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Levels of nitrate, nitrite and nitrosamines in model sausages during heat treatment and *in vitro* digestion – The impact of adding nitrite and spinach (*Spinacia oleracea* L.)

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ABSTRACT

Nitrite derivatives react with endogenous precursors forming N-nitrosamines associated with development of colorectal cancer. The present study aims to investigate the formation of N-nitrosamines in sausage during processing and *in vitro* gastrointestinal digestion after adding sodium nitrite and/or spinach emulsion. The INFOGEST digestion protocol was used to simulate the oral, gastric, and small intestinal phases of digestion, and sodium nitrite was added in the oral phase to mimic the input of nitrite from saliva as it has shown to affect the endogenous formation of N-nitrosamines. The results show that the addition of spinach emulsion, in spite of it being a source of nitrate, did not affect the nitrite content in either batter, sausage, or roasted sausage. The levels of N-nitrosamines increased with the added amount of sodium nitrite, and further formation of some volatile N-nitrosamines was observed during roasting and *in vitro* digestion. In general, N-nitrosamine levels in the intestinal phase followed the same trend as in the undigested products. The results further indicate that nitrite present in saliva may cause a significant increase in N-nitrosamine levels in the gastrointestinal tract and that bioactive components in spinach may protect against the formation of volatile N-nitrosamines both during roasting and digestion.

1. Introduction

Processed meat intake has been associated with an increased risk of colorectal cancer (CRC), and in 2015, the International Agency for Research on Cancer (IARC) classified high consumption of processed meat as carcinogenic to humans (IARC 2015). Several pathways have been proposed to explain this association, including dietary intake and endogenous formation of potent carcinogenic N-nitrosamines (NAs). Nitrite is added to processed meat to prevent the growth of spoilage bacteria and pathogens such as *Clostridium botulinum*, as well as to develop a salty flavour and red/pink colour. Nitrite once added to meat undergoes different fates, and part of it may be converted to nitrosation agents. NAs are formed through nitrosation of secondary amines during e.g. heat treatment and storage (De Mey et al 2017, Herrmann et al. 2015a) or endogenously in the digestive tract (Sander 1967, Engemann et al 2013). Because of the association between consumption of processed meat and increased risk of cancer, it is of interest to both consumers and producers that healthier alternatives to the traditional products are developed.

NAs belong to a class of organic compounds containing an amino group and an N-nitroso group and they are the structurally simplest of all carcinogens. NAs are not carcinogenic as such but have to be metabolically activated by cytochrome P450 enzymes (Lijinsky 1987). It is suggested that activation takes place by the hydroxylation of the carbon in the α position to the N-nitroso group. Created through this pathway electrophilic intermediates may form DNA adducts causing DNA mutation that may lead to the development of CRC (Lijinsky 1987; Wong et al. 2005).

Around 20 different NAs have been detected in processed meat products containing nitrite. They can be divided into volatile (VNA) and non-volatile (NVNA) compounds. Some of these compounds are classified by the IARC into groups 2A (probably carcinogenic to humans), 2B

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(possibly carcinogenic to humans), and 3 (not carcinogenic to humans), and part of them still have an unknown carcinogenic status (IARC, 1978). Structures, IUPAC names, abbreviations, and IARC classification of NAs relevant to this study are provided in Fig. 1. N-nitrosodiethylamine (NDEA) and N-nitrosodimethylamine (NDMA), among the most frequently found VNAs in processed meat, have been evaluated as probably carcinogenic (group 2A) (IARC, 1978). According to the EFSA report regarding the safety of curing salts usage, the mean concentrations of NDMA in processed meat on the European market range between 0.7 and 2.7 μg kg^{-1}, whereas the reported amount of NDEA range from 0.04 to 0.9 μg kg^{-1} (EFSA, 2017). Other often present VNAs are N-nitrosopyrolidine (NPYR) and N-nitrosopiperidine (NPIP) evaluated as highly likely to be carcinogenic (group 2B) (IARC, 1978). There are only a few studies on the occurrence and genotoxicity of NVNAs (Tricker & Kubacki 1992, Massey et al. 1991, Herrmann et al. 2015a, Niklas et al. 2022). Some of the nitroso-compounds belonging to this group, such as N-nitrosoproline (NPRO) and N-nitrosohydroxyproline (NHPRO) are not likely to cause cancer (EFSA 2017). However, due to insufficient data, the carcinogenic status of compounds such as N-nitroso-thiazolidine-4-carboxylic acid (NTCA) and N-nitroso-2-methyl-thiazolidine-4-carboxylic acid (NMTCA) is unknown (EFSA 2017).

Although processed meat intake is linked to increased cancer risk (IARC, 2015) it is still unknown to what extent the presence of NAs formed during nitrite curing can explain observed phenomena. Tricker (1997) suggested that endogenous formation is the primary source of exposure to NAs. In the human body, endogenous exposure is the result of the nitrosation of ingested secondary amines under acidic conditions prevailing in the stomach. Intake of fresh "unprocessed", as well as "processed" meat, contributes to the endogenous formation of NAs due to the availability of NO-metabolites and secondary amines throughout the gastrointestinal tract (Vermeer et al., 1998; Zeilmaker et al. 2010).

Exposure to nitrite via nitrite-containing food such as processed meat is a minor part. In fact, the main source of exposure to nitrite is indirectly through the reduction of dietary nitrate from vegetables (e.g. lettuce, celery, spinach). Nitrate once absorbed is actively transported to the saliva gland by ~ 20–25%, where between 5 and 36% of it is reduced to nitrite by oral bacteria (EFSA 2017 and references therein). Although the consumption of vegetables may increase endogenous nitrosation due to the intake of nitrate, no association has been found between consumption of vegetables and increased risk of CRC. On the contrary, a high intake of vegetables may lower the cancer risk. In 2007, the World Cancer Research Fund/American Institute for Cancer Research stated evidence as "convincing to probable" that diets high in vegetables and/or fruits protect against cancers of the mouth and pharynx, esophagus, lung, stomach, colon and rectum, larynx, pancreas, breast, and bladder (WCRF/AICR 2007). In that respect, it has been shown that nitrosation reactions forming NAs can be inhibited by antioxidative compounds in vegetables such as *L*-ascorbic acid and polyphenols (Habermeyer et al.; 2015; Herrmann et al. 2015b; Rocha et al 2014). Therefore, it may be beneficial to add antioxidant-rich vegetables to processed meat products.

Spinach (*Spinacia oleracea* L.) is high in nitrate content, as well as rich in vitamins and secondary metabolites, including flavonoids, phenolic acids, and carotenoids acting as potential antioxidants (Bergman et al., 2001; Melendez-Martinez et al., 2022). High-pressure homogenization (HPH) is a green technology applied in the food industry to improve the nutritional and technological quality of food products, such as the physical stability of fruit and vegetable juices and oil-in-water emulsions. Several studies report good retention of health-related phytochemicals, as well as increased release and potentially increased bioavailability (Kirkhus et al. 2019; Yong et al., 2021).

The aim of the present study was to investigate how the addition of nitrite and a spinach HPH-produced emulsion to processed meat affects the endogenous formation of NAs. NA levels in model sausages were studied during processing (heat treatment) and *in vitro* gastrointestinal digestion to assess the importance of nitrite and nitrate concentrations within the food matrix for human exposure to NAs. Furthermore, the contribution of nitrate from spinach to salivary nitrite and endogenous

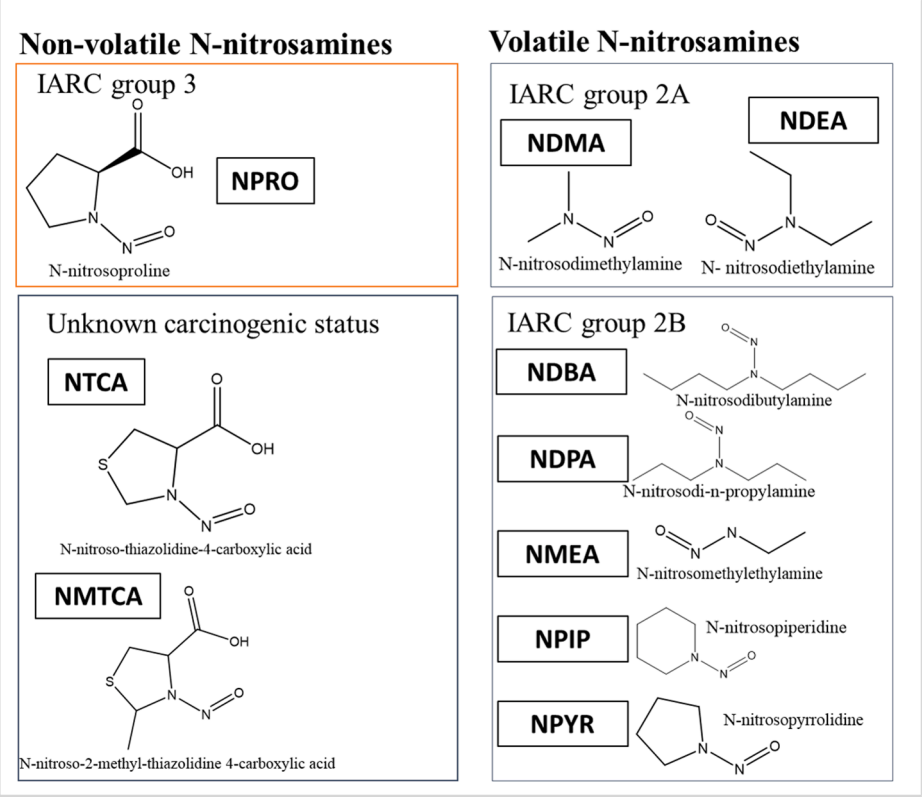

Fig. 1. Structures and IUPAC names of non-volatile and volatile N-nitrosamines relevant to the present study together with IARC classification.

nitrosation was evaluated in a separate experiment.

2. Materials and methods

2.1. Materials

The meat ingredients were purchased from a commercial slaughterhouse Furuseth AS (Dal, Norway). The spinach (*Spinacia oleracea*) was a commercially blanched and deep-frozen product (product code EPD: 1132950) obtained from Norrek Dypfrys AS, Larvik, Norway. Refined rapeseed oil was from Rema Koin AS, purchased at REMA1000 store, Norway, with an expiration date more than a year ahead. Sodium benzoate (≥99%, FCC, FG 532–32-1), pepsin (porcine, P7000), pancreatin (porcine, P1750), and bile extract (bovine/ovine, B8381) were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Sodium hydroxide (>98%) and sodium nitrite (>99.0%) were obtained from Sigma Aldrich (Merck KGaA, Darmstadt, Germany), sodium nitrate (purity > 99.99%) from Fluka-Analytical/Honeywell International Inc. (NC, USA), and solid phase extraction (SPE)-columns, e.g. Chromabond, C18 EC 500 mg/6 mL, from Macherey-Nagel GmbH&CoKG (Düren, Germany). Acetonitrile was purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland). Formic acid (purity 98–100%), and methanol (purity ≥ 99.9%) were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The pure standards of non-volatile and volatile N-nitrosamines were purchased from Toronto research chemicals (Toronto, Canada) and Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), respectively. All compounds included in the method scope are presented in Fig. 1. The internal standards N-nitrosodimethylamine-d6 (NDMA-d6) and N-nitrosopyrrolidine-d8 (NPYR-d8) were purchased from Sigma-Aldrich (Merck KGaA, Germany), and CDN isotopes Inc (Quebec, Canada) respectively. The purity of all standards was ≥ 98%. All chemicals were of analytical grade.

2.2. Preparation of spinach emulsion using high-pressure homogenization (HPH)

A spinach emulsion containing rapeseed oil was produced at the food pilot plant of Nofima, Ås, Norway, by high-pressure homogenization (HPH) mainly as described in Kirkhus et al. (2019). The frozen blocks (approx. 4x3x3 cm^3) of chopped spinach (5 kg) were thawed at low temperature in a saucepan with 20% water (w/w), simmering for 15 min (80–90 °C) with the lid on. The content was then cooled on ice to approximately 15 °C, and the amount of evaporated water was replenished with preboiled water before the addition of rapeseed oil (10%, w/w) and sodium benzoate (0.1%, w/w). The mixture was further homogenized in a Wilfa blender 1400S (Wilfa AS, Oslo, Norway) for 2.5 min at speed 1–2 (pre-emulsion). To remove larger particles, the pre-emulsion was sieved using a separator with a strainer of 0.5 mm mesh (Robot Coupe C80, Robot Coupe USA Inc.). The emulsion was produced by HPH using a Panda PLUS 2000 (GEA Mechanical Equipment, GEA Niro Soavi S.p.A., Parma, Italy) at 1000 bar. The emulsion was collected and mixed before packing in trays sealed with film using a packing machine and stored at 4 °C in the dark for the sausage production the next day. Samples of the emulsion were frozen in tubes with screw caps (45 mL) at −20 °C for nitrate and nitrite analysis.

2.3. Preparation of sausages

Model sausages were produced at a food pilot plant (Nofima, Ås, Norway) with strictly controlled process conditions. All batter recipes (Table 1) were balanced to give 20% (w/w) total fat and 10% (w/w) protein. The recipes were based on pork trimmings (23% fat, 16% protein), beef trimmings (14% fat, 18.5% protein), pork backfat (without skin, 70% fat, 6.5% protein), 6.0% potato flour, 0.1% polyphosphate, 1.8% $NaCl/NaNO_2$ and 0.2% spices (including white pepper). The content of beef and pork in each recipe varied to fulfil the criteria of constant fat and protein and was approximately 30% pork trimmings and 22% beef trimmings. No antioxidants, like ascorbic acid, were added during sausage production. In short, the meat trimmings were minced and mixed with salt and other dry ingredients before fat and water/spinach emulsion were added to produce a sausage batter that was stuffed in plastic casings, cooked at 80 °C for 30 min and cooled. Additionally, natural smoke from beech chips was added before cooking of the sausages.

A 2^2 full factorial design (Table 1) was used to determine the effects of adding nitrite ion (350 mg/kg sausage) and/or a spinach emulsion (30%, replacing the water content in the sausage recipe) (see section 2.2.). To obtain similar fat content and fatty acid profile in all model sausages, sausages without spinach emulsion were added 3% rapeseed oil. The sausages (final product) and samples of the sausage batter were vacuum-packed and stored at −40 °C until analysed. To study the effect of heat treatment, newly produced sausages were chopped into approx. 1 cm pieces and roasted in an oven at 160 °C for 30 min. Sausages were weighed before and after the heat treatment to estimate weight loss. After roasting, the sausages were cooled and stored at −40 °C until analysed.

Analyses of protein content, total fat and fatty acid composition, sodium, and calcium in the sausages (final products) were performed using accredited methods at Eurofins (Eurofins Food & Feed Testing Norway AS, Moss, Norway).

2.4. In vitro digestion

Model sausages were subjected to a static *in vitro* digestion model simulating the oral-, gastric- and duodenal phases. The model is based on the EU Cost Action 1005 INFOGEST harmonized digestion method with standardized electrolyte solutions for the preparation of simulated salivary, gastric- and intestinal fluids (Minekus et al., 2014; Brodkorb et al., 2019). The sausages were thawed and chewed in a kitchen mincer (CombiMax700, Braun GmbH, Kronberg, Germany), and 2.0 g were placed in tubes with 2 mL of an electrolyte solution containing salivary amylase (50 U mL^{-1}) and kept at 37 °C during shaking. The gastric phase was simulated by adding 4.0 mL of an electrolyte solution containing pepsin (4000 U mL^{-1}). The pH was adjusted to 3.0 with 10 M HCl before incubation in a rotary incubator at 215 rpm (Innova® 40/40R, New Brunswick Scientific, Edison, NJ, USA) at 37 °C for 120 min. To simulate the intestinal phase, tubes were added 8 mL of simulated duodenal fluid containing 0.07 mM $NaHCO_3$, porcine pancreatin and bile resulting in a bile salt concentration of 10 mM and pancreatin concentration of 1.25 mg mL^{-1} in the final volume. After adjustment to pH 7.0 with 10 M NaOH, the samples were incubated at 37 °C and 215 rpm for 80 min in

Table 1
Design and compositions of the model sausages. SAFA = saturated fatty acids, MUFA = monounsaturated fatty, PUFA = polyunsaturated fatty acids, GAE = gallic acid equivalents, and CP = center point.

Recipe	Nitrite (mg kg^{-1})	Spinach emulsion %	Protein %	Total fat %	SAFA %	MUFA %	PUFA %	Sodium g kg^{-1}	Calcium mg kg^{-1}	Total phenolics mg GAE 100 g^{-1}
1	0	0	11.6	21.6	7.5	10.9	2.9	8.2	54	29.1
2	350	0	11.4	21.9	7.5	10.9	2.9	8.4	65	27.8
3	0	30	11.4	21.6	7.3	11.0	3.0	8.5	390	39.2
4	350	30	11.5	21.5	7.3	11.0	3.1	7.8	330	36.3
CP	175	15	11.6	21.7	7.3	11.0	3.0	7.7	200	32.5

the intestinal phase. After withdrawal, all samples were immediately heat-treated at 90 °C for 10 min to stop the enzyme activity and then centrifuged at 4000 rpm for 10 min. Aliquots of the supernatant (micellar phase) were stored at −40 °C until the analysis of nitrosamines.

To investigate the potential effect of nitrite in saliva on nitrosamine formation in the gastrointestinal tract, nitrite (4.5 mg L^{-1} in simulated saliva) was added in the oral phase during *in vitro* digestion of sausage recipes 1 and 3 (see Table 1). All *in vitro* digestion experiments were run in parallel.

2.5. Analysis of total phenolics (TP)

Sausages were thawed overnight at 4 °C and casings removed before blending the sample into a homogenous sample in a food processor (CombiMax700, Braun GmbH, Kronberg, Germany). Phenolic compounds were extracted from duplicate sample aliquots (10.0 g) with methanol (20 mL) by homogenization in a Polytron, PT3100 homogenizer (Kinematica AG, Littau, Switzerland) at 28,000 rpm for 30 s. The extracts were centrifuged at 39,200*g* for 10 min at 4 °C (Avanti J-26 XP Centrifuge, Beckman Coulter, USA), after which the supernatants were collected, and the pellet was re-extracted as above with 70% methanol in water (20 mL). The two pooled supernatants of each sample were combined, and the volume was made up to 50 mL with 70% methanol. TP was determined using the Folin–Ciocalteu colourimetric method as previously described (Aaby et al 2020). Methanolic extracts (0.2 mL), in technical duplicates, were mixed with Folin–Ciocalteu reagent (1.0 mL) and incubated for 3 min before the addition of 7.5% sodium carbonate (0.8 mL). The mixture was incubated for 30 min at 20–22 °C in the dark before absorbance was measured at 765 nm (Spectrostar Nano, BMG-Labtech). TP content was expressed as gallic acid equivalents (GAE) in mg per 100 g.

2.6. Analyses of nitrate and nitrite

The contents of nitrate and nitrite in spinach (commercially blanched-frozen), spinach emulsion, sausage batter, sausage (final product), and roasted sausage (see 2.3) were determined by ion chromatography-UV detection. Homogenized samples (5.0 g) were weighed into Falcon centrifuge tubes and 50 mL of 1 mM sodium hydroxide was added. The samples were ultra-sonicated for 15 min in an ultrasonication bath (Branson 5510 from VWR Int., Søborg, DK) and then centrifuged for 10 min at 3600 rpm (Sigma 3–18 K, Buch & Holm A/S, Herlev, DK). Two mL of sample was transferred to a C18-SPE column 500 mg/6 cc, then a plastic cap with a hole (Biolab A/S, Risskov, DK) was placed at the column, and by using a 10 mL disposable plastic syringe (Becton Dickinson S.A., UK) the sample was pressed through the column into an HPLC vial (PP plastic, Mikrolab Aarhus A/S, DK). Calibration standards in the range of 0.25–100 μg mL^{-1} were made in 1 mM sodium hydroxide from 1 mg mL^{-1} sodium nitrite and sodium nitrate. The calibration standards were prepared on the day of the analyses. The ion chromatography-UV analysis was performed on an Agilent instrument with HP1100 series with gradient pump, degasser, autosampler, and UV-DAD detector. The autosampler was set at 10 °C. The ions of 25 μL injections were separated using a Dionex IonPac^{TM} AG11 guard column and a Dionex IonPac ^{TM} AS11 separation column (Thermo Fischer Scientific, Roskilde, DK). The elution gradient of eluent A (deionized water) and B (50 mM sodium hydroxide) was: 0–10 min 10% B, 10–20 min. 100% B, 20–25 min 10% B. UV-detection at 225 nm. Recoveries in a batch of 50 mg kg^{-1} sodium nitrite and 150 mg kg^{-1} sodium nitrate were 94% and 99%, respectively. Reference material 6600 mg kg^{-1} nitrate had a recovery of 103% (*Rucola*, fapas®, Fera, York, UK).

2.7. Analysis of nitrosamines

For the measurement of NAs in sausage batter and model sausages (unroasted and roasted), NAs were extracted from homogenized samples according to the analytical method developed by Niklas et. al. 2022. A mixture of water and acetonitrile 1:1 v/v with formic acid (1% v/v) was used as an extraction solvent. Non-volatile compounds were analysed in a fraction before phase separation containing both water and acetonitrile. Volatile compounds were analysed in the organic phase obtained after phase separation induced by the addition of 5 g ammonium formate per 20 mL of solvent mixture and purified with d-SPE sorbent primary secondary amine (PSA) (100 mg pre 1 mL extract).

For the extraction of NAs from digested samples, i.e., the soluble fraction (supernatant/ micellar phase) of the intestinal phase, separate extraction procedures were used for volatile and non-volatile N-nitrosamines. The sample was divided into two parts. For extraction of volatile compounds, 6 mL supernatant was transferred to a 15 mL polypropylene (PP) tube (Sarstedt, Numbrecht, Germany) and internal standards (ISTD) (NDMA-d6 and NPYR-d8) were added. Then, 6 mL of acetonitrile was added, and the tube was shaken on Geno/Grinder (SPEX Sample Prep. 2010) (10 min, 1500 rpm). The sample was sonicated (Branson 5510 from VWR Int., Søborg, DK) for 1 h in a water bath (50 °C). After sonication, 2 g of ammonium formate was added to the sample, the tube was shaken again on Geno/Grinder until salt dissolved (approx. 1 min, 750 rpm) and centrifuged (Thermo Scientific, Multifuge X3 FR) (5 min, 4500*g*). The upper layer (organic phases) was transferred to a new tube (6 mL) and frozen until solid (-80 °C, approx. 30 min). The thawed extract was purified by the addition of 600 mg PSA and 6 mL of n-heptane. The sample was shaken on Geno/Grinder (3 min, 1000 rpm) and centrifuged (5 min, 4500*g*). The upper n-heptane layer was aspirated to waste, and 5 mL of the acetonitrile phase was transferred to a glass tube (size 5 mL) containing 100 μL of water. The organic solvent was evaporated under a gentle stream of nitrogen (at 30 °C ± 0.5) (Supertherm, Mikrolab Aarhus A/S) to a volume of 0.1 mL and adjusted to 0.5 mL by the addition of Milli-Q water. The sample was once more frozen until solid, thawed, and centrifuged (10 min, 13,100 g (Eppendorf, mini spin plus). 200 μL of the extract was filtered (GE Healthcare Life Sciences Whatman, Mini-UniPrep, syringeless filters, polypropylene (PP) membrane, pore size 0.2 μm) and analysed. Extraction of non-volatile compounds was performed from 4 mL of the remaining intestine supernatant. Due to the limited amount of available material, <4 mL was for some samples employed, but the proportions for the rest of the procedure were then adjusted accordingly. Four mL of supernatant was mixed with 4 mL of Milli-Q water in 15 mL tube by shaking on Geno/Grinder (SPEX Sample Prep. 2010) (10 min, 1500 rpm). Then, the sample was sonicated for 1 h in a water bath (50 °C) (Branson 5510 from VWR Int., Søborg, DK). After sonication, the sample was loaded on preconditioned SPE column Oasis MAX 6 cc (Waters Corp., Milford MA-USA). SPE column was conditioned with 2 mL of methanol and then 2 mL of ultra-pure water. After loading the sample, the SPE column was washed with 3 mL of acetonitrile. Non-volatile N-nitrosamines were eluted with acidified acetonitrile (2% formic acid in v/v). Three mL of the acetonitrile phase was transferred to a glass tube (size 5 mL) containing 100 μL of water and evaporated to a volume of 0.1 mL. Volume was adjusted to 0.5 mL with Milli-Q water. Then the extract was frozen until solid, defrosted, and centrifuged (10 min, 14,000 rpm) (Eppendorf, mini spin plus). The extract was filtered (GE Healthcare Life Sciences Whatman, Mini-UniPrep, syringeless filters, PP membrane, pore size 0.2 μm) and analysed.

The separation was performed on UPLC Ultimate 3000 (Thermo Scientific) equipped with a reversed-phase Poroshell 120 Phenyl-Hexyl column (150 × 2.1 mm, particle size 2.7 μm, Agilent Technologies) as described by Niklas et al. (2022) in the gradient of Milli-Q water with formic acid (0.1% v/v) (Mobile phase A) and methanol (Mobile phase B). Detection was performed in Multiple Reaction Monitoring (MRM) mode using EVOQ ELITE (Bruker, Billerica, MA, USA) equipped with ESI source for the analysis of the NVNA, or APCI source for the analysis of the VNA, and parameters were held as described by Niklas et al. (2022). Transitions with collision energies used for NAs detection as well as

criteria applied for identification are described in Niklas et al. (2022). Quantitation of compounds (expressed as μg kg^{-1} sausage) was based on bracketing calibration curves. The limit of detection was defined as the lowest concentration of NAs in a calibration solution with S/N higher than 3.

2.8. Design of experiments (DOE) and statistical analyses

Statistical analyses of designed experiments were performed with Unscrambler® v 10.3 (Camo Inc., Norway) to establish the effects of added nitrite and spinach emulsion. Significant ($p < 0.05$) main effects and interaction effects were analysed by classical DOE analysis using multiple linear regression (MLR) and Scheffé formulas (Scheffé 1958).

3. Results and discussion

3.1. Composition of model sausages

The experimental design and nutritional information of the model sausages are shown in Table 1. Nitrite and spinach emulsion were added to a basic recipe typical for Norwegian barbecue and dinner sausages, though the recipe did not contain ascorbic acid and the levels of nitrite were higher than allowed in sausage production. As expected, there were no significant differences in the contents of fat, protein, and sodium between the various recipes. However, recipes 3 and 4, as well as the center point (CP), contained higher levels of total phenolics and calcium, both increasing linearly with the addition of spinach emulsion (regression statistics, $p < 0.01$).

3.2. Contents of nitrite and nitrate in model sausages during processing

As seen from the results presented in Fig. 2, nitrite was only detected in sausage samples where nitrite ion was added, at either 350 mg kg^{-1} or 175 mg kg^{-1} (CP). EU regulation (No 1129/2011) limits the maximum amount of added sodium nitrite in commercial sausage batter to 150 mg kg^{-1}, which is equal to 100 mg kg^{-1} of nitrite ion. This means that both the high level and the CP level of added nitrite in the present study were above the legal limit. The concentrations of nitrite ions decreased during processing for recipes with the highest nitrite additions; 137–142 mg kg^{-1} in batter decreasing to 113–118 mg kg^{-1} after cooking of sausage (heat treatment 80 °C for 30 min) and decreasing further to 38–41 mg kg^{-1} after roasting (160 °C for 30 min). When prepared with 175 mg nitrite kg^{-1} (CP), the sausage batter, sausage, and roasted sausage contained 58, 48, and 17 mg kg^{-1}, respectively. Thus, the levels of nitrite in the different preparations were halved when the added amount of nitrite was halved. Although the addition of nitrite in this study was higher than the legal limit, the final levels in the model sausages (48–118 mg kg^{-1} sausage and 17–41 mg kg^{-1} mg roasted sausage) were in the same range as levels reported for Italian wurstel sausages (12.9–103.0 mg kg^{-1}) in a recent publication of Berardi et al. (2021). During the processing steps a major part of the nitrite disappeared, and the oxidation product nitrate increased (Fig. 2). From the recipe with high nitrite addition without spinach emulsion (recipe 2), it can be estimated that only ~ 12% of the nitrite ions were recovered as nitrate ions (adjusted for molar weights). The blanched-frozen spinach product contained 349 mg kg^{-1} nitrate ions and 4 mg kg^{-1} nitrite ions, while the spinach emulsion contained 223 mg kg^{-1} nitrate ions and 2 mg kg^{-1} nitrite ions after one day of storage at 4 °C. Meat has naturally a low content of nitrate, and the sausage recipe without nitrite or spinach (recipe 1) had a nitrate content < 7 mg kg^{-1}. Roasted and non-roasted sausages that contained 30% spinach emulsion had concentrations of ~ 74 mg kg^{-1} nitrate ions which is in accordance with the contributions from 30% spinach emulsion and meat. The addition of 30% spinach emulsion did not affect the nitrite levels in batter or in sausage or roasted sausage. For example, the recipe with 30% spinach emulsion and no added nitrite, did not contain nitrite.

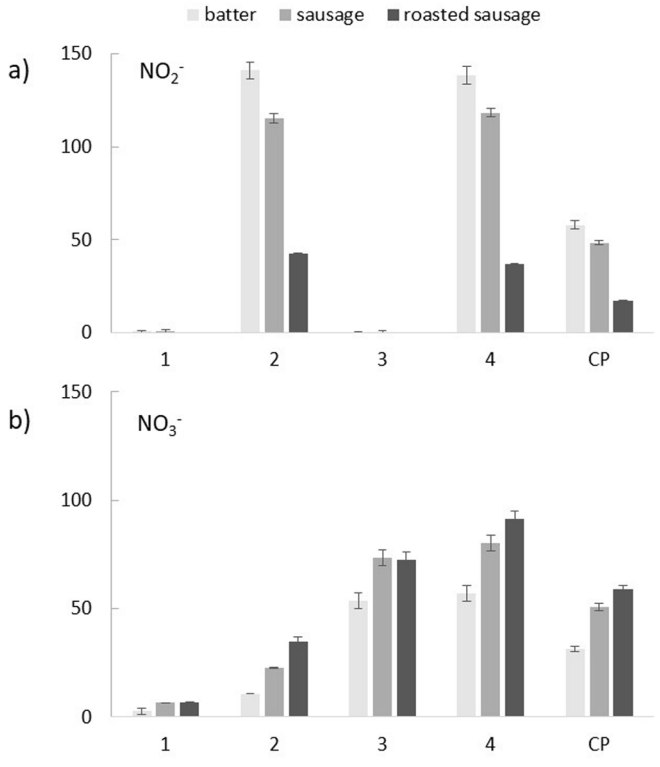

Fig. 2. Contents (mg kg^{-1}) of a) nitrite (NO_2^-) and b) nitrate (NO_3^-) (mg kg^{-1}) in batter, sausage, and roasted sausage of recipes 1 (no nitrite), 2 (nitrite), 3 (no nitrite, 30% spinach emulsion), 4 (nitrite, 30% spinach emulsion) and CP (see Table 1). Error bars indicate the standard deviation.

3.3. Contents of nitrosamines in model sausages during processing and after in vitro digestion

NAs are mainly formed during the nitrosation of secondary amines. Therefore, the contents of NAs in meat products depend not only on the amount of nitrite but also on specific precursors that need to be present. In meat products, the presence of biogenic amines and other protein degradation products are considered to be important sources of amine precursors. Among the protein degradation products, secondary amines such as dimethylamine are supposed to be directly nitrosated. However, biogenic amines, such as spermidine, spermine, cadaverine, and putrescine, which contain only primary amine groups, are suggested to undergo deamination and cyclization reactions to form corresponding nitrosatable secondary amines (De Mey et al., 2017). Besides the presence of precursors, the formation of NAs may be affected by processing conditions such as pH, temperature, and time, as well as the presence of compounds that occur naturally in meat, e.g. haem iron or intentionally added compounds during manufacturing.

Contents of NAs were measured in batter and model sausages before and after roasting, as well as after *in vitro* digestion (Fig. 3, Table S1 and S2). Generally, the volatile nitrosamines (VNAs) were found at low concentrations < 1 μg kg^{-1}, except NPYR which was found in the roasted sausages at concentrations up to 4 μg kg^{-1}. VNAs frequently found were NDMA and NPYR, and occasionally NPIP and NDBA were detected, whereas NDEA, NDPA and NMEA were not detected in any of the analysed samples. NDBA (0.1–0.5 μg kg^{-1}) was only present in sausages with nitrite addition. NDMA was found in the highest concentrations in samples with high nitrite addition (recipes 2 and 4). NDMA was formed during sausage production (0.2–0.3 μg kg^{-1}) and roasting (0.5–0.6 μg kg^{-1}) probably because heat treatment increased the nitrosation to some extent. Mottram et al. (1977) found considerably higher amounts of NDMA fried meat and suggested that the optimum temperature for the

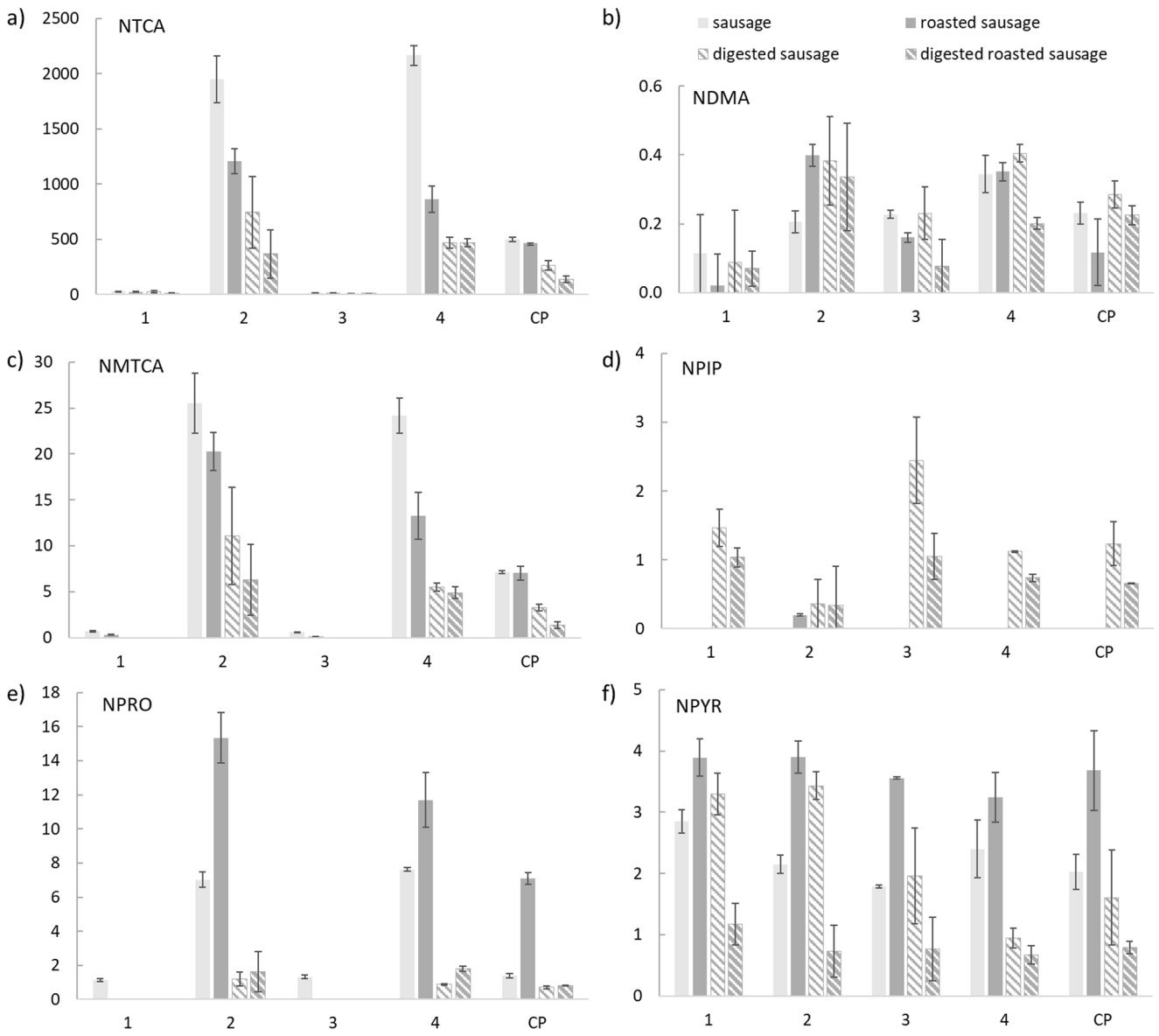

Fig. 3. a-f. Contents of N-nitrosamines (μg kg^{-1}) in sausages, roasted sausages, digested sausages, and digested roasted sausages for recipes 1 (no nitrite), 2 (nitrite), 3 (no nitrite, 30% spinach emulsion), 4 (nitrite, 30% spinach emulsion) and CP (see Table 1). Error bars indicate the standard deviation.

formation of NDMA is between 150 °C and 200 °C. Although at 150 °C, about 80% of these compounds were present in the vapour (Mottram et al. 1977), as NDMA has a boiling point of 154 °C. Thus, even though NDMA content was higher after roasting, still some loss by e.g. evaporation may have occurred. The levels of NPYR did not increase with the nitrite addition, which was also reported by Herrmann et al. (2015b). Moreover, NPYR increased upon heat treatment (roasting) roughly to the same extent as reported by Li et al., 2012 (~1.3 times), who found an additional formation of NPYR in sausages during pan frying or deep frying at temperature 150 °C. Formation of NPYR was observed in all sausage recipes including no nitrite or spinach emulsion added (recipe 1). Other authors (Herrmann et al. 2015b, De Mey et al. 2017) found that the formation of NPYR is not correlated with the amount of added nitrite but with applied temperature during processing. NPYR was suggested to be formed in cured meat at high temperatures either by decarboxylation of NPRO or through the nitrosation of proline present abundantly in collagen (de Mey et al., 2017). Furthermore, Hotchkiss et al. (1985) suggested that during cooking release of nitrogen oxide from proteins or other nitrosating species bound to lipids can occur and hereby give rise to nitrosation agents that can further react with protein precursors forming NAs. Those observations can explain the presence of NPYR in roasted sausages prepared without nitrite.

The non-volatile nitrosamines (NVNAs) were all found to increase with the ingoing amount of nitrite, as also reported by Herrmann et al. (2015b). NTCA was found in all samples including those where no nitrite was added, however much higher concentrations of NTCA were present in nitrite cured sausages (1950–2200 μg kg^{-1}). NMTCA was also found in most of the samples, although at significantly lower levels ranging from 0.1 to 0.6 μg kg^{-1} in samples without nitrite addition compared to 10 to 28 μg kg^{-1} in samples where nitrite was added. NTCA and NMTCA contents were highly correlated ($R^2 = 0.96$). The amounts of NTCA and NMTCA appear to be closely related to the amount of added nitrite, and their levels decreased during roasting. These observations are in accordance with available literature suggesting that N-nitrosated amino acids undergo thermal decarboxylation (Tricker & Kubacki 1992). In contrast, NPRO, which also contains a carboxylic group, showed increased levels after roasting, the reason for this could be direct nitrosation of proline released from collagen (de Mey et al., 2017).

During *in vitro* digestion, the amounts of NTCA, NMTCA, and NPRO were reduced possibly because of the decarboxylation. In contrast, the

levels of NDMA and NPIP increased. Especially NPIP, which was not detectable in most of the sausage samples, was detected in all digested samples (roasted and non-roasted) and did not follow the same pattern as other NAs. White and black pepper contain comparable amounts of piperine and piperidine, which are confirmed to be precursors of NPIP (de Mey et al., 2017). NPIP can be formed in the presence of white or black pepper and nitrosation agent by either the oxidative cleavage of the amide bond of piperine or direct nitrosation of available piperidine. Herrmann et al. (2015b), found that NPIP content in sausages increased with increasing black pepper addition. In the present study, NPIP was not detected in the sausages, possibly because there was no fermentation step and thus piperine was not available for further nitrosation. During digestion, oxidative cleavage of the amide bond of piperine may occur resulting in the release of piperine which could be nitrosated to NPIP. Higher content of NPIP was found in digested sausage than in roasted digested sausage (Fig. 3). The reason for this could be that roasting caused a decrease in the nitrite content (see Fig. 2), thus less nitrite can be converted into nitrosating agents. Furthermore, it may be assumed that the highest added amounts of nitrite to sausage 2 and 4 to some extent prevented meat degradation to biogenic amines due to less microbiological activity, which could explain why those samples formed less endogenous NPIP. Skibsted (2011) suggested that meat proteins can be a reservoir for NO and nitrosating agents which could explain the presence of these reactants during the digestion of sausages without added nitrite and spinach emulsion.

Less NAs were found in the samples with added spinach emulsion. Spinach is a rich source of many health-promoting compounds with antioxidant activity, such as ascorbate and phenolic compounds (Table 1). The presence of those could mitigate the endogenous formation of nitrosating agents (Herrmann et al. 2015b). Classical DOE analysis showed significant effects of both nitrite and spinach emulsion on NA levels in undigested and digested sausages (Fig. 3, Table 2). The addition of nitrite increased the levels of all NAs (except NPYR) in undigested sausages, both unroasted and roasted. A similar increase in NAs with added nitrite was observed in digested sausages in the intestinal phase, except for NPIP and NPYR which were rather reduced. The addition of spinach emulsion reduced the levels of NVNAs (NTCA, NMTCA) in unroasted digested sausages, indicating some inhibitory effects of spinach constituents during *in vitro* digestion. A reduction in NVNAs (NPRO, NTCA, and NMTCA) was also observed in roasted sausages with added nitrite and spinach emulsion compared to those containing only nitrite, but only in undigested sausage (Fig. 3). This may be explained by bioactive components in spinach acting as antioxidants and thereby inhibiting the formation of NAs. When these components act during roasting, they may be depleted and therefore not contribute to a lowering of the NA formation during digestion. When a significant effect of spinach emulsion was observed there was also a significant interaction effect (Table 2) implying that the effect of spinach was highest in sausages with added nitrite. The results further indicate that the addition of spinach emulsion may increase levels of NDMA (in undigested unroasted sausage) and NPIP (in digested unroasted sausage), probably because nitrate in the spinach acted as a nitrosation agent under the given conditions.

3.4. Effect of salivary nitrite on endogenous formation of nitrosamines during in vitro digestion of model sausages

To further study endogenous NA formation, an experiment was performed where nitrite was added to the oral phase (4.5 mg of nitrite per L of simulated saliva) of the *in vitro* digestion model. The resulting concentration of nitrite per gram sausage was 4.5 μg g^{-1}. The dosage was based on EFSA (2017) which estimated that 1–9% (on average 5%) of ingested nitrate may re-enter the gastrointestinal tract after circulatory passage and bacterial reduction to nitrite in the mouth. The experiment was performed with the two model sausages that were not added nitrite; recipe 1 (0% spinach emulsion) and recipe 3 (30% spinach emulsion). The aim was to assess the contribution to endogenous NA formation of nitrite derived indirectly from spinach nitrate, as well as the influence of potential bioactive components in spinach. The determination of NAs in the soluble fraction of the intestinal phase reflects the amount of NAs available for uptake in the body. Results from the *in vitro* digestion of recipes 1 and 3, with and without the addition of nitrite in the oral phase, are shown in Fig. 4.

Extensive formation of NDMA, NPIP, NTCA, NMTCA and NPRO was observed when nitrite was added to simulated saliva during *in vitro* digestion, showing that in the gastrointestinal tract nitrosation agent is the limiting factor for this NAs formation and that amino precursors are available during digestion. Ohshima & Bartsch, 1981; Ohshima, O'Neill, Friesen, Béréziat, & Bartsch, 1984 conducted human *in vivo* experiments to demonstrate endogenous nitrosation. Proline was proved to be nitrosated to non-carcinogenic NPRO, which could be concluded because NPRO is excreted unchanged in the urine of human subjects. Also, NTCA and NMTCA were found, proving that nitrosation can occur endogenously. Precursors for endogenous nitrosation to NTCA and NMTCA could be thiazolidine 4-carboxylic acid and 3-methylthazolidine 4-carboxylic acid originating from cysteine (Ohshima et al. (1984). Cysteine is a non-essential amino acid being synthesized in the human body and is also present in meat. Cysteine can react with formaldehyde or acetaldehyde forming thiazolidine 4-carboxylic acid and 3-methylthazolidine 4-carboxylic acid. Cysteine is suggested to be a part of the meat-related Maillard reactions, e.g. the Strecker degradation of cysteine reacting with a dicarbonyl releases formaldehyde and acetaldehyde, which cysteine can condensate into thiazolidine-4 carboxylic acids; or in the Strecker degradations reactions of cysteine with dicarbonyls via intermediates form thiazolidine-4 carboxylic acids (Mottram 2007, Shakoor et al. 2022). Formaldehyde and acetaldehyde derive from the liquid smoke added to the sausages. Generally, smoking seems to favour the formation of NVNA, especially NTCA (Tricker & Kubacki 1992, Herrmann et al. 2015a). Constituents of smoke may reduce some other nitrosamines, e.g. NDMA and NPYR, perhaps because thiazolidine

Table 2
Statistical analyses of designed experiments. Main and interaction effects ($p < 0.05$) of nitrite (350 mg/kg) and spinach emulsion (30%) on nitrosamine levels in sausages after processing and *in vitro* digestion. A positive effect is denoted by "+" ($p < 0.05$), "++" ($p < 0.01$, "+++" ($p < 0.005$), and a negative effect by "-" ($p < 0.05$), "- -" ($p < 0.01$) and "- - -" ($p < 0.005$), whereas "ns" is not significant.

Processing/digestion	Nitrite/spinach	NDMA	NPIP	NPYR	NPRO	NTCA	NMTCA
Sausages	Nitrite	+ + +		ns	+ + +	+ + +	+ + +
	Spinach emulsion	++		ns	ns	ns	ns
Sausages, roasted	Nitrite	+ + +	+ + +	ns	+ + +	+ + +	+ + +
	Spinach emulsion	ns	− − −	ns	–	–	–
	Interaction effect		spinach × nitrite		spinach × nitrite	spinach × nitrite	spinach × nitrite
***In vitro* digested sausages**	Nitrite	+ + +	− −	–	+ + +	+ + +	+ + +
	Spinach emulsion	ns	+	ns	ns	− −	− − −
	Interaction effect					spinach × nitrite	spinach × nitrite
***In vitro* digested roasted sausages**	Nitrite	+ + +	–	ns	+ + +	+ + +	+ + +
	Spinach emulsion	ns	ns	Ns	ns	ns	+ + +

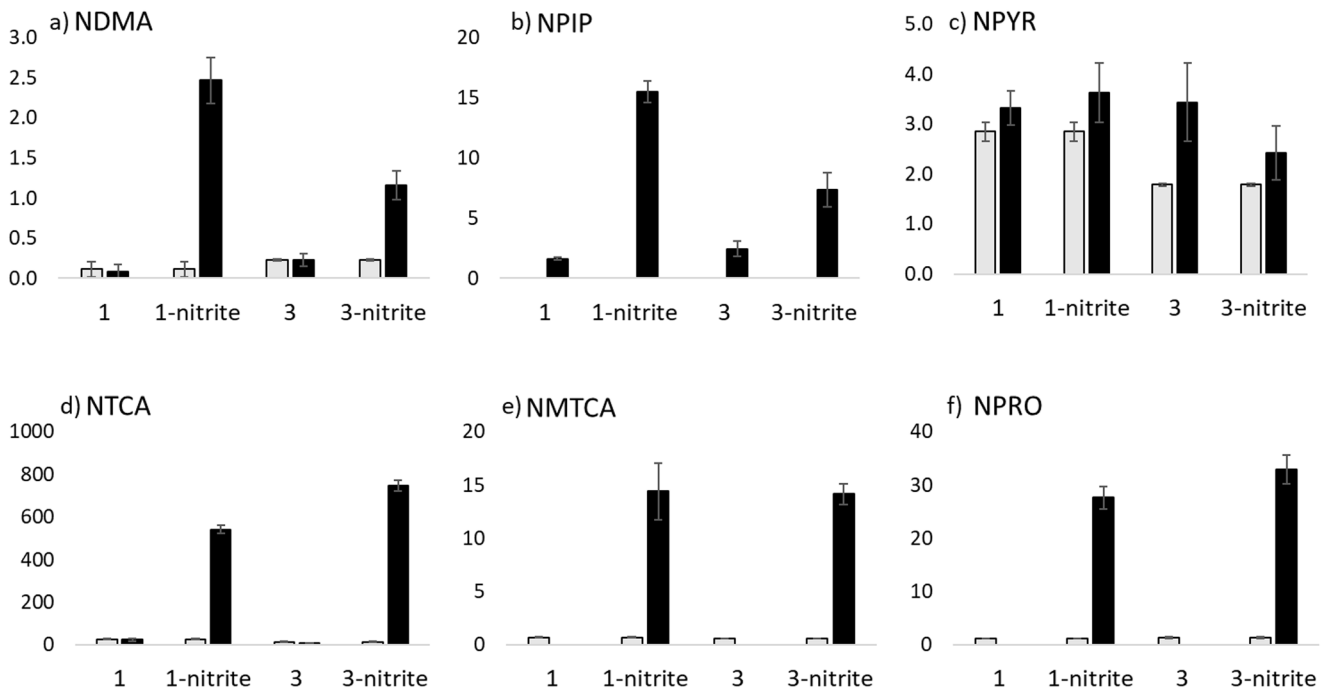

Fig. 4. a-f. Contents of NAs (μg kg^{-1}) in model sausages (recipes 1 and 3, Table 1) before (light grey bar) and after *in vitro* digestion (black bar) with (1-nitrite, 3-nitrite) or without (1 and 3) added salivary nitrite (corresponding to nitrate contents of spinach). Concentrations of NAs are calculated as relative to in model sausages. Error bars indicate the standard deviation.

4-carboxylic acids compete for the nitrosating agent and are more easily nitrosated to NTCA.

Zeisel et al. (1985) demonstrated that dimethylamine can be endogenously formed, and further nitrosated to NDMA. It was suggested that the dietary source of dimethylamine could result from the breakdown product of creatine (Blau, 1961) or the transmethylation of methylamine derived from sarcosine or glycine (Zeisel et al. 1985). The aforementioned compounds are naturally present in raw meat.

As described in the previous section of this manuscript, piperine and piperidine present in pepper can be released during digestion under the acidic environment of the gastric phase and the presence of digestive enzymes might have released precursors from the pepper, which contributed to NPIP formation. In this experiment, salivary nitrite delivered the nitrosation agent needed to form NPIP from available precursors.

Spinach constituents may affect NA formation. From Fig. 4 it appears that while NTCA, NMTCA, and NPRO were not reduced when spinach was present, NDMA and NPIP were. Herrmann et al. (2015b) found that vitamin C (ascorbic acid) prevented the formation of NPIP and NPRO, but not NTCA and NMTCA. The observed inhibitory effect on the formation of NDMA and NPIP in the presence of spinach could be related to the antioxidant activity of ascorbic acid or other bioactive compounds, e.g. polyphenols, naturally present in the spinach emulsion. As shown, the concentrations of polyphenols (Table 1) were found to increase linearly with spinach content in sausages. The well-known vitamin C's instability during heat treatments (Wang et al., 2017) may reduce the amount present in the spinach emulsion and model sausages, but still, a certain amount will be present and act as an antioxidant. In addition, Tsao (2010) suggests that phytochemicals especially polyphenols are predominant contributors to antioxidant activity of fruits and vegetables. In a review, Corpet (2011) discussed different possibilities for making the consumption of meat safer; one of the suggestions was to increase the calcium content to reduce the adverse effect of haem iron. The spinach emulsion included in the recipes of sausages prepared in the present study contains preventive compounds such as phenolics and vitamin C, but it also significantly increased the calcium contents in the sausages (Table 1). Regarding the effect of calcium, Pierre et al. (2013) suggested that calcium may chelate the iron in haem, thus preventing the endogenous formation of nitroso compounds. In addition, spinach is rich in nitrate which may also affect NA formation during digestion. The rapidly absorbed nitrate will however pass the circulatory system and part of the nitrate in the saliva will be transformed to salivary nitrite by mouth bacteria before re-entering the gastrointestinal system where it may be converted to the nitrosation agent at low pH in the stomach. Moreover, bacteria e.g. enterobacteria can reduce nitrate to nitrite and thereby exhibit nitrosating activity (Calmels et al 1985). However, this will mainly take place in the large intestine hosting the gut microbiota, and since nitrate is rapidly and almost completely absorbed (bioavailability 92%) in the small intestine (Mensinga et al., 2003), only a small amount will reach the large intestine. Since the gastric transit time of solid food is usually $\geq$ 2 h (Minekus et al., 2014), salivary nitrite derived from nitrate in the spinach is less likely to contribute to endogenously formed NAs during the sausage meal.

4. Conclusions

This is the first study investigating *in vitro* endogenous formation of eight VNA and three NVNA.

After *in vitro* digestion the NA levels in the soluble fraction of the intestinal phase follow the same trend as NAs in the respective sausage products, with the highest amounts of NAs found in sausages with high nitrite addition. NVNAs decreased during *in vitro* digestion, whereas an increase in the VNAs was observed during the digestion of sausages with added nitrite. Formation of VNA during digestion may partly be related to decarboxylation of NVNA to homolog carcinogenic VNAs under enzymatic activity. The results further indicate that nitrite in saliva can cause a significant increase in NA levels during *in vitro* digestion. However, there is also evidence that components in spinach (phenolics, calcium) may mitigate the formation of VNAs both during roasting as well as during digestion.

CRediT authorship contribution statement

Agnieszka A. Niklas: Methodology, Validation, Formal analysis, Investigation, Interpretation of results, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Grethe Iren A. Borge:** Conceptualization, Conceptualization, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Rune Rødbotten:** Conceptualization, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Ingunn Berget:** Conceptualization , Data curation, Investigation, Writing – original draft , Writing – review & editing. **Mette H.B. Müller:** Conceptualization, Writing – review & editing. **Susan S. Herrmann:** Methodology, Validation, Writing – review & editing. **Kit Granby:** Methodology, Validation, Formal analysis, Investigation, Data curation, Interpretation of results, Writing – original draft, Writing – review & editing, Visualization. **Bente Kirkhus:** Project administration, Conceptualization, Funding acquisition, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112595.

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