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## Food Control

journal homepage: [www.elsevier.com/locate/foodcont](http://www.elsevier.com/locate/foodcont)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 ( $\leq 0.8 \mu\text{g kg}^{-1}$ ), whereas the mean levels of the NVNA were considerably higher ( $\leq 118 \mu\text{g kg}^{-1}$ ). Slightly higher mean levels were indicated for the Belgian products (i.e.  $\text{VNA} \leq 1.5 \mu\text{g kg}^{-1}$  and  $\text{NVNA} \leq 270 \mu\text{g kg}^{-1}$ ). The sums of VNA were higher than  $10 \mu\text{g kg}^{-1}$  for one Danish sample and two Belgian samples. Levels of up to 2000 and  $4000 \mu\text{g kg}^{-1}$  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.

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## 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<sup>-1</sup> 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<sup>-1</sup> and 150 mg kg<sup>-1</sup>, respectively. The common EU legislation (Directive 2006/52/EC) allows an addition of 150 mg kg<sup>-1</sup> 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; Rywotycy, 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<sup>-1</sup>) 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.

		N-nitrosodi-methylamine						N-nitroso-morpholine						N-nitroso-methylethylamine						N-nitroso-pyrrolidine						N-nitroso-diethylamine						N-nitroso-piperidine						N-nitroso-sarcosine						N-nitroso-proline						N-nitroso-methylaniline						N-nitroso-thiazolidine-4-carboxylic acid						N-nitroso-2-methyl-thiazolidine-4-carboxylic acid					
Compound name		N-nitrosodi-methylamine						N-nitroso-morpholine						N-nitroso-methylethylamine						N-nitroso-pyrrolidine						N-nitroso-diethylamine						N-nitroso-piperidine						N-nitroso-sarcosine						N-nitroso-proline						N-nitroso-methylaniline						N-nitroso-thiazolidine-4-carboxylic acid						N-nitroso-2-methyl-thiazolidine-4-carboxylic acid					
Abbreviation		NDMA						NMOR						NMEA						NPYR						NDEA						NPIP						NSAR						NPRO						NMA						NTCA						NMTCA					
	N <sup>3</sup>	No.	Mean	det.	Max	No.	Mean	det.	Max	No.	Mean	det.	Max	No.	Mean	det.	Max	No.	Mean	det.	Max	No.	Mean	det.	Max	No.	Mean	det.	Max	No.	Mean	det.	Max	No.	Mean	det.	Max	No.	Mean	det.	Max	No.	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																																				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				0.6	1	0.6		1.1	1	1.1	33	9	99	3.2	8	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		0.2	1	0.2					0.04	1	0.04		0.4	1	0.4	4.0	5	2.0		0.3	2	0.3	9	5	25	4.4	5	12																							
Ham	8	2.0	3	3.0		0.03	1	0.03		0.2	4	0.4		1.2	7	2.3		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	8	23																							
Salami <sup>2</sup>	24	1.6	15	4.0		0.5	1	0.5		0.2	1	0.2		2.1	10	3.4		0.3	2	0.3					49	2	82		17	3	30		0.4	2	0.5	292	5	723	14	5	34																										
Filet, smoked	5	1.1	4	1.8						0.2	1	0.2		4.0	4	13		0.3	2	0.3																																															
Mean content (positive results, all products) <sup>4</sup>		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) <sup>4</sup>	70	0.7				0.01				0.04				0.8				0.04						0.02			1.5			2.3			0.1			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 <sup>1</sup>	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 <sup>2</sup>	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) <sup>4</sup>		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) <sup>4</sup>	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 calculating 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  $\geq 99.9\%$ , Fluka-Analytical) were purchased from Sigma–Aldrich Co. (St Louis, MO, USA). The pure standards of *N*-nitrososarcosine (NSAR), *N*-nitrosohydroxyproline (NHPR), *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 (Ausborg, 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  $\geq 98\%$  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  $\times$  2.1 mm, 3  $\mu$ m 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  $\mu$ L.

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  $<1 \mu\text{g kg}^{-1}$ , though with some exceptions. LOD for NDBA and NTCA in kassler were 1.7 and 2.1  $\mu\text{g kg}^{-1}$ , respectively; LOD for NDMA and NTCA in bacon were 3.3 and 4.9  $\mu\text{g kg}^{-1}$ , respectively; and LOD for NPYR, NTCA and NMTCA in salami were 17.1, 18.4 and 9.4  $\mu\text{g kg}^{-1}$ , 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  $\mu\text{g kg}^{-1}$ , 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  $\mu\text{g/kg}$ . 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 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<sup>-1</sup>. 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<sup>-1</sup> (assigned value 143 mg kg<sup>-1</sup>) and for sodium nitrite it was 189 mg kg<sup>-1</sup> (assigned value 178 mg kg<sup>-1</sup>).

### 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<sup>-1</sup> (Table 1). The most frequently found VNA were NDMA and NPYR. The levels of NDMA ranged from non-detectable to 4 µg kg<sup>-1</sup>. The levels of NPYR ranged from non-detectable to 13 µg kg<sup>-1</sup>.

NDMA was detected in >50% of the samples of bacon, salami and smoked filet. NPYR was detected in ≥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<sup>-1</sup> (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 Spiegelhalder, Eisenbrand, and Preussmann (1980) reported 32% and 11% of such products (*N* = 395) to contain detectable levels (>0.5 µg kg<sup>-1</sup>) of NDMA and NPYR, respectively. Levels above 5 µg kg<sup>-1</sup> 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. In the present study no levels of NDMA above 5 µg kg<sup>-1</sup> occurred in the Danish samples but in one of the twenty Belgian samples (5%). NPYR occurred at concentrations above 5 µg kg<sup>-1</sup> 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<sup>-1</sup>), NPYR (1.4 and 0.9 µg kg<sup>-1</sup>), NPIP (1.1 and 0.9 µg kg<sup>-1</sup>) and NDEA (0.7 and 0.3 µg kg<sup>-1</sup>) 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<sup>-1</sup>). 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<sup>-1</sup> 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<sup>-1</sup>. This smoked filet sample also contained the highest level of NPRO (30 µg kg<sup>-1</sup>) 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<sup>-1</sup>) 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<sup>-1</sup>), i.e. in meat sausage (luncheon meat) and boiled ham. The highest level of NMTCA (39 µg kg<sup>-1</sup>) 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<sup>-1</sup> and 98 µg kg<sup>-1</sup>, 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* ≥ 0.95 or *P* ≥ 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<sup>-1</sup> nitrite will result in higher average NA levels than if 60 mg kg<sup>-1</sup> 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<sup>-1</sup> 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<sup>-1</sup>, except for one sample of smoked filet for which the sum amounted to 15.0 µg kg<sup>-1</sup>. For the samples on the Belgian market the sum of VNA were all ≤3.6 µg kg<sup>-1</sup>, 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  $10 \mu\text{g kg}^{-1}$  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  $-20^\circ\text{C}$  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  $-20^\circ\text{C}$ .

The nitrite ( $\text{NaNO}_2$ ) levels ranged from  $<3 \text{ mg kg}^{-1}$  to  $36 \text{ mg kg}^{-1}$  with a mean content of  $6.0 \text{ mg kg}^{-1}$ . The nitrate ( $\text{NaNO}_3$ ) levels ranged from  $<5 \text{ mg kg}^{-1}$  to  $124 \text{ mg kg}^{-1}$  with a mean value of  $27.7 \text{ mg kg}^{-1}$ . 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  $-20^\circ\text{C}$ . When calculating the mean values the  $<\text{LOD}$  values were set to 0.5 times the LODs (i.e.  $0.5 \times 3 \text{ mg kg}^{-1}$  for  $\text{NaNO}_2$  and  $0.5 \times 5 \text{ mg kg}^{-1}$  for  $\text{NaNO}_3$ ).

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

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 ( $\text{NaNO}_2$ ) levels in the Belgian samples ranged from  $0.3 \text{ mg kg}^{-1}$  to  $25 \text{ mg kg}^{-1}$  with a mean content of  $4.0 \text{ mg kg}^{-1}$ . The nitrate ( $\text{NaNO}_3$ ) levels ranged from  $1.5 \text{ mg kg}^{-1}$  to  $178 \text{ mg kg}^{-1}$  with a mean value of  $32.9 \text{ mg kg}^{-1}$ . 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.

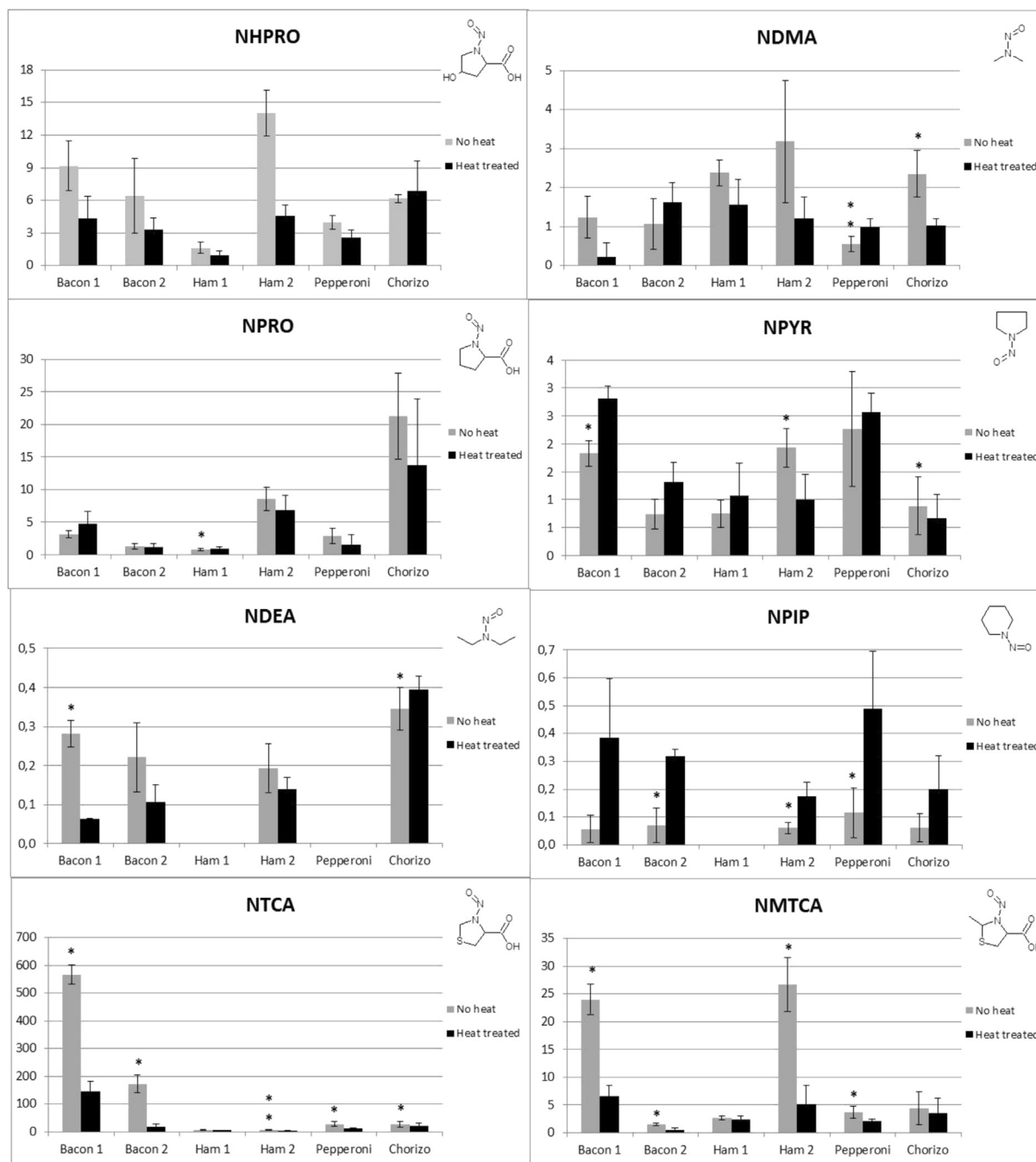
#### 3.3. Heat treatment

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  $\mu\text{g kg}^{-1}$  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.

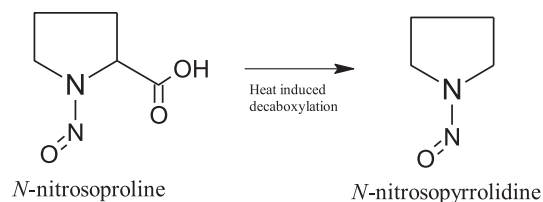
It was only for NPIP, NTCA and NMTCA that the same effect of heat treatment was observed for all products. The levels of NPIP increased for all products with a factor of three to five, though only significantly for “bacon 2”, “ham 2” and pepperoni. The levels of NTCA were significantly lower after the 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  $\leq 0.05$  or  $\leq 0.01$ ).

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 ( $0.5\text{--}2.0\text{ }\mu\text{g kg}^{-1}$ ) and up to 8 times increase of NPYR (from  $1.9$  to  $15\text{ }\mu\text{g kg}^{-1}$ ) (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  $200\text{ }^{\circ}\text{C}$  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  $217\text{ }^{\circ}\text{C}$  (at  $721\text{--}760\text{ mmHg}$ ), 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 ( $214\text{ }^{\circ}\text{C}$ ) 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.

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