the limiting factor for the formation of NMTCA. In

235   sausages prepared with 150 mg kg^{-1} nitrite, which is the amount of nitrite allowed to be added to sausages for

236   the common European market (https://webgate.ec.europa.eu/sanco_foods), NPIP (Figure 1C), NHPRO,

237   NPRO, NTCA and NMTCA (Figure 1A) were found in levels of approximately 2, 10, 40, 70 and 25 µg kg^{-1},

238   respectively. NSAR was at LOD if more than 150 mg kg^{-1} nitrite was added, and by further increasing the

239   nitrite level a clear increase in the NSAR level was found (Figure 1C). The levels of NDMA and NPYR were

240   found relatively unaffected by the increase in added nitrite. The levels of NDMA and NPYR remained at or

241   below 2 µg kg^{-1}, which is at the limit of quantification (LOQ) for the method applied (Herrmann, Duedahl-

242   Olesen & Granby, 2014). Increasing the level of nitrite was also found by others to have a limited effect on

243   the level of NDMA (Drabik-Markiewicz, Dejaegher, De Mey, Kowalska, Paelinck & Vander Heyden, 2011).

244   If the sausages were further prepared by pan frying (Figure 1B 1C) the levels of NSAR, NPIP (Figure 1D),

245   NTCA and NMTCA (Figure 1B) increased by up to about 2, 2, 1.5 and 4 times, respectively. For NTCA the

246   difference in the content between the not fried (Figure 1A) and the fried sausages (Figure 1B) increased with

247   increasing amount of ingoing nitrite. This resulted in a more linear correlation between added nitrite and

248   NTCA level and with a steeper slope than found for the not fried sausages. For these fried sausages a slightly

249   higher level of NDMA and NPYR were indicated for the sausages prepared with 60 or 100 mg kg^{-1} nitrite

than in those prepared without nitrite (Figure 1D). In the sausages prepared with 150 mg kg^{-1} nitrite the

levels of NPIP (Figure 1D), NHPRO, NPRO, NTCA and NMTCA (Figure 1B) amounted to 2.6, 10, 40, 70

and 80 µg kg^{-1}, thus frying induced an increase in the NPIP (2.6 µg kg^{-1}) and the NMTCA (80 µg kg^{-1})

levels.

The levels of NDMA and NPYR remained at or below 2 µg kg^{-1} also in the fried sausages. NTCA levels of

up to 140 µg kg^{-1} were detected in the sausages after frying. Thus relatively high levels of NTCA may be

produced when sausages are fried until a center temperature of 100°C. The high levels of NTCA reported for

smoked meat products (Massey, Key, Jones & Logan, 1991) may be at least partly attributed to the heat

treatment (60-80°C) which is also performed during traditional hot smoke processing (Fellows, 2009).

However if heated to a temperature of 250°C for approximately 10 min. studies performed at our laboratory

have shown that the levels of both NTCA and NMTCA decrease (Herrmann, Duedahl-Olesen & Granby,

2015). This decrease may be caused by heat induced decarboxylation of NTCA and NMTCA to NTHz and

NMTHz, respectively. Though according to Mandagere *et al.* (1987) the levels of NTHz also decrease during

frying of bacon.

Only slight differences in the NA levels were observed between sausages frozen immediately after

preparation (without drying process)(t_0), immediately after drying (t_1) and sausages frozen after drying and

24 hours of storage at 5°C (t_2)(Figure 2). Though, the levels of NTCA were affected by the drying process,

i.e. increased from approximately 10 µg kg^{-1} (t_0) to 55 and 85 µg kg^{-1} (t_1) in sausages prepared with 150 and

350 mg nitrite kg^{-1}. Storage for 24 hours at 5°C (t_2) did not further affect the levels of NTCA.

The present study show that when the ingoing amount of nitrite increases, the levels of most NA also

increase. Only for NDMA and NPYR this relationships was not found. In general however the results for

NDMA and NPYR in the present study can only be indicative because the levels of these two NA were at the

LOQ level and therefore associated with higher uncertainties.

Role of erythorbic acid, ascorbyl palmitate, fat content, black pepper and tripolyphosphate (second setup)

Figure 3 A1-E1 shows the main effects, i.e. the effect of the individual factors, on the NA levels in sausages. Of the five factors studied in the factorial design it was found that the two antioxidants, erythorbic acid and ascorbyl palmitate, had the highest impact on the levels of NA (Figure 3 A1, B1, D1 and E1). In general the increasing the level of or adding antioxidants lowered the levels of NAs in the sausages. The levels of NSAR, NDMA and NPYR were at the limit of determination (LOD) or LOQ and the observed effects are therefore associated with great uncertainty. No figures have therefore been generated for these three NA since the observed effects can only be indicative. Mottram, Patterson, Edwards and Gough (1977) showed however that NDMA formation is inhibited by ascorbate. They produced an NDMA level of 100 µg kg^{-1} pork meat by fortifying meat with dimethylamine (100 ppm) and curing it in brine with 1000 ppm NaNO$_2$. By also adding 2000 ppm of ascorbate to the brine the level of NDMA decreased to <1 µg kg^{-1} (Mottram, Patterson, Rhodes & Gough, 1975).

In the present setup the levels of NPIP (Figure 3 C1) was not reduced by increasing the level of erythorbic acid or adding ascorbyl palmitate. Nor did the levels of NSAR and NDMA seem to be affected by the presence of erythorbic acid and ascorbyl palmitate. For NPIP the low level of erythorbic acid of 250 mg kg^{-1} though seems to provide the full inhibitory effect. This is indicated by the approximately 60% reduction in the NPIP levels observed for sausages prepared with 1000 mg kg^{-1} erythorbic acid compared to no erythorbic acid in the setup four (Figure 5 C1).

No significant effects were induced by increasing the fat content from 12% to 25%, though a slight increase in the levels of NDMA and NPYR, as well as a decrease in the levels of NSAR and NMTCA, was indicated. The slightly higher levels of NDMA and NPYR are in agreement with the results of e.g. Mottram *et al.* (1977) who found that NDMA and NPYR formation was primarily occurring in the lipid phase of bacon. Several mechanism for this preferential formation in the lipid phase has been presented; higher temperature during frying than in the lean part with a higher water content, a different chemical environment favoring nitrosation (Mottram, Patterson, Edwards & Gough, 1977) which could give a higher solubility of both

299   nitrogen oxide (NO) and oxygen in the lipid phase (Liu, Miller, Joshi, Thomas & Lancaster, 1998) resulting

300   in higher levels of nitrifying species as e.g. N_2O_2.

301   The level of NPIP increased from approximately 0.1 to 0.4 µg kg^{-1} when increasing the amount of black

302   pepper from 1.25 to 5.0 g kg^{-1} sausage meat (Figure 3 C1). Though, the effect was not significant. However

303   if applying the same analysis and data treatment to the same type of sausages stored for additionally four

304   days at 5°C before freezing, the level of NPIP was significantly higher in the sausages with the high amount

305   of black pepper than in the sausages with the low amount (data not shown). Besides the higher level of NPIP

306   only minor differences in the NA levels were observed for the sausages stored for 24 hours and those stored

307   for 5 days at 5°C. When preparing the sausages with 5.0 g of black pepper per kg sausage meat and without

308   any antioxidants the levels of NPIP were in the order of 2.0 (setup one) to 2.7 µg kg^{-1} (setup four). The

309   present study supports, that NPIP in processed meat products originates or partly originates from the use of

310   black pepper. Yurchenko & Mölder (2007) also suggested that black pepper may be the main source of

311   NPIP.

312   The level of NMTCA was also significantly increased by an increase in the amount of black pepper (Figure 3

313   E1). A pepper induced increase was also indicated for NTCA (Figure 3 D1). NTCA (Ratner & Clarke, 1937)

314   and NMTCA are formed by the condensation of formaldehyde or acetaldehyde with cysteine followed by

315   nitrosation. The formation of these two NA may therefore be limited by the availability of the aldehydes,

316   cysteine or the actual precursors, i.e. thiazolidine 4-carboxylic acid (TCA) and 2-methylthiazolidine 4-

317   carboxylic acid (MTCA).

318   In meat the presence of the amino acid cysteine ought not to be a limiting factor for the formation of NTCA

319   or NMTCA. TCA occurs in plants at varying levels (Suvachittanont, Kurashima, Esumi & Tsuda, 1996) why

320   pepper may be a source of TCA (Figure 6). Formaldehyde is ubiquitous in the environment and exists at low

321   levels in most living organisms as a metabolic intermediate however black pepper also contains substances

322   (e.g. piperine) which can liberate formaldehyde. Larger amount of formaldehyde is liberated during

323   combustion processes and therefore also produced during wood smoking. High levels of NTCA occur in

324   smoked meat products. The formation of NTCA therefore seems to be limited by the availability of

325   formaldehyde. Formation of NMTCA seems to be less related to the smoking process (Herrmann, Duedahl-

326   Olesen & Granby, 2015, Massey, Key, Jones & Logan, 1991) and dependent on other constituent(s). As

327   mentioned earlier we performed some preliminary tests on a simpler meat system using minced pork meat, to

328   which only water, nitrite and sodium chloride were added. In this simple meat system we found that the

329   formation of both NTCA and NMTCA were not limited by another factor than nitrite, because saturation

330   curves were observed with increasing ingoing amount of nitrite (data not shown).

331   The addition of tripolyphosphate resulted in no significant main effects (Figure 3 A1-E1).

332   In Figure 3 A2-E2 are the observed interactions presented as interaction plots. If the lines in the interaction

333   plots are parallel it indicates no interaction between the two factors in question (indicated below and in the

334   right side of the figure). Only one significant interaction was observed in this setup. If the level of erythorbic

335   acid was high then the effect of also adding ascorbyl palmitate on the NPRO level (Figure 3 B2) was very

336   limited, whereas if the level of erythorbic acid was low adding ascorbyl palmitate did provide further

337   inhibition. This interaction was also indicated for the other NA. From the interaction plots it also appears that

338   the distance between the two lines are generally greatest for erythorbic acid which very nicely illustrates that

339   of the tested factors erythorbic acid exhibits the largest effect on the NA levels.

340   Based on the result of this second setup we concluded that black pepper increases the levels of at least two

341   NA of which one is known to be carcinogenic. Besides the ingoing amount of nitrite, erythorbic acid is the

342   factor with the highest impact on the NA levels. Ascorbyl palmitate may contribute to the inhibition of NA

343   and it was therefore chosen to further examine the effect of combining the two antioxidants at different levels

344   (third setup).

345   **Mitigation by erythorbic acid and/or ascorbyl palmitate (third setup)**

346   The results of this third setup are illustrated as surface plots (Figure 4). As can be seen from these surface

347   plots the levels of NHPRO, NPRO, NPIP and NTCA decrease with increasing amount of erythorbic acid

348   (396, 500, 750, 1000 and 1104 mg kg^{-1}). The levels of NDMA, NSAR and NPYR were also in this setup up

349   low (~1 µg kg^{-1}) in all thirteen samples and the results obtained for these NA are therefore associated with

350   great uncertainty and surface plots have therefore not been generated for these two NA. The present study

351   demonstrate that the levels of NA in sausages may be reduced by adding erythorbic acid and that the extent

352   of the inhibition increases with increasing amount of erythorbic acid, at least up to the highest tested level of

353   1104 mg kg^{-1}. The effect of concurrent presence of ascorbyl palmitate (26, 150, 450, 750 and 874 mg kg^{-1})

354   however seems to have the opposite effect, i.e. to stimulate the formation of NA or to counter act the effect

355   of erythorbic acid.

Role of haem and iron (fourth setup)

357   Haem has been suggested to play an essential role in the endogenous formation of NA linked with high

358   consumption of red and processed meat (Bingham, Hughes & Cross, 2002, Cross, Pollock & Bingham, 2003,

359   Pierre *et al*., 2013). Lunn *et al*. (2007) also found that in an aqueous solution no nitrosation of morpholine

360   occurred even at elevated levels of nitrite, however if haem iron was also added NMOR was produced (Lunn

361   *et al*., 2007). Calcium, which can chelate the iron in haem, was by Pierre *et al*. (2013) found to prevent a

362   haem induced increase in endogenous formation of nitroso compounds in humans (Pierre *et al*., 2013). Iron

363   plays an essential role in lipid peroxidation processes occurring in meat and antioxidants as

364   ascorbate/erythorbic acid inhibit these unwanted processes (Igene, King, Pearson & Gray, 1979, Ladikos &

365   Lougovois, 1990).

366   The results of the study are presented in Figure 5. The levels of NSAR, NDMA and NPYR were in all the

367   tested combinations at or below the LOQ of the method. The observed effects on the levels of these three NA

368   are therefore associated with high uncertainty and the results for these are not included in Figure 5. The

369   observations are however described as indicative results in the following.

370   No significant effects were observed by supplementing the sausage meat with haem (myoglobin)(Figure 5

371   A1-E1). However a slight reduction in the levels of NPIP (Figure 5 C1), NSAR and NPYR were indicated.

372   This reduction may be the result of increased competition for the nitrosating species because more NO was

373   bound to the added haem. Slight indications of calcium counteracting this inhibiting effect were observed for

374   NHPRO (Figure 5 A2), NDMA and NPYR.

375   The levels of NHPRO and NMTCA were found to increase significantly by adding Fe (III)(Figure 5 A1 and

376   E1). An increase was also indicated for NTCA (Figure 5 D1) and NPYR. For the remaining NA no effect

377   was observed by adding Fe(III). Erythorbic acid was also in this setup found to reduce the NA levels (Figure

378   5 A1-E1) except for NDMA and NPYR. The reduction was found to be significant for NHPRO, NPRO,

379   NPIP and NTCA. Interaction between iron and erythorbic acid was indicated for NTCA and NMTCA,

380   though only significantly for NMTCA (Figure 5 E2). Addition of Fe(III) canceled the otherwise inhibiting

381   effect of erythorbic acid (Figure 5 D2, E2). The fact that erythorbic acid does not result in a decrease in the

382   levels of NTCA and NMTCA if the sausage meat has also been supplemented with Fe(III) may indicate, that

383   the degree of oxidative stress is too severe for the added amount of prooxidant to have an effect, or that the

384   inhibitory effect of adding erythorbic acid is counteracted by an increasing level of Fe(II).

385   Thus the present study does not indicate that haem is a catalyst for the formation of NA in meat product as

386   has been suggested for endogenous formation. It does however indicate that free iron may stimulate the

387   formation of NA in meat and that the effect of adding antioxidants as erythorbic acid which normally reduces

388   the levels of NA is diminished or prevented by the elevated iron level. This effect was especially clear for

389   NTCA and NMTCA. The formation of NTCA, NMTCA was also prevented to a lesser extent by just the

390   presence of erythorbic acid than was NHPRO, NPRO and NPIP. The levels of these three NA were reduced

391   by approximately 60-75% by the addition of the 1000 mg kg^{-1} erythorbic acid.

392   The observed interaction between Fe and erythorbic acid may indicate that the formation of NTCA and

393   NMTCA are linked to oxidative processes occurring in the meat. Oxidation of phosphor lipids actually

394   results in the formation of many different aldehydes (Esterbauer, Schaur & Zollner, 1991) including

395   formaldehyde (Farmer & Mottram, 1990) and perhaps also acetaldehyde (Figure 6). Lipid oxidation

396   processes are promoted by heat and prolonged storage under aerobic conditions. Storage for 24 hours of

397   uncooked sausage meat at room temperature and aerobic conditions resulted in four times higher levels of

398   NTCA (10 compared to 40 µg kg^{-1}) and NMTCA (3 compared to 12 µg kg^{-1}) than if the same samples were

399   stored at 5 degrees in a tight container. A fourfold higher level of NTCA and NMTCA by a temperature

400   increase of 15°C corresponds well to a general temperature coefficient by a factor of 2 for a 10°C increase in

401   temperature which has been found to apply to biological and chemical reactions in general. The higher levels

402   produced in the sample stored at room temperature under aerobic may have resulted in more lipid oxidation.

403   Smoke is though also a significant source of aldehydes (Ikins *et al*., 1988) why the highest levels of NTCA

404   are found in smoked products (Herrmann, Duedahl-Olesen & Granby, 2015, Sen, Baddoo & Seaman, 1986).

405   Several aldehydes may occur in the products but e.g. formaldehyde and acetaldehyd can upon reaction with

406   cysteine from the meat and subsequent nitrosation produce NTCA and NMTCA, respectively (Ohshima &

407   Bartsch, 1984). The saturation curves observed for the formation of NTCA and NMTCA in relation to added

408   nitrite in the minced meat model, as described earlier (data not shown), may thus indicate that the amount of

409   precursors was limited. This may be due to a low degree of lipid peroxidation and/or that ingredients added

410   to the sausages, but not to the minced meat model, contain the relevant precursors (Figure 6).

Conclusion

412   From the present results it can be concluded that there is a positive correlation between the amount of

413   ingoing nitrite and the levels of the targeted NA formed in sausages prepared from minced pork meat. An

414   exception was NDMA and NPYR of which the formation seemed unaffected by increasing amount of nitrite.

415   Among several factors which can easily be controlled during the production, erythorbic acid was the factor

416   which affected the levels of most NA in nitrite preserved sausages. The levels of most NA were found to be

417   inversely proportional with increasing amounts of erythorbic acid (396-1104 mg kg^{-1}). By preparing the

418   sausages with 1000 mg kg^{-1} erythorbic acid the levels of the different NA were reduced by 20 to 75%. The

419   formation of NPIP was found to be closely linked to the amount of black pepper added. Adding ascorbyl

420   palmitate, a fat soluble antioxidant, provided no additional protection from NA formation than a high level of

421   erythorbic acid (1000 mg kg^{-1}) alone. Free iron and not haem stimulated the formation of some NA

422 (NMTCA and NHPRO and maybe also NTCA) in sausages. Addition of free iron counteracted the inhibitory

423 effect of erythorbic acid on the formation of NTCA and NMTCA.

Acknowledgements

References

428 Bianchi, E. (1971). Polyphosphates and meat. *Annales Des Falsifications Et De L'Expertise Chimique*,

429 64(693), 1-4.

430 Bingham, S. A., Hughes, R., & Cross, A. J. (2002). Effect of white versus red meat on endogenous N-

431 nitrosation in the human colon and further evidence of a dose response. *Journal of Nutrition*, 132(11), 3522-

432 3525.

433 Combet, E., Paterson, S., Iijima, K., Winter, J., Mullen, W., Crozier, A., Preston, T., & McColl, K. E. L.

434 (2007). Fat transforms ascorbic acid from inhibiting to promoting acid-catalysed N-nitrosation. *Gut*, 56(12),

435 1678-1684.

436 Cross, A. J., Pollock, J. R. A., & Bingham, S. A. (2003). Haem, not protein or inorganic iron, is responsible

437 for endogenous intestinal N-nitrosation arising from red meat. *Cancer research*, 63(10), 2358-2360.

438 De Mey, E., De Klerck, K., De Maere, H., Dewulf, L., Derdelinckx, G., Peeters, M., Fraeye, I., Vander

439 Heyden, Y., & Paelinck, H. (2014). The occurrence of N-nitrosamines, residual nitrite and biogenic amines

440 in commercial dry fermented sausages and evaluation of their occasional relation. *Meat Science*, 96(2, Part

441 A), 821-828.

442 Drabik-Markiewicz, G., Dejaegher, B., De Mey, E., Kowalska, T., Paelinck, H., & Vander Heyden, Y.

443 (2011). Influence of putrescine, cadaverine, spermidine or spermine on the formation of N-nitrosamine in

444 heated cured pork meat. *Food Chemistry*, 126(4), 1539-1545.

445 Drabik-Markiewicz, G., Van den Maagdenberg, K., De Mey, E., Deprez, S., Kowalska, T., & Paelinck, H.

446 (2009). Role of proline and hydroxyproline in N-nitrosamine formation during heating in cured meat. *Meat*

447 *Science*, 81(3), 479-486.

448 Esterbauer, H., Schaur, R., & Zollner, H. (1991). Chemistry and Biochemistry of 4-Hydroxynonenal,

449 Malonaldehyde and Related Aldehydes. *Free Radical Biology and Medicine*, 11(1), 81-128.

450 Farmer, L. J., & Mottram, D. S. (1990). Interaction of lipid in the maillard reaction between cysteine and

451 ribose: The effect of a triglyceride and three phospholipids on the volatile products. *Journal of the science of*

452 *food and agriculture*, 53(4), 505-525.

453 Fellows, P. J. (2009). *Food processing technology - Principles and practice* (third ed.), Woodhead

454 Publishing, UK.

455 Herrmann, S. S., Duedahl-Olesen, L., & Granby, K. (2015). Occurrence of volatile and non-volatile N-

456 nitrosamines in processed meat products and the role of heat treatment. *Food Control*, 48, 163.

457 Herrmann, S. S., Duedahl-Olesen, L., & Granby, K. (2014). Simultaneous determination of volatile and non-

458 volatile nitrosamines in processed meat products by liquid chromatography tandem mass spectrometry using

459 atmospheric pressure chemical ionisation and electrospray ionisation. *Journal of Chromatography A*,

460 1330(0), 20-29.

461 Hill, M. J., Massey, R. C., Shuker, D. E. G., Leach, S., Tricker, A. R., Preussmann, R., & Rowland, J. R.

462 (1988). Nitrosamines. Toxicology and microbiology. 169pp. ISBN 0-89573-605-0.

463   IARC monographs on the evaluation of carcinogenic risks to humans (1978). Some N-Nitroso Compounds.

464   *IARC monographs on the evaluation of carcinogenic risks to humans*. 17.

465   Igene, J. O., King, J. A., Pearson, A. M., & Gray, J. I. (1979). Inflence of heme pigments, nitrite, and non-

466   heme iron on development of Warmed-Over Flavor (WOF) in cooked meat. *Journal of Agricultural and*

467   *Food Chemistry*, 27(4), 838-842.

468   Ikins, W. G., Gray, J. I., Mandagere, A. K., Booren, A. M., Pearson, A. M., Stachiw, M. A., & Buckley, D. J.

469   (1988). Contribution of wood smoke to in vivo formation of N-nitrosothiazolidine-4-carboxylic acid: Initial

470   studies. *Food and Chemical Toxicology*, 26(1), 15-21.

471   Janzowski, C., Eisenbrand, G., & Preussmann, R. (1978). Occurrence of N-nitrosamino acids in cured meat

472   products and their effect on formation of N-nitrosamines during heating. *Food and cosmetics toxicology*,

473   16(4), 343-348.

474   Ladikos, D., & Lougovois, V. (1990). Lipid oxidation in muscle food a review. *Food Chemistry*, 35(4), 295-

475   314.

476   Li, L., Shao, J., Zhu, X., Zhou, G., & Xu, X. (2013). Effect of plant polyphenols and ascorbic acid on lipid

477   oxidation, residual nitrite and N-nitrosamines formation in dry-cured sausage. *International Journal of Food*

478   *Science & Technology*, 48(6), 1157-1164.

479   Li, L., Wang, P., Xu, X., & Zhou, G. (2012). Influence of Various Cooking Methods on the Concentrations

480   of Volatile N-Nitrosamines and Biogenic Amines in Dry-Cured Sausages. *Journal of Food Science*, 77(5),

481   C560-C565.

482   Linseisen, J., Kesse, E., Slimani, N., Bueno-de-Mesquita, H., Ocké, M., Skeie, G., Kumle, M., Iraeta, M. D.,

483   Gómez, P. M., Janzon, L., Stattin, P., Welch, A., Spencer, E., Overvad, K., Tjønneland, A., Clavel-Chapelon,

484   F., Miller, A., Klipstein-Grobusch, K., Lagiou, P., Kalapothaki, V., Masala, G., Giurdanella, M., Norat, T., &

485   Riboli, E. (2002). Meat consumption in the European Prospective Investigation into Cancer and Nutrition

486   (EPIC) cohorts: results from 24-hour dietary recalls. *Public health nutrition*, 5(6), 1243-1258.

487   Liu, X., Miller, M., Joshi, M., Thomas, D., & Lancaster, J. (1998). Accelerated reaction of nitric oxide with

488   O-2 within the hydrophobic interior of biological membranes. *Proceedings of the National Academy of*

489   *Sciences of the United States of America*, 95(5), 2175-2179.

490   Lunn, J. C., Kuhnle, G., Mai, V., Frankenfeld, C., Shuker, D. E. G., Glen, R. C., Goodman, J. M., Pollock, J.

491   R. A., & Bingham, S. A. (2007). The effect of haem in red and processed meat on the endogenous formation

492   of N-nitroso compounds in the upper gastrointestinal tract. *Carcinogenesis*, 28(3), 685-690.

493   Mandagere, A. K., Gray, J. I., Ikins, W. G., Booren, A. M., & Pearson, A. M. (1987). An Investigation into

494   Glucose as a Potential Precursor of N-Nitrosothiazolidine in Bacon. *Journal of Food Science*, 52(5), 1147-

495   1148.

496   Massey, R. C., Key, P. E., Jones, R. A., & Logan, G. L. (1991). - Volatile, non-volatile and total N-nitroso

497   compounds in bacon. - *Food Additives and Contaminants*(- 5), - 585.

498   Mey, E., Maere, H., Dewulf, L., Paelinck, H., Sajewicz, M., Fraeye, I., & Kowalska, T. (2014). Assessment

499   of the N-nitrosopiperidine formation risk from piperine and piperidine contained in spices used as meat

500   product additives. *European Food Research and Technology*, 238(3), 477-484.

501   Mottram, D. S., Patterson, R. L. S., Edwards, R. A., & Gough, T. A. (1977). The preferential formation of

502   volatile N-nitrosamines in the fat of fried bacon. *Journal of the science of food and agriculture*, 28(11),

503   1025-1029.

504   Mottram, D. S., Patterson, R. L. S., Rhodes, D. N., & Gough, T. A. (1975). Influence of ascorbic acid and ph

505   on the formation of n-nitrosodimethylamine in cured pork containing added dimethylamine. *Journal of the*

506   *science of food and agriculture*, 26(1), 47-53.

507 Ohshima, H., & Bartsch, H. (1984). Monitoring endogenous nitrosamine formation in man. *IARC scientific*

508 *publications* (59), 233-246.

509 Pierre, F. H. F., Martin, O. C. B., Santarelli, R. L., Taché, S., Naud, N., Guéraud, F., Audebert, M., Dupuy,

510 J., Meunier, N., Attaix, D., Vendeuvre, J., Mirvish, S. S., Kuhnle, G. C. G., Cano, N., & Corpet, D. E.

511 (2013). Calcium and α-tocopherol suppress cured-meat promotion of chemically induced colon

512 carcinogenesis in rats and reduce associated biomarkers in human volunteers. *The American Journal of*

513 *Clinical Nutrition*, 98(5), 1255-1262.

514 Ratner, S., & Clarke, H. T. (1937). The Action of Formaldehyde upon Cysteine. *Journal of the American*

515 *Chemical Society*, 59(1), 200-206.

516 Rywotycki, R. R., & Ryszard, Y. (2002). The effect of selected functional additives and heat treatment on

517 nitrosamine content in pasteurized pork ham. *Meat Science*, 60(4), 335-339.

518 Santarelli, R. L., Vendeuvre, J., Naud, N., Tache, S., Gueraud, F., Viau, M., Genot, C., Corpet, D. E., &

519 Pierre, F. H. F. (2010). Meat Processing and Colon Carcinogenesis: Cooked, Nitrite-Treated, and Oxidized

520 High-Heme Cured Meat Promotes Mucin-Depleted Foci in Rats. *Cancer Prevention Research*, 3(7), 852-

521 864.

522 Sebranek, J. G., & Fox, J. B. (1985). A review of nitrite and chloride chemistry: Interactions and

523 implications for cured meats. *Journal of the science of food and agriculture*, 36(11), 1169-1182.

524 Sen, N. P., Baddoo, P. A., & Seaman, S. W. (1986). N-Nitrosothiazolidine and N-Nitrosothiazolidine-4-

525 Carboxylic Acid in Smoked Meats and Fish. *Journal of Food Science*, 51(3), 821-825.

526 Sen, N. P., Donaldson, B., Seaman, S., Iyengar, J. R., & Miles, W. F. (1976). Inhibition of nitrosamine

527 formation in fried bacon by propyl gallate and L-ascorbyl palmitate. *Journal of Agricultural and Food*

528 *Chemistry*, 24(2), 397-401.

529   Sen, N. P., Donaldson, B., Charbonneau, C., & Miles, W. F. (1974). Effect of additives on the formation of

530   nitrosamines in meat curing mixtures containing spices and nitrite. *Journal of Agricultural and Food*

531   *Chemistry*, 22(6), 1125-1130.

532   Skibsted, L. H. (2011). Nitric oxide and quality and safety of muscle based foods. *Nitric Oxide*, 24(4), 176-

533   183.

534   Suvachittanont, W., Kurashima, Y., Esumi, H., & Tsuda, M. (1996). Formation of thiazolidine-4-carboxyiic

535   acid (thioproline), an effective nitrite-trapping agent in human body, in Parkia speciosa seeds and other

536   edible leguminous seeds in Thailand. *Food Chemistry*, 55(4), 359-363.

537   Tricker, A. R., Pfundstein, B., Theobald, E., Preussmann, R., & Spiegelhalder, B. (1991). Mean daily intake

538   of volatile N-nitrosamines from foods and beverages in West Germany in 1989-1990. *Food and Chemical*

539   *Toxicology*, 29(11), 729-732.

540   Yurchenko, S., & Mölder, U. (2007). The occurrence of volatile N-nitrosamines in Estonian meat products.

541   *Food Chemistry*, 100(4), 1713-1721.
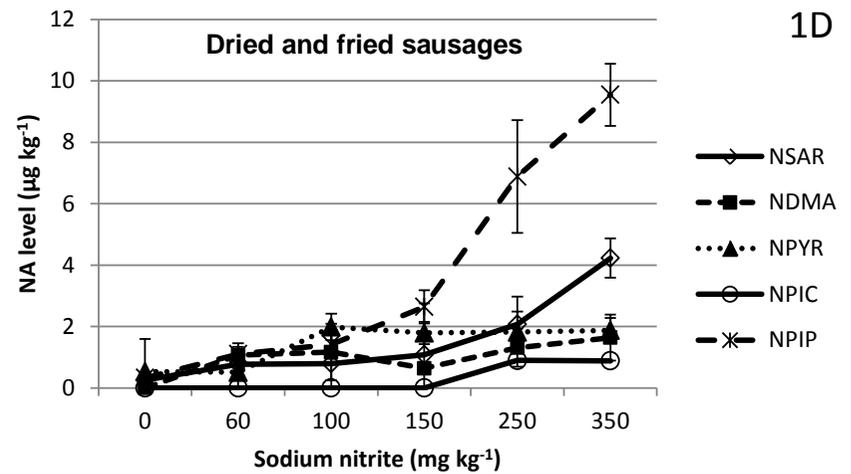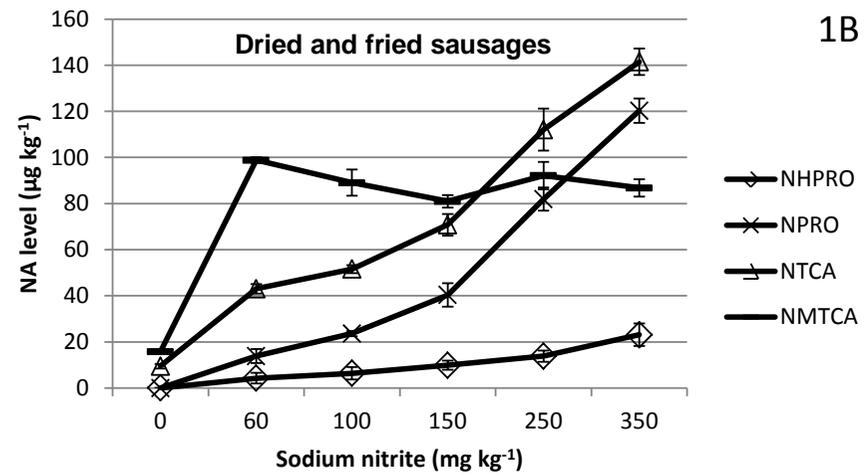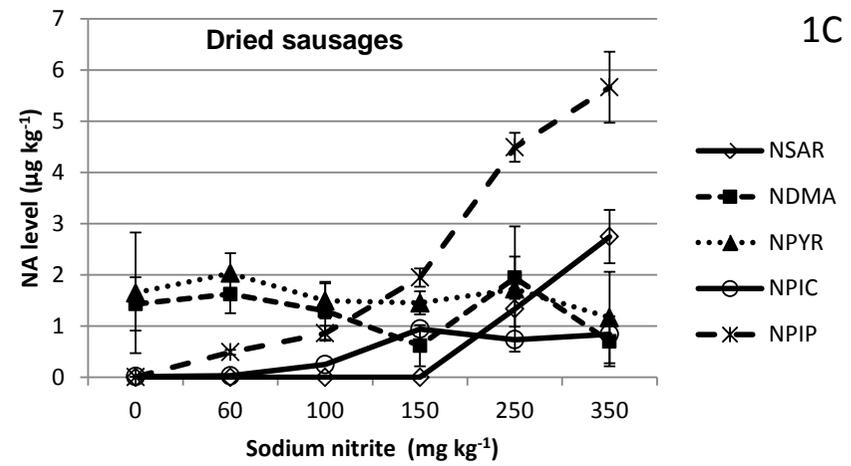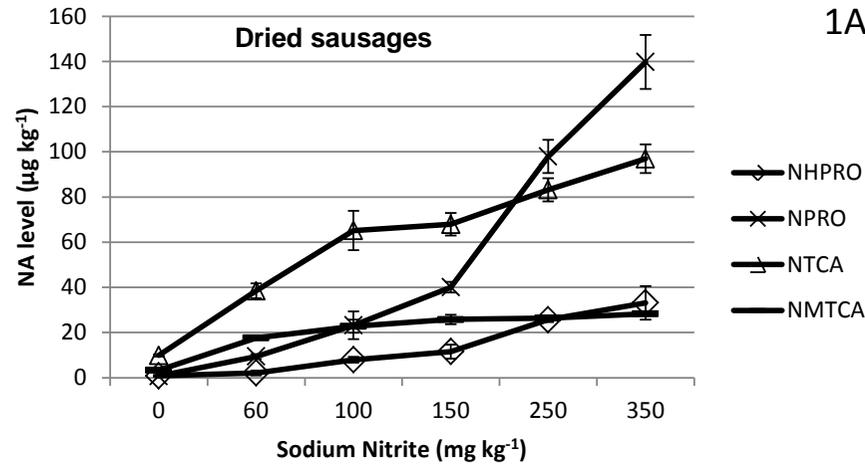
Figure 1: Level of NA in sausages prepared with 0, 60, 200, 150, 250 or 350 mg kg^{-1} sodium nitrite. The NVNA occurred at high levels (1A and 1B), whereas the VNA occurred at lower levels (1C and 1D) in sausages analysed after drying and 24 hours of storage at 5°C (1A and 1C) and sausages which were also further prepared by pan frying for 10 minutes until a core temperature of 100°C (1B and 1D). The levels found in the fried sausages have been corrected for weight loss during frying, i.e. presented in µg kg^{-1} sausage before frying.
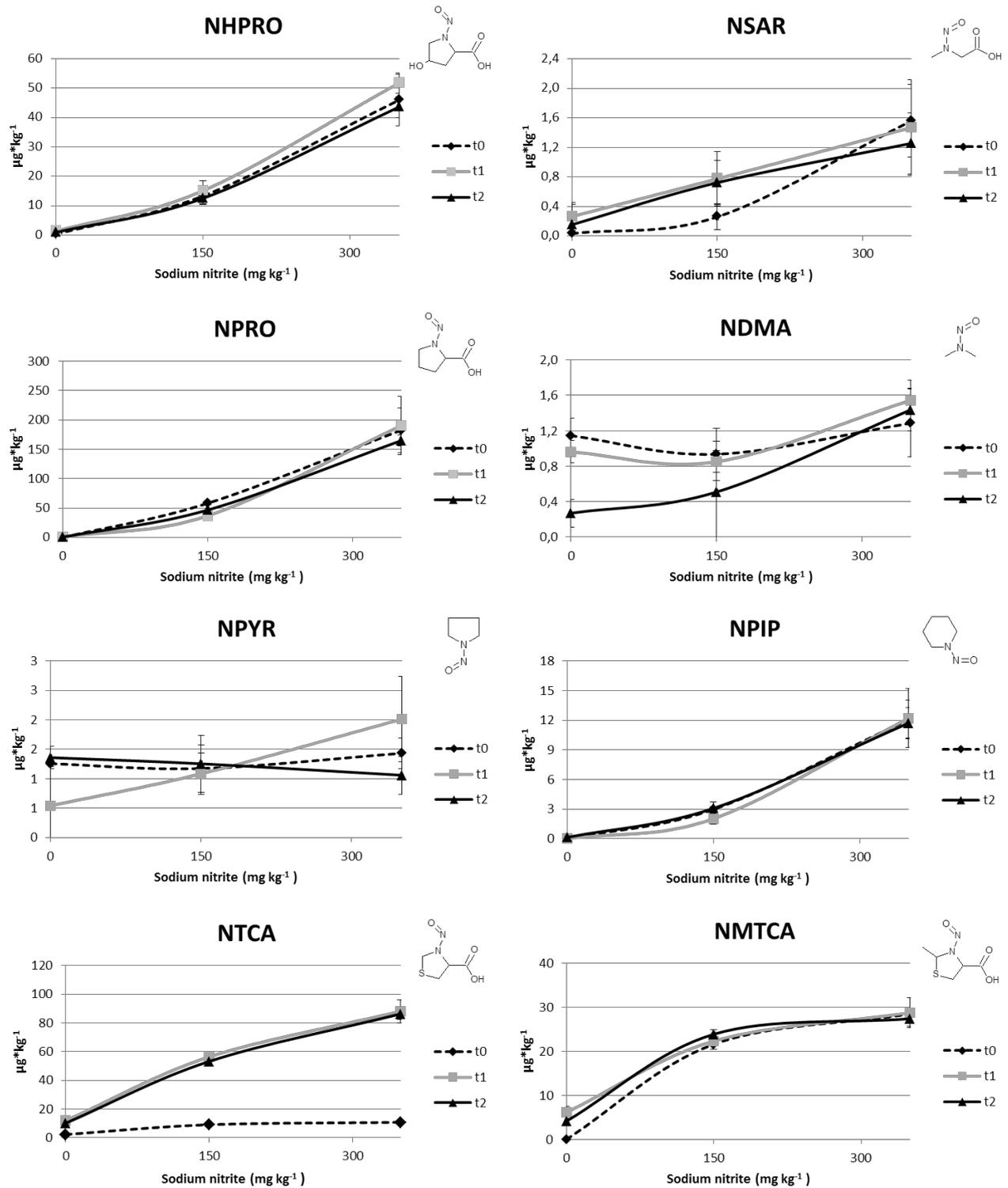
Figure 2: N-nitrosamine levels in sausages prepared with sodium nitrite (0, 150 or 350 mg kg^{-1}) without drying (t$_0$) with drying for 50 minutes at 70°C (t$_1$) and further storage for 24 hours at 5°C (t$_2$). All samples were analysed in triplicate and the error bars show the standard deviation on the results.
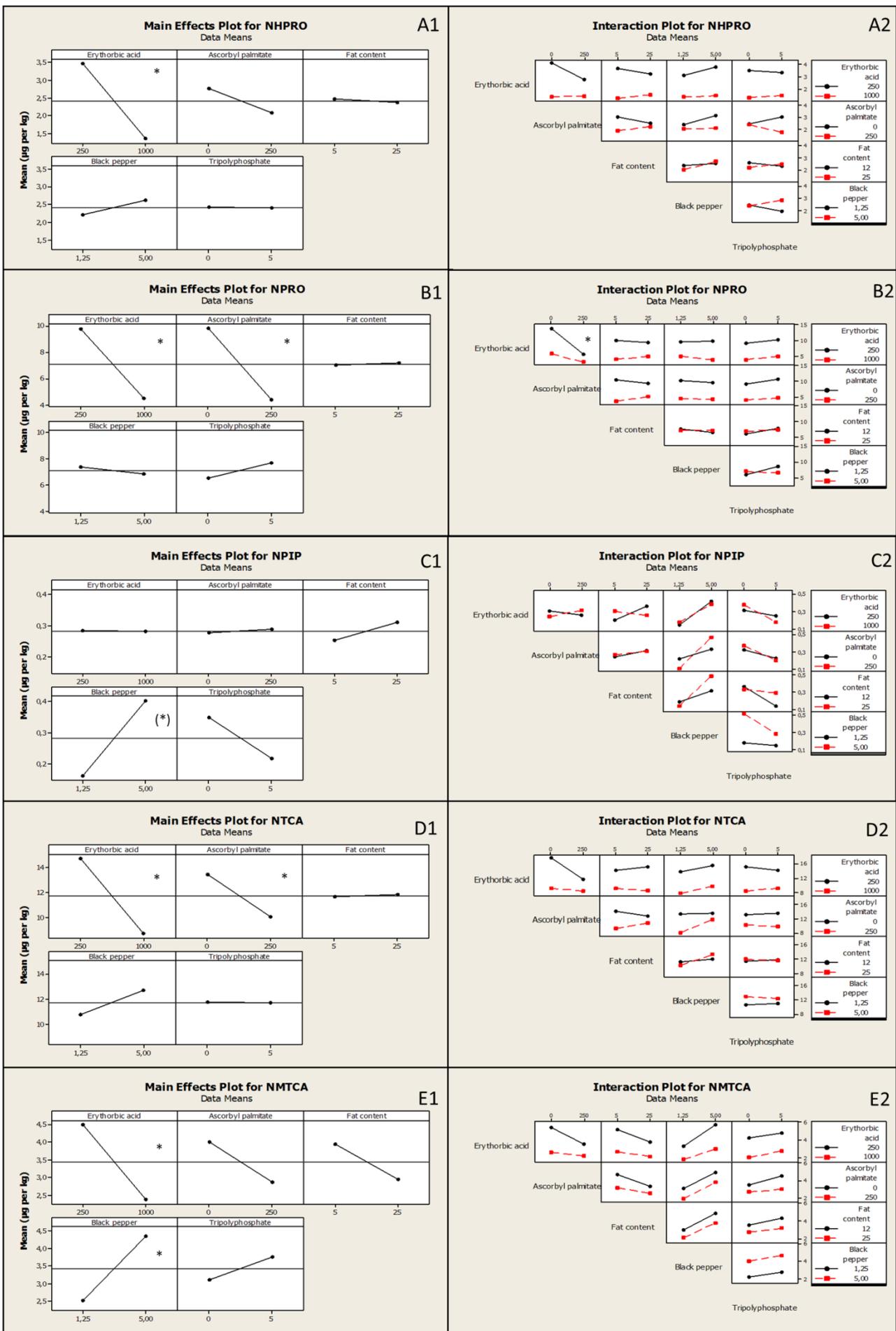
Figure 3: Main effects (A1-E1) and interactions (A2-E2) of erythorbic acid (250 or 1000 mg kg^{-1}), ascorbyl palmitate (0 or 250 mg kg^{-1}), fat content (~12 or ~25%) and black pepper (1.25 or 5 g kg^{-1}) on the N-nitrosamine levels in sausages prepared with 150 mg kg^{-1} sodium nitrite and stored for 24 hours at 5°C. Data analysis was performed using Minitab (version 6). * : the effect or interaction is statistical significant (95% confidence level). (*): the effect was significant in sausages stored for five days (unpublished data) instead of 24 hours at 5°C.
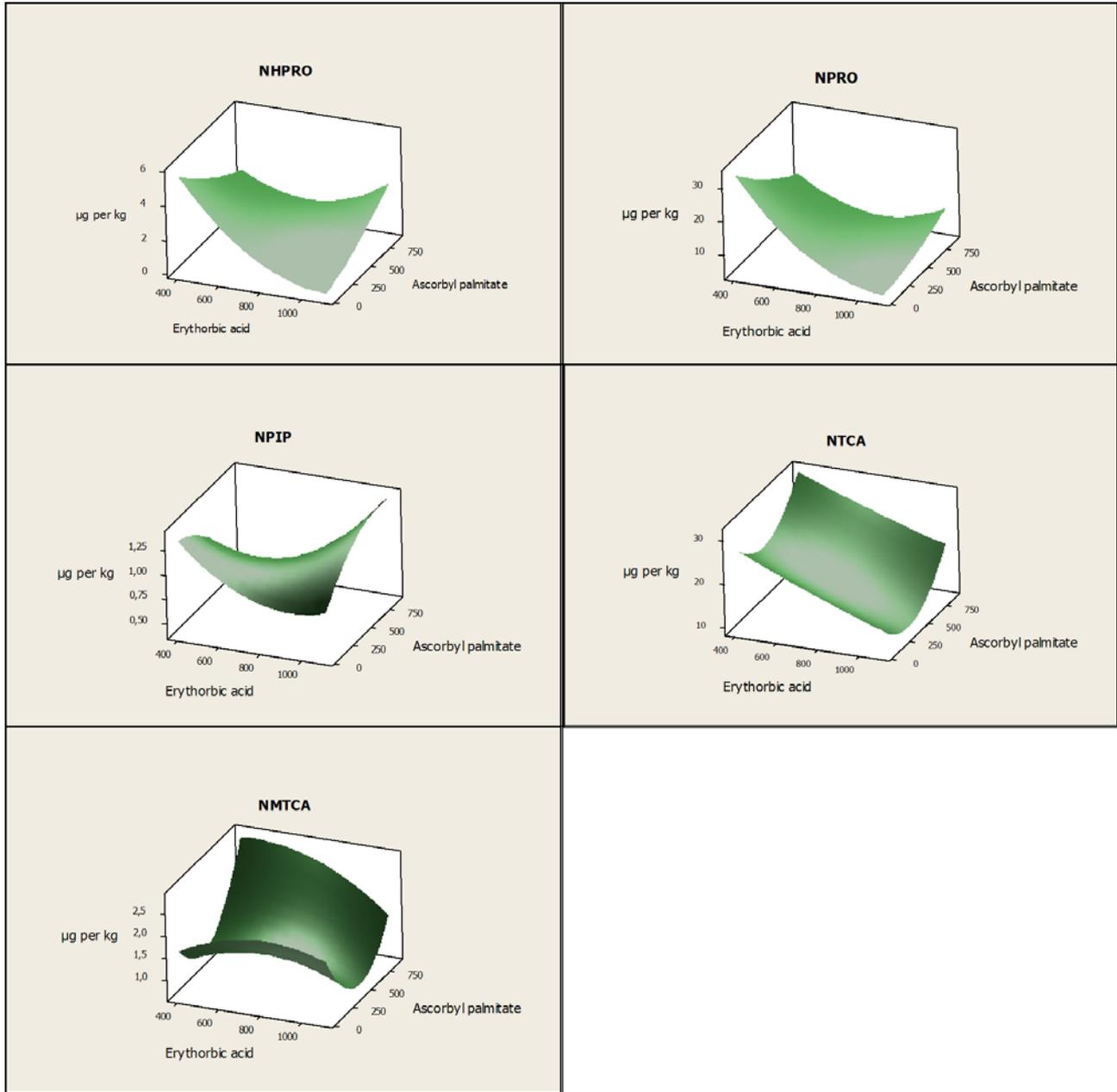
Figure 4: Response surface plots for the effect of erythorbic acid (396, 500, 750, 1000 and 1104 mg kg^{-1}) and ascorbyl palmitate (26, 150, 450, 750 and 874 mg kg^{-1}) on the NA levels in sausages prepared with sodium nitrite (150 mg kg^{-1}).
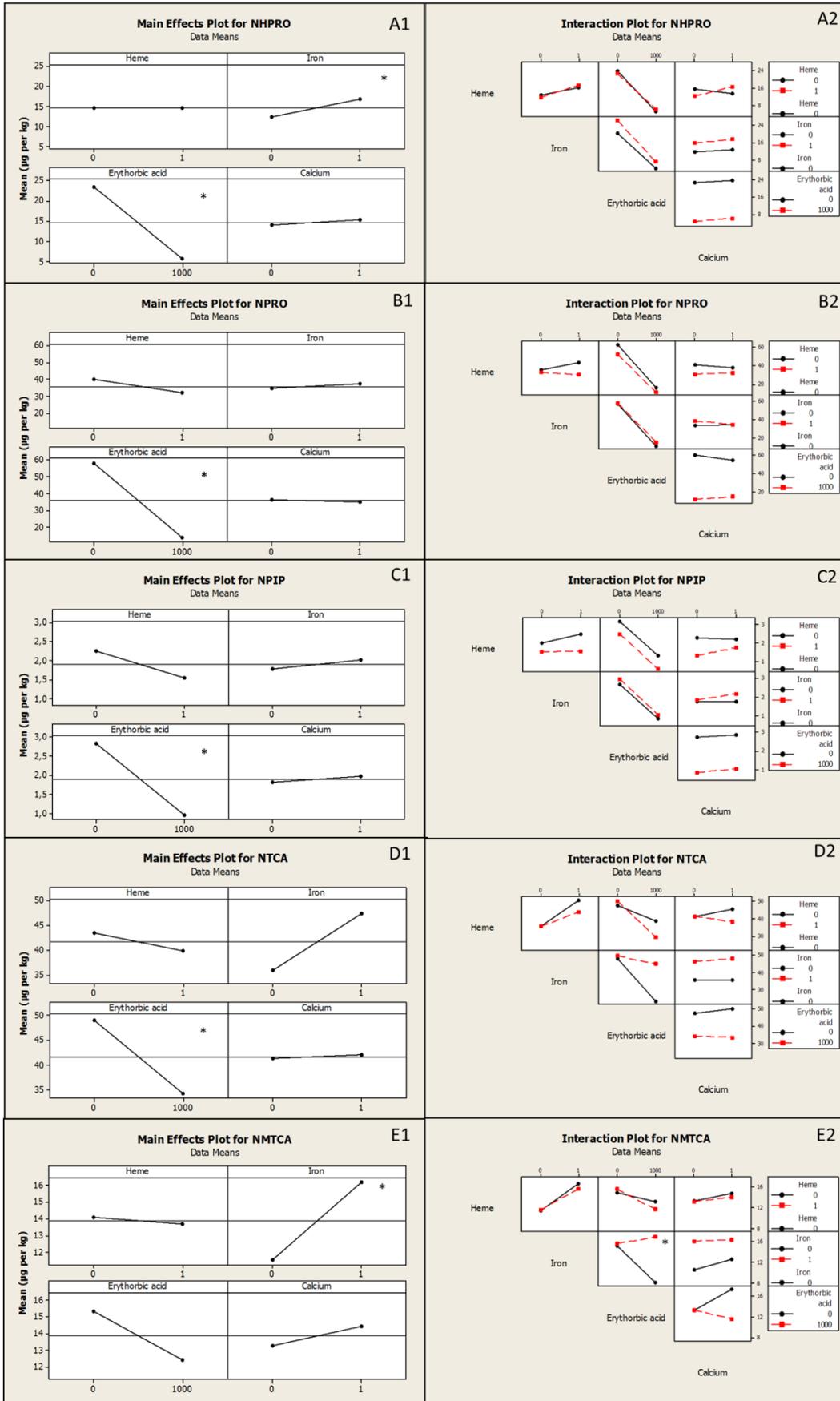
Figure 5: Main effects (A1-E1) and interactions (A2-E2) of heme as myoglobin (0 or 1.5 g kg^{-1}), iron as iron(III)sulphate hydrate (0 or 36 mg kg^{-1}), erythorbic acid (0 or 1000 mg kg^{-1}) and calcium as calcium carbonate (0 or 6 g kg^{-1}) on the N-nitrosamine levels in sausages prepared with 150 mg kg^{-1} sodium nitrite and stored for 24 hours at 5°C. Data analysis was performed using Minitab (version 6). * : the effect or interaction is statistical significant (95% confidence level).

Manuscript I

Dietary exposure to volatile and non-volatile *N*-nitrosamines from processed meat products in Denmark

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1. Abstract

Recent epidemiological studies have shown a positive association between cancer incidence and high intake of processed meat. N-nitrosamines (NA) in these products have been suggested as one potential causative factor. Most volatile NA (VNA) are classified as probable human carcinogens, whereas the carcinogenicity for the majority of the non-volatile NA (NVNA) remains to be elucidated. Both the Danish adults (15-75 years) and children (4-6 years) consume 30 g of processed meat per day (95th percentile), primarily consisting of sausages, salami, pork flank (spiced and boiled) and ham. This consumption results in an exposure to NVNA of 33 and 90 ng kg bw^{-1} day^{-1} for adults and children, respectively. The exposure to VNA is significantly lower amounting to 0.5 and 1.6 ng kg bw^{-1} day^{-1} for adults and children, respectively. Based on a BMDL$_{10}$ of 29 µg kg bw^{-1} day^{-1} a MOE value of ≥17000 was derived for the exposure to NA known to be carcinogenic (VNA including NSAR), indicating an exposure of low concern. The exposure to the NVNA is substantially higher and if found of toxicological significance the exposure may be of concern.

Highlights:

- The exposure to VNA and one NVNA classified as probable human carcinogens for nitrite preserved meat is low
- The exposure to the NVNA is substantially higher than the VNA exposure
- Ham, salami and sausages are the primary sources to the NA exposure from processed meat products
- NA in processed meat may contribute to the observed association between cancer and consumption of processed meat

Keywords: nitrite curing; *N*-nitroso compounds; dietary exposure; risk assessment;

2. Introduction

Several epidemiologic studies show associations between consumption of red and processed meat and increased risk of e.g. colorectal cancer (Santarelli et al., 2008), stomach cancer (Larsson et al., 2006), pancreatic cancer (Larsson and Wolk, 2012) but also with increased risk of cardiovascular diseases and other causes of death (Rohrmann et al., 2013). The association was stronger for high consumption of processed meat than for high consumption of red meat in several of these studies. In 2007 the scientifically based

evidence led the World Cancer Research Fund to recommend that consumption of processed meat should be avoided whereas the Danish food authorities recommend limiting the consumption of processed meat (www.foedevarestyrelsen.dk). Rohrmann et al. (2013) estimated that consumption of more than 20 g of processed meat per day increased the mortality rate (Rohrmann et al., 2013).

Processed meat often is signified by the use of nitrite (E 249 – E 250) or nitrate (E 251-E 252) for preservation, salting and for some products also smoking. Meat products preserved with nitrite and/or nitrate are associated with the occurrence of *N*-nitrosamines (NA), of which many are genotoxic and classified as probable human carcinogens (IARC, 1998). The so called volatile NA (VNA), which include e.g. *N*-nitrosodimethylamine (NDMA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosopiperidine (NPIP) and *N*-nitrosodiethylamine (NDEA), occurs generally at low levels <5 µg kg^{-1} but levels up to 20 µg kg^{-1} has been reported (Hill et al., 1988;Massey et al., 1991). NDEA has been evaluated as the most potent carcinogen among the known meat related VNA (Peto et al., 1984). The non-volatile NA (NVNA), which include the *N*-nitrosamino acids, e.g. *N*-nitrosohydroxyproline (NHPRO), *N*-nitrosoproline (NPRO), *N*-nitrososarcosine (NSAR), *N*-nitroso-thiazolidine-4-carboxylic acid (NTCA), *N*-nitroso-2-methyl-thiazolidine-4-carboxylic acid (NMTCA), generally occur at significantly higher levels than the VNA, *i.e.* up to several thousand microgram per kilo (Tricker and Kubacki, 1992;Massey et al., 1991;Herrmann et al., 2014a). With the exception of NHPRO and NPRO the carcinogenicity of the NVNA's are poorly elucidated (Tricker et al. 1991). For NTCA and NMTCA a literature study only revealed three *in vitro* genotoxicity studies of limited scope (Lin and Gruenwedel, 1990;Negishi et al., 1991;Umano et al., 1984) for NTCA and no studies for NMTCA. Thus, the toxicological significance of several of the NVNA cannot be evaluated because of insufficient data.

Estimations of the dietary exposure to NAs are available in the literature though only for the VNA; NDMA, NPYR and in a few cases NPIP. Studies on the dietary exposure to NDMA published from 1978 to 1990 have been reviewed by Tricker et al. (1991)(Table 1). Since then others have published results on dietary exposure to NDMA, NPYR and NPIP (Table 1). The estimated exposures to VNA from all foods range from 80 ng day^{-1} (NDMA only) for the Finish population (1990), to 900 ng day^{-1} (NDMA, NPYR, NPIP) for the German (1980) and 1000 ng day^{-1} (NDMA, NPYR) for the Swish population (1992) (Tricker et al., 1991)(Table 1). According to the estimates performed for the German population in 1980 and in 1990 the VNA exposure level was reduced to one third during this period. This was primarily due to a decrease in the levels of NDMA in beer but also due to a decrease in the content in meat products. Because of lower levels of NDMA in beer, meat products became the primary dietary source of NDMA. Meat products were the second largest source of dietary NPYR and NPIP only superseded by spices (Tricker et al., 1991). The exposures to NDMA, NPYR and NPIP from meat products were estimated to be 90, 1.6 and 5.5 ng day^{-1}, respectively (Germany 1990). Another important source of VNA are fish products, being the major source

2

of NDMA exposure in some countries (Tricker et al., 1991). Recently the exposure of the Dutch population to NDMA from food and beverages was estimated to range from 2 to 2701 ng day^{-1} with a mean of 109 ng day^{-1} (Keszei et al., 2013)(Table 1). Thus, meat products, which will include processed meat, are in most cases the main source of VNA. So far insufficient data exist on the occurrence of the NVNA in order to perform an estimation of the dietary exposure. It was however suggested by Tricker and Preussmann (1991) to be in the order of 10-100 μg day^{-1} (Tricker and Preussmann, 1991), thus significantly higher than the estimated dietary exposure to VNA. Since the 1970s the levels of NA in processed meat have decreased, because of the introduction of regulation and restriction on the use of nitrite and nitrate and because it has become more common to also add antioxidants which limit the NA formation. However the dietary exposure to NA from processed meat may still pose a health risk due to the increased consumption of processed meat in several European countries including Denmark (Linseisen et al., 2002).

The Danish population´s exposure to VNA and NVNA from processed meat products has so far not been assessed. Primarily due to concern for the formation of NA the Danish authorities have found it necessary to maintain national provisions imposing stricter limits on the use of nitrites than the EU. These national provisions allow the addition of maximum 60 mg kg^{-1} to most meat products intended for the Danish market. The common EU legislation (Directive 2006/52/EC) allow the addition of 150 mg kg^{-1} meat. This exception expire in May 2015 and in order to evaluate what the consequence will be for the Danish population if more nitrite may be added during meat processing, an evaluation of the exposure levels, as it is now, is necessary. Recently we performed a survey on the occurrence of VNA as well as NVNA in processed meat products on the Danish market (Herrmann et al., 2014a). Survey data from the 70 analyzed samples can provide a preliminary estimation of the exposure to VNA and NVNA from processed meat products for the Danish populations.

The aim of the present study is therefore to estimate the exposure to VNA and NVNA from processed meat products for the Danish population based on results of the recently performed Danish survey and data on consumption of processed meat products by the Danish population as well as for the high consumers, the 95th percentile. Risk characterization is performed by estimating the margin of exposure (MOE) to NA, based on the ratio between a bench mark dose level (BMDL$_{10}$) and the estimated dietary exposures.

3. Methods

Contents of NAs in processed meat products

Result from a recently performed survey on the occurrence of NAs in 70 samples processed meat products available on the Danish market, were applied for the exposure assessment. Details on this study are described elsewhere (Herrmann et al., 2014a). The NA contents of the samples were determined using a recently

103 developed method allowing for the quantification of eight VNA and five NVNA (Herrmann et al., 2014b).

104 The results of the survey are presented as the mean content in Table 2. Both the mean of all positive findings

105 as well as the mean of all samples analysed are presented. The latter mean values are the values applied for

106 the exposure assessment. The non-detects were in this case set to zero.

Exposure assessment

108 Intakes of NA via processed meat products were estimated using two representative groups, *i.e.* Danish

109 children 4-6 years of age and Danish Adults 15-75 years of age. The exposure level for the 6-14 year old

110 children was not calculated because the consumption of processed meat is comparable to the consumption by

111 the 4-6 year olds (Table 3) and the latter group will therefore be the most exposed group of the two.

112 Consumption data from the Danish National Survey of Diet and Physical Activity (DANSDA)(Pedersen et

113 al., 2010) was used for estimation of the NA exposure for both groups. In this survey consumption of food

114 and drink was recorded for seven consecutive days from a representative sample of 2700 Danes aged 4-75.

115 The consumption data for each individual participant in the survey was available for the dietary estimation

116 performed in the present work. The types of processed meat products traditionally preserved with nitrite

117 included in the survey were: Ham (specific recording of either boiled and smoked ham, boiled and canned

118 ham or boiled and sliced ham), bacon, salami, sausages, medister sausage (raw sausage, Danish speciality),

119 pork flank (spiced and boiled), meat sausage (pork based luncheon meat), smoked pork filet, kassler

120 (smoked, boiled pork saddle), salted meat (pork, luncheon meat), chicken breast (boiled, luncheon meat).

121 More details on how the survey and handling of the consumption data were performed are available in

122 Pedersen *et al*., 2010.

Risk characterisation

125 In order to evaluate the significance of an estimated dietary exposure to genotoxic compounds the exposure

126 basically compared with the dose leading to a specified incidence of tumor formation in experimental

127 animals. The larger the margins between the effect dose level and the actual exposure level (margins of

128 exposure, MOE) the lower is the concern. An internationally recognised toxicological reference point is the

129 Benchmark Dose Lower bound limit (BMDL), which represent the exposure level were an increase in the

130 incidences of the effect (at 10% in case of animal experiments) is smaller than the specified Benchmark

131 Response with a confidence of 95% (Zeilmaker et al., 2010). The $BMDL_{10}$ may be derived from dose-

132 response data from several long-term carcinogenicity studies instead of one extensive study. EFSA has for

133 genotoxic compounds expressed that if the $BMDL_{10}$ is 10,000 times higher than the exposure (*i.e.* MOE of

134 10,000) the exposure is of low concern (EFSA, European Food Safety Authority, 2012).

4. Results and discussion

Processed meat consumption and occurrence data

The 95th percentile of the mean consumption of processed meat consumption in Denmark derived from DANSDA (Pedersen et al., 2010) is summarized in Table 3. The amounts of processed meat consumed by the three different age groups are all about 30 g per person per day. If the body weights (bw), of the three groups are taken into account, the 4-6 year olds (bw 18 kg) and the 6-14 year olds (bw 30 kg) consume about three and two times more processed meat per day than the 15-75 year olds (bw 72 kg), respectively. Thus the 4-6 year olds has the highest exposure because of the lower bodyweight. The types of processed meat consumed by the three groups are similar (Table 3). Sausages contribute most to the total consumption (25-30%) and salami accounts for the second largest fraction for both groups of children (13-20%)). Pork flank (4-5 years) or ham (6-14 and 15-75 years) is the third most consumed product. Thus the high consumers, defined by the 95th percentile, of Danish population consume on average 1.5 times the amount (20 g) reported to affect the mortality (Rohrmann et al., 2013).

According to data from the European Prospective Investigation into Cancer and Nutrition (EPIC) the total consumption of processed meat product by the Danish population is similar to the consumption by the French adults, though only about half of that reported for the German and Norwegian adults. Sausages also account for the major part of the processed meats consumed by the French, German and Norwegian adults (Linseisen et al., 2002). A higher consumption of sausages accounts for the major part of the higher total consumption by the German and Norwegian adults. According to the survey summarised in Table 2 the NVNA are detected in nearly all samples taken from the Danish market. The mean levels of the individual NVNA were ≤118 µg kg^{-1}, highest for NTCA. NTCA and NMTCA are found at concentration levels up to 4030 µg kg^{-1} and 39 µg kg^{-1}, respectively.

VNA are also detected in several samples though at considerably lower levels. The mean levels of the individual VNA in samples from the Danish market are ≤0.8µg kg^{-1}. Of the NA targeted only *N*-nitrosodibutylamine is not found. NDBzA and NDPA were detected in a few samples and NHPRO were detected in about 40% of the samples. However, the contents of these three NA could not be quantified with enough certainty using the developed method and contents of these have therefore not been included in the exposure calculation.

Exposure assessment

The estimated mean exposure and the 95th percentile for the individual NAs and the sum of NVNA and VNA are presented in Figure 1A/1C and 1B/1D, respectively.

The 4 to 5 years old children have the highest consumption of processed meat (g kg bw^{-1} day^{-1}) and therefore also the highest exposure to NVNA and VNA, *i.e.* 90 ng kg bw^{-1} day^{-1} (mean 37 ng kg bw^{-1} day^{-1}) and 1.1 ng

5

kg bw^{-1} day^{-1} (mean 0.45 ng kg bw^{-1} day^{-1}), respectively (Figure 1A and 1B). The total exposure to NVNA and VNA for adults was 33 and 0.34 ng kg bw^{-1} day^{-1} (mean 13 and 0.13 ng kg bw^{-1} day^{-1}), respectively (Figure 1C and 1D). NTCA accounted for about 90% of the total exposure to NVNAs for both children and adults. NMTCA and NPRO accounted for about 5 and 2% of the NVNA exposure, respectively. For the VNAs, NPYR and NDMA accounted for about 50% and 40% of the total exposure to VNA for both children and adults, respectively (Figure 1B and 1D). The classical VNA (NDMA, NPYR, NPIP, NDEA) accounted for >90% of the exposure to VNAs. The exposure levels estimated in the present work for the VNA are in the same order of magnitude as the exposure levels reported by others (Table 1)(Keszei et al., 2013;Tricker et al., 1991). The exposure levels estimated for the NVNA though seems to be lower than the intake suggested by Tricker and Preussmann (1991). Though other NVNA than those included in the present study has been identified in processed meat (Sen et al., 1993;Janzowski et al., 1978;Tricker and Kubacki, 1992) so the estimated exposure might be underestimated.

Ham and salami accounted for about 75% of the exposure to NVNA (Figure 2A and 2C). Salami, ham and sausages accounted for approximately 70 and 80% of the exposure to VNA for adults and children, respectively (Figure 2B and 2D). Thus, the present results indicate that ham, salami and sausages are the primary meat source of NA for the Danish population. In Germany a higher intake of NA would be expected, partly due to the greater consumption of processed meat and partly due to the less restrictive EU regulation on the use of nitrite for meat preservation allowing for more nitrite to be added than the Danish provisions. The more nitrite is added during the processing the levels of NA generally increase (Herrmann et al. unpublished data)(Gry et al., 1983). The primary meat source of NA is most likely sausages for the German and Norwegian populations since they consume 4-7 times more sausages than the Danish population (Linseisen et al., 2002).

Risk assessment

The NA are relatively stable compounds but are activated metabolically, via hydroxylation catalysed by enzymes of the cytochrome P450 family, and thereby become carcinogenic. NDMA and NDEA are the most studied NA in regard to toxicity. Both NDMA and NDEA are carcinogenic in all of the animals they have been tested. The target organs are liver, respiratory tract and kidney (IARC, 1978). Because the NA needs to be metabolically activated to become carcinogenic the target organs are those with activity of the P450 enzyme with affinity for the relevant NA. E.g. NDMA is readily metabolised in the rat liver, less in rat kidney and lung, and consequently liver tumours is the primary end point in rats (Shank, 1975). Total liver tumours were found to be the most sensitive endpoint for rats exposed to NDMA (Zeilmaker et al., 2010). NPYR is non-carcinogenic in the rat oesophagus whereas NPIP is a potent rat oesophagus carcinogen. The activity of several P450 enzymes including the P4502A subfamily are in humans associated with polymorphism and are inducible by several xenobiotic (Su and Ding, 2004). Thus the degree of which NA

are metabolically activate may vary between individuals and result in variation in susceptibility to NA exposure. It is assumed that the more polar the NA are rapidly excreted unchanged and therefore less likely to be activated. This is part of the reason why the hydroxylated NA are less potent carcinogens than their un-substituted NA (Lijinsky 1987, 301-356).

A large number of studies on the carcinogenicity of the NA are available. Tough the majority of these are smaller studies performed during the 1960s and 1970s. In general these studies show that long-term exposure of rats to NDMA or NDEA at levels of around 4 mg kg bw^{-1}day^{-1} leads to the development of tumours in up to 100% of the test animals (IARC, 1978). In general studies of sufficient quality and extent to allow for the estimation of Bench Mark Dose (BMD) are limited. However, a comprehensive chronic administration study performed with 16 different concentrations of NDMA in water given to rats was performed by Peto et al. (Peto et al., 1984). This study has been used by several researchers to define a BMDL value (Table 4) e.g. by Dybing et al. (2008) and Zeilmarker et al. (2010) to estimate a BMDL$_{10}$ value for NDMA. Zeilmaker et al. 2010 derived a BMDL$_{10}$ of 29 µg kg bw^{-1} day^{-1} for NDMA chronic exposure when using total liver tumours as the most sensitive marker. This BMDL$_{10}$ value is in good agreement with the BMDL$_{10}$ of 27 µg kg bw^{-1} day^{-1} applied by the Scientific Committee on Consumer Safety (Scientific Committee on Consumer Safety, 2012) . Dybing et al. (2008) on the other hand derived a BMDL$_{10}$ value of 62 µg kg bw^{-1} day^{-1} when using incidence of liver cell tumours as marker.

The different VNA are different in their carcinogenic potency. NDMA, NPYR and NPIP all affected the same endpoint, *i.e.* total liver tumours but with descending potency with NDMA being the most potent of the three (Peto et al., 1984). In the present study the BMDL$_{10}$ of 29 µg kg bw^{-1} day^{-1} was conservatively chosen for the combined risk assessment for the total of VNA and NSAR. Except for NDEA the VNA and especially NSAR are less potent carcinogens than NDMA.

With an VNA exposure level of 1.1 ng kg bw^{-1} day^{-1} and NSAR of 0.6 ng kg bw^{-1} day^{-1} (total carcinogenic NA of 1.7 ng kg bw^{-1} day^{-1}) for children and a BMDL$_{10}$ of 29,000 ng kg bw^{-1} day^{-1} the MOE is 17,000 for NA originating from processed meat and thus higher than 10,000. By applying the same approach for adults the MOE is found to be 45,000 for NA originating from processed meat.

Hence the present risk assessment of the exposure to VNA from processed meat products is that it may be considered of low concern, a conclusion which is in accordance with the evaluation by the European Food Safety Authority (EFSA). However, the VNA are genotoxic compounds and a "no effect level" may therefore not exist. Even small exposure levels may still have a genotoxic effect (Dybing et al., 2008). Further, it should also be cautioned that the results from this study are based on fairly limited number of results on the occurrence of NA in processed meat products.

Assuming that only the VNA are of toxicological relevance, there may still be reason to be concerned about the occurrence of NA in processed meats, even though the present study indicates that the NA exposure from processed meat on the Danish market is of low concern based on the estimated MOE. Firstly, the population is also exposed to NA from other sources than processed meat. Exposure data from other sources than processed meat are needed in order to make a complete risk assessment for the population. Secondly, as mentioned earlier the carcinogenic potential of the majority of the NVNA are unknown or only very limited information is available. These NVNA can occur in much higher concentrations than the VNA and in order to fully assess the risk of the NA exposure form processed meat products further toxicological studies are needed on the NVNA. Thirdly, other unidentified carcinogenic NA might be produced when the conditions allow for the formation of the known NA

5. Conclusion

The Danish population consume as a 95th percentile on average 30 g of processed meat per day primarily consisting of sausages, salami, pork flank (spiced and boiled) and ham. The exposure to VNA by the consumption of processed meat was found to be low (0.34-1.1 ng kg bw^{-1} day^{-1}), whereas the exposure to NVNA was considerably higher (33-90 ng kg bw^{-1} day^{-1}). Adults (15-75 year old) and children (4-6 year old) consume almost the same amount of processed meat per day resulting in a higher exposure for children because of their lower body weight. The calculated MOE ($\geq$17000) for the VNA exposure indicate that this is of low concern. In order to assess the significantly higher exposure to the NVNA the carcinogenic potential of these NA needs to be elucidated.

Acknowledgement

References

Dybing, E., O'Brien, J., Renwick, A.G. and Sanner, T., 2008. Risk assessment of dietary exposures to compounds that are genotoxic and carcinogenic—An overview. *Toxicol. Lett.* 180, 110-117 doi: http://dx.doi.org.globalproxy.cvt.dk/10.1016/j.toxlet.2008.05.007.

EFSA, European Food Safety Authority, 2012. Statement on the applicability of the Margin of Exposure approach for the safety assessment of impurities which are both genotoxic and carcinogenic in substances added to food/feed, EFSA Scientific Committee.

Gry, J., Rasmussen, N.J.D., Jensen, W.K., Brandt, I.G. and Fabech, B., 1983. Investigations on effects of nitrite in commercially prepared Danish cured meat products. The Federation of Danish Pig Producers and Slaughterhouses & The National Food Agency of Denmark. ISBN 8750344595, 9788750344599.

Habermeyer, M. and Eisenbrand, G., 2008. N-Nitrosamines, Including N-Nitrosoamino acids and Potential Further Nonvolatiles, in: Stadler, R.H. and Lineback, D.R., Process-Induced Food Toxicants: Occurrence, Formation, Mitigation, and Health Risks. John Wiley & Sons, Inc., pp. 363-386. DOI: 10.1002/9780470430101.ch4a.

Herrmann, S.S., Duedahl-Olesen, L. and Granby, K., 2014a. Occurrence of volatile and non-volatile N-nitrosamines in processed meat products and the role of heat treatment. Accepted for publication in *Food Control* .

Herrmann, S.S., Duedahl-Olesen, L. and Granby, K., 2014b. Simultaneous determination of volatile and non-volatile nitrosamines in processed meat products by liquid chromatography tandem mass spectrometry using atmospheric pressure chemical ionisation and electrospray ionisation. *Journal of Chromatography A* 1330, 20-29 doi: http://dx.doi.org.globalproxy.cvt.dk/10.1016/j.chroma.2014.01.009.

Hill, M.J., Massey, R.C., Shuker, D.E.G., Leach, S., Tricker, A.R., Preussmann, R. and Rowland, J.R., 1988. Nitrosamines. Toxicology and microbiology. 169pp. ISBN 0-89573-605-0.

IARC, 1978. IARC monographs on the evaluation of carcinogenic risks to humans. Some N-Nitroso Compounds. IARC monographs on the evaluation of carcinogenic risks to humans. 17.

Janzowski, C., Eisenbrand, G. and Preussmann, R., 1978. Occurrence of N-nitrosamino acids in cured meat products and their effect on formation of N-nitrosamines during heating. *Food Cosmet. Toxicol.* 16, 343-348 doi: http://dx.doi.org.globalproxy.cvt.dk/10.1016/S0015-6264(78)80005-4.

Keszei, A.P., Goldbohm, R.A., Schouten, L.J., Jakszyn, P. and van, d.B., 2013. Dietary N-nitroso compounds, endogenous nitrosation, and the risk of esophageal and gastric cancer subtypes in the Netherlands Cohort Study. *Am. J. Clin. Nutr.* 97, 135-146.

Larsson, S.C. and Wolk, A., 2012. Red and processed meat consumption and risk of pancreatic cancer: meta-analysis of prospective studies. *Br. J. Cancer,* 1-5.

Larsson, S.C., Bergkvist, L. and Wolk, A., 2006. Processed meat consumption, dietary nitrosamines and stomach cancer risk in a cohort of Swedish women. *International Journal of Cancer* 119, 915-919.

Lin, I.N.C. and Gruenwedel, D.W., 1990. Mutagenicity and Cytotoxicity of N-Nitrosothiazolidine-4-Carboxylic Acid. *Food Addit. Contam.* 7, 357-368.

Linseisen, J., Kesse, E., Slimani, N., Bueno-de-Mesquita, H., Ocké, M., Skeie, G., Kumle, M., Iraeta, M.D., Gómez, P.M., Janzon, L., Stattin, P., Welch, A., Spencer, E., Overvad, K., Tjønneland, A., Clavel-Chapelon, F., Miller, A., Klipstein-Grobusch, K., Lagiou, P., Kalapothaki, V., Masala, G., Giurdanella, M., Norat, T. and Riboli, E., 2002. Meat consumption in the European Prospective

Investigation into Cancer and Nutrition (EPIC) cohorts: results from 24-hour dietary recalls. *Public Health Nutr.* 5, 1243-1258.

Massey, R.C., Key, P.E., Jones, R.A. and Logan, G.L., 1991. - Volatile, non-volatile and total N-nitroso compounds in bacon. *Food Addit. Contam.*8, 5, 585-598.

Negishi, T., Shiotani, T., Fujikawa, K. and Hayatsu, H., 1991. The genotoxicities of N-nitrosamines in Drosophila melanogaster in vivo: the correlation of mutagenicity in the wing spot test with the DNA damages detected by the DNA-repair test. *Mutation Research/Environmental Mutagenesis and Related Subjects* 252, 119-128.

Pedersen, A.N., Fagt, S., Groth, M.V., Christensen, T., Biltoft-Jensen, A., Matthiessen, J., Andersen, N.L., Kørup, K., Ygil, K.H., Hinsch, H., Saxholt, E. and Trolle, E., 2010. Danskernes kostvaner 2003-2008. ISBN: 978-87-92158-67-3 also available at www.food.dtu.dk, last accessed 27-10-2014.

Penttila, P., Rasanen, L. and Kimppa, S., 1990. Nitrate, Nitrite, and N-Nitroso Compounds in Finnish Foods and the Estimation of the Dietary Intakes. Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung 190, 336-340 doi: 10.1007/BF01184503.

Peto, R., Gray, R., Brantom, P. and Grasso, P., 1984. Nitrosamine carcinogenesis in 5120 rodents: chronic administration of sixteen different concentrations of NDEA, NDMA, NPYR and NPIP in the water of 4440 inbred rats, with parallel studies on NDEA alone of the effect of age of starting (3, 6 or 20 weeks) and of species (rats, mice or hamsters). *IARC Sci. Publ.* , 627-665.

Rohrmann, S., Overvad, K., Bueno-de-Mesquita, H.B., Jakobsen, M.U., Egeberg, R., Tjønneland, A., Nailler, L., Boutron-Ruault, M., Clavel-Chapelon, F., Krogh, V., Palli, D., Panico, S., Tumino, R., Ricceri, F., Bergmann, M.M., Boeing, H., Li, K., Kaaks, R., Khaw, K., Wareham, N.J., Crowe, F.L., Key, T.J., Naska, A., Trichopoulou, A., Trichopoulos, D., Leenders, M., Peeters, P.H.M., Engeset, D., Parr, C.L., Skeie, G., Jakszyn, P., Sánchez, M., Huerta, J.M., Redondo, M.L., Barricarte, A., Amiano, P., Drake, I., Sonestedt, E., Hallmans, G., Johansson, I., Fedirko, V., Romieux, I., Ferrari, P., Norat, T., Vergnaud, A.C., Riboli, E. and Linseisen, J., 2013. Meat consumption and mortality - results from the European Prospective Investigation into Cancer and Nutrition. *BMC Medicine* 11.

Santarelli, R.L., Pierre, F. and Corpet, D.E., 2008. Processed Meat and Colorectal Cancer: A Review of Epidemiologic and Experimental Evidence. *Nutr. Cancer* 60, 131-144 doi: 10.1080/01635580701684872.

Scientific Committee on Consumer Safety, 2012. Opinion on Nitrosamines and Secondary Amines in Cosmetic Products. SCCS/1458/11.

Sen, N., Baddoo, P. and Seaman, S., 1993. Studies on the Occurrence and Formation of 2-(Hydroxymethyl)-N-Nitrosothiazolidine-4-Carboxylic Acid (Hmntca) and 2-(Hydroxymethyl)-N-Nitrosothiazolidine (Hmnthz) in various Cured Smoked Meats, Fish and Cheese. *J. Sci. Food Agric.* 61, 353-356 doi: 10.1002/jsfa.2740610311.

Shank, R.C., 1975. Toxicology of N-nitroso compounds. *Toxicol. Appl. Pharmacol.* 31, 361-368 doi: http://dx.doi.org/10.1016/0041-008X(75)90257-4.

Su, T. and Ding, X., 2004. Regulation of the cytochrome P450 2A genes. *Toxicol. Appl. Pharmacol.* 199, 285-294 doi: http://dx.doi.org/10.1016/j.taap.2003.11.029.

Tricker, A.R. and Kubacki, S.J., 1992. - Review of the occurrence and formation of non-volatile N-nitroso compounds in foods. *Food Addit. Contam.* 9, 1, 39-69.

Tricker, A.R., Pfundstein, B., Theobald, E., Preussmann, R. and Spiegelhalder, B., 1991. Mean daily intake of volatile N-nitrosamines from foods and beverages in West Germany in 1989-1990. *Food and Chemical Toxicology* 29, 729-732 doi: http://dx.doi.org/10.1016/0278-6915(91)90180-F.

Tricker, A.R. and Preussmann, R., 1991. Carcinogenic N-nitrosamines in the diet: occurrence, formation, mechanisms and carcinogenic potential. *Mutation Research/Genetic Toxicology* 259, 277-289 doi: DOI: 10.1016/0165-1218(91)90123-4.

Umano, K., Shibamoto, T., Fernando, Y.S. and WEI, C.I., 1984. Mutagenicity of 2-hydroxyalkyl-N-nitrosothioazolidines. *Food and Chemical Toxicology* 22, 253-260.

Zeilmaker, M.J., Bakker, M.I., Schothorst, R. and Slob, W., 2010. Risk Assessment of N-nitrosodimethylamine Formed Endogenously after Fish-with-Vegetable Meals. *Toxicological Sciences* 116, 323-335.

Table

Table 1: Dietary exposure levels for *N*-nitrosamines estimated by others. The exposure values are for adults unless other ways stated.

Compound	Exposure level	Food source (Population)	Reference
NDMA	0.1 µg day^{-1}	Food and beverages (Netherlandes)	Keszei et al., 2013
NDMA	*0.03 µg day^{-1} *0.04 µg day^{-1} (children 1 year old)	Fish/vegetables meals (Netherlandes)	Zeilmarker et al. 2010
NDMA	0.08 µg day^{-1}	Food (Finland)	Penttila et al. 1990
Sum of NDMA, NPYR and NPIP	0.9 µg day^{-1}	Food (Germany 1980)	Tricker et al. 1991
Sum of NDMA, NPYR and NPIP	0.3 µg day^{-1}	Food (Germany 1990)	Tricker et al. 1991
NDMA	0.09 µg day^{-1}	Meat products (Germany in 1990)	Tricker et al., 1991
NPYR	0.0016 µg day^{-1}	Meat products (Germany in 1990)	Tricker et al., 1991
NPIP	0.0055 µg day^{-1}	Meat products (Germany in 1990)	Tricker et al., 1991
Sum of NDMA and NPYR	*1 µg day^{-1}	Food (Switzerland)	Lutz and Schlatter 1992
VNA	0.3-1 µg day^{-1}	Food (with cured meats and beer as major sources) (Western countries in average)	Tricker & Preussmann 1991
NVNA	~10-100 µg day^{-1}	Food (Western countries in average)	Tricker & Preussmann 1991

*In order to have all the values presented with the same unit the values for a 1 year old and adults have been multiplied with 9.5 kg or 75 kg, respectively.

Table

Table 2: Mean levels of VNA (uncolored columns) and NVNA (grey columns) ($\mu g\ kg^{-1}$) in processed meat products on the Danish market. The mean of all quantifiable contents and mean of all contents including non-detects each compound.

Compound name	Abbreviation	Mean content (positive findings)[1]	Mean content (all findings)[1]
N-nitrosodimethylamine	NDMA	1.3	0.7
N-nitrosomorpholine	NMOR	0.1	0.01
N-nitrosomethylethylamine	NMEA	0.3	0.04
N-nitrosopyrrolidine	NPYR	2	0.8
N-nitrosodiethylamine	NDEA	0.3	0.04
N-nitrosopiperidine	NPIP	0.1	0.02
N-nitrososarcosine	NSAR	20	1.5
N-nitrosoproline	NPRO	4.3	2.3
N-nitrosomethylaniline	NMA	0.47	0.1
N-nitroso-thiazolidine-4-carboxylic acid	NTCA	120	118
N-nitroso-2-methyl-thiazolidine-4-carboxylic acid	NMTCA	8.1	7.3

1: The means are based on all positive findings also those below LOQ if the retention times and ion ratios matched those of the standard solutions or quality control samples. Non-detects were not included when calculating the means for the individual product groups nor when calculation the mean of "positive results, all products". When calculating the means of "all results, all products" non-detects were set to zero.

Table

Table 3: Consumption of processed meat products (gram day[-1] person[-1] for Danish children and adults as mean and at the 95th percentile, derived from the Danish National Survey of Diet and Physical Activity (DANSDA) (Dietary habits in Denmark 2003-2008)(Pedersen et al., 2010).

g/day (mean)	4-5 years (bw: 18 kg)	6-14 years (bw: 30 kg)	15-75 years (bw: 72 kg)
Sausages	2.0	1.6	1.3
Salami	1.2	1.3	1.1
Pork flank, spiced, boiled (Luncheon meat)	0.9	0.8	1.0
Medister sausage (Danish special product)	0.6	0.4	0.4
Ham	0.9	1.1	1.6
Kassler (Luncheon meat)	0.6	0.5	0.8
Salted meat (Luncheon meat)	0.3	0.3	0.5
Meat sausage (Luncheon meat, pork based)	0.3	0.3	0.4
Chicken and Turkey filet (Luncheon meat)	0.1	0.2	0.1
Bacon	0.1	0.2	0.4
Filet, pork, smoked	0.2	0.2	0.3
Total	6.9	6.5	7.5
Total 95th percentile	16.2	16.8	20.2
Largest daily consumption	22.0	56.6	53.0

BW: body weight

Table 4: Bench mark dose levels (BMDL) applied previously in the literature for carcinogenic risk assessment

compound	$BMDL_{10}$	Based on what	Reference
NDMA	62 µg kg bw^{-1} day^{-1}	Incidence of liver cell tumours in male colworth wistar rats	BMDL value set by Dybing et al. 2008 based on toxicological study by Petro et al. 1991
NDMA	29 µg kg bw^{-1} day^{-1}	Total liver tumors in male colworth wistar rats	BMDL value set by Zeilmaker et al. 2010 based on toxicological study by Petro et al. 1991
NDMA	27 µg kg bw^{-1} day^{-1}	fatal liver neoplasm (rat)	Scientific Committee on Consumer Safety, 2012
NPYR	160 µg kg bw^{-1} day^{-1}	total liver tumor (rat)	Scientific Committee on Consumer Safety, 2012
NDEA	18 µg kg bw^{-1} day^{-1}	Total liver tumors (rat)	Scientific Committee on Consumer Safety, 2012

The T25 approach is defined as the chronic dose rate (usually expressed in units of mg per kg bodyweight per day) which will give tumors at a specific tissue site in 25% of the animals after correction for spontaneous incidence and within the standard life time of the species (Dybing et al., 1997).
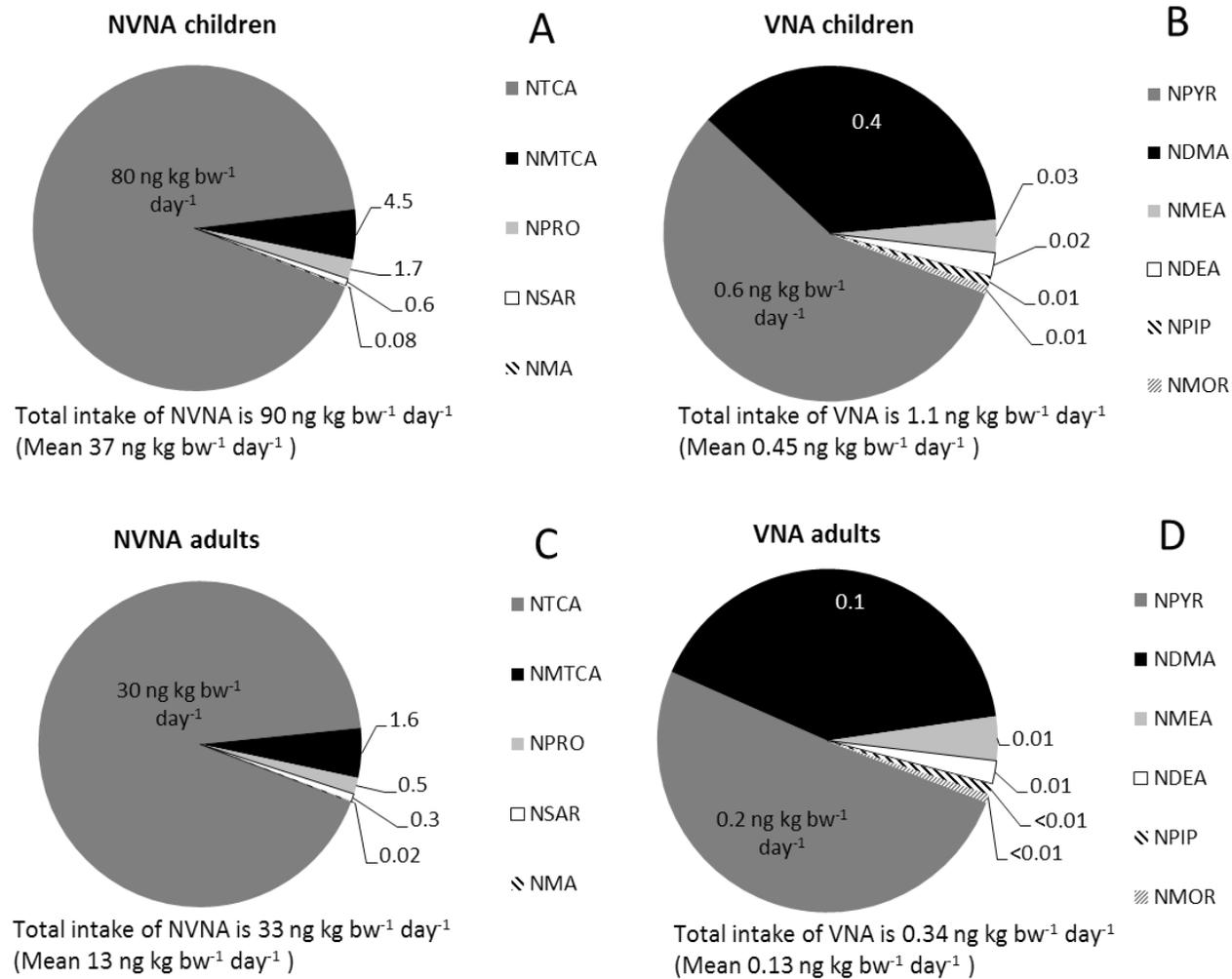
Figure 1: Exposure level (ng kg bw^{-1} day^{-1})(95th percentile) to NVNA (Figure A and C) and VNA (B and D) by consumption of processed meat products for children (4-5 years of age)(Figure A and B) and adults (15-75 years of age)(Figure C and D).
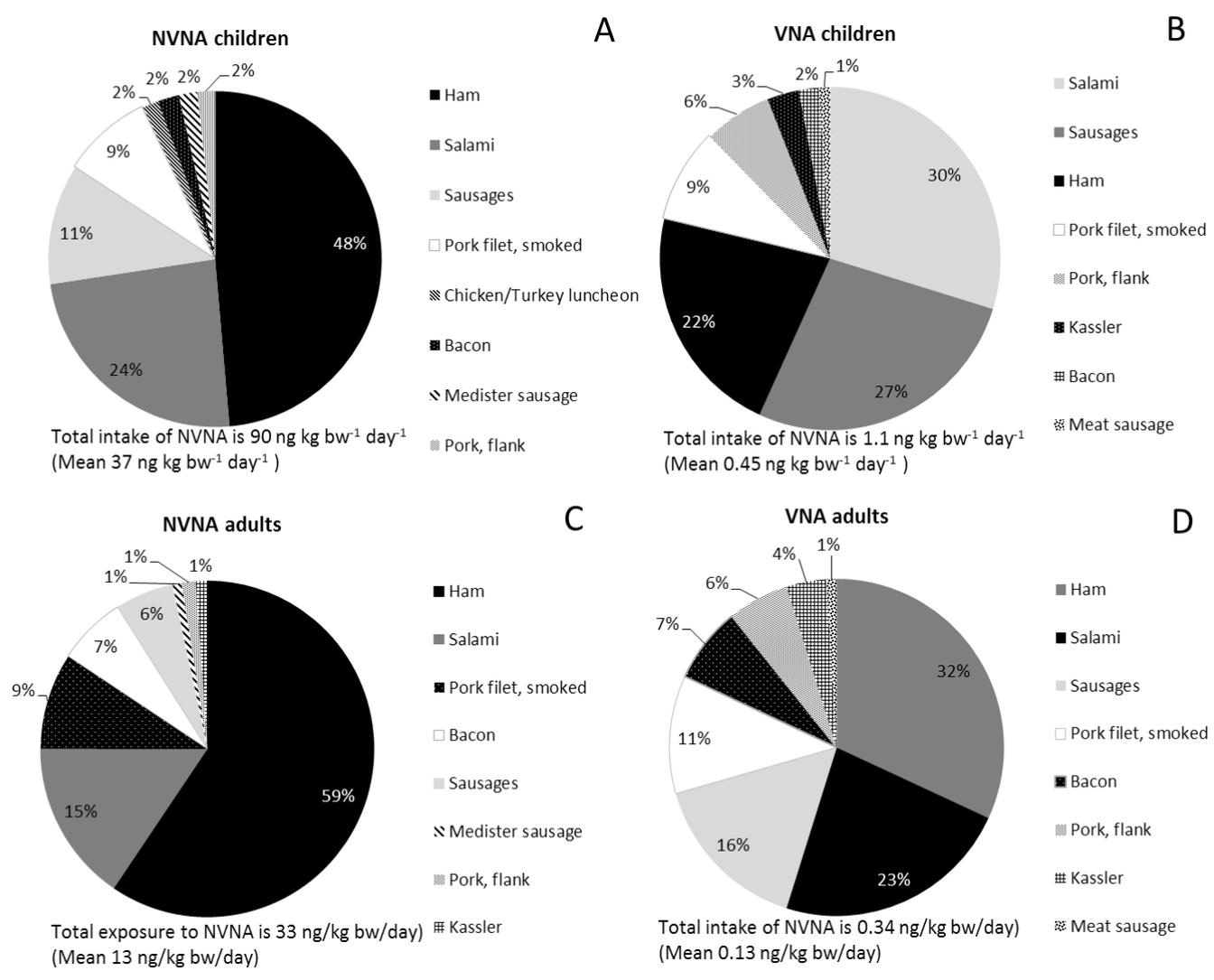
Figure 2: Processed meat products contributing to the exposure to NVNA (Figure A and C) and VNA (Figure B and D)for children (4-5 years of age) (Figure A and B) and adults (15-75 years of age)(Figure C and D).