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Evaluation of the influence of proline, hydroxyproline or pyrrolidine in the presence of sodium nitrite on *N*-nitrosamine formation when heating cured meat

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ABSTRACT

N-nitrosamines are meant to be probable or possible carcinogenic components, possibly formed out of a reaction between nitrite and N-containing substances such as amino acids and secondary amines. Nitrite is often used for processing meat products because of its colouring and antimicrobial properties.

During this experimental setup, the influence of proline, hydroxyproline or pyrrolidine on *N*-nitrosamine formation in meat samples was evaluated. The *N*-nitrosamines concentrations were measured with gas chromatography-thermal energy analyzer. Only the concentrations of *N*-nitrosodimethylamine and *N*-nitrosopyrrolidine were found above the limit of detection in a number of tested experimental conditions. The concentration of these two *N*-nitrosamines was modelled as a function of temperature and nitrite concentration for different situations (presence or absence of added natural N-containing meat components). It could be concluded that proline and pyrrolidine promoted the formation of *N*-nitrosopyrrolidine. It could also be confirmed that the higher the temperature of the meat processing procedure and the higher the sodium nitrite amounts added, the higher were the yields of the respective *N*-nitrosamines.

1. Introduction

Meat is an essential component in an everyday human diet, as it is a source of proteins (amino acids), vitamins and minerals. Curing meat was originally meant to prolong the freshness and safety of the meat products for the consumers. Nowadays, these products make part of a wide assortment of comestibles served during for instance a sandwich lunch. Meat industry uses different kinds of preservatives, yet those most frequently applied are nitrites and nitrates, which serve this purpose very well. Sodium nitrite and sodium nitrate are used as curing agents in meat, not only as preservatives (as they decrease the risk of botulism poisoning), but also for colour and flavor formation [1–3]. However, sodium nitrite plays also a considerable role in the formation of carcinogenic *N*-nitrosamines [4]. *N*-nitrosamines are formed in the reaction between a nitrosating agent and a substance having an amino group, and their formation can be a result of a chemical and/or a microbial reaction [5,6]. Formation of *N*-nitrosamines is a complex process and appearance of these compounds in meat products depends on various different parameters associated with conditions of preparation, storage, and/or thermal processing of meat [7,8]. The presence of the *N*-nitroso group containing compounds in cured meat has been acknowledged more than 40 years ago and the results of numerous analytical studies have shown that in cured meat the following compounds are most frequently encountered: *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosodibutylamine (NDBA), *N*-nitrosopiperidine (NPIP) and *N*-nitrosopyrrolidine (NPYR) (Fig. 1 shows the chemical structures of (a) *N*-nitrosodimethylamine, (b) *N*-nitrosodiethylamine, (c) *N*-nitrosodibutylamine, (d) *N*-nitrosopiperidine and (e) *N*-nitrosopyrrolidine). In 1978, the International Agency for Research on Cancer (IARC) classified a number of *N*-nitrosamines with respect of the cancer risk for humans [4]. According to the classification of carcinogenic compounds, NDMA and NDEA belong to the group of the probable carcinogens, whereas NDBA, NPIP and NPYR belong to the group of the possible carcinogens [9]. Meat industry

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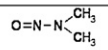
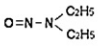
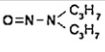
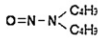
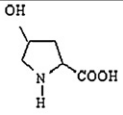
Compound	Molecular structure
N-Nitrosodimethylamine	
N-Nitrosodiethylamine	
N-Nitrosodipropylamine	
N-Nitrosobutylamine	
N-Nitrosopiperidine	
N-Nitrosopyrrolidine	
Proline	
Hydroxyproline	
Pyrrolidine	

Fig. 1. Chemical structures of the volatile *N*-nitrosamines, the added amino acids and pyrrolidine.

is responsible for the delivery of safe products to the market, and recently, it tends to limit the amounts of nitrites and nitrates added to processed meat by replacement of these compounds with other substances not promoting carcinogenic *N*-nitrosamines' formation [10,11]. The permitted amount of nitrite in cured meat products has been regulated by the European Customs Inventory of Chemical Substances which prescribes a maximum addition of sodium nitrite at a level of 150 mg kg^{-1} processed meat [12]. However, in most cases, the amount added to meat products usually is 120 mg kg^{-1}.

Various analytical techniques have been used for the determination of volatile *N*-nitrosamines. Subsequent tests show the usefulness and popularity of chromatographic techniques, used in combination with selective detectors. In order to obtain optimal analysis conditions, not only appropriate sample-preparation parameters, consisting of several steps such as isolation, clean-up and concentration, are required, but also the optimal parameters for detection are crucial [13]. For the determination of volatile nitrosamines in preserved meat [14], fried bacon [15,16] and salami [3], gas chromatography coupled to an extremely selective detector, i.e. thermal energy analyzer (TEA) has been described as the analysis technique the most commonly used. In seldom cases, the combination of GC with an alkali flame ionization detector (AFID) has been cited in the literature for the quantification of *N*-nitrosamines, yet resulting in chromatograms less clean then those obtained by the TEA detector [17]. Mass spectrometry (MS) has been recommended by IARC as the most reliable technique because of the structural information resulting from this kind of analyses [8,9].

Scientific attention is mostly focused on the volatile *N*-nitrosamines, which are easier to isolate and identify than the non-volatile. Nevertheless, less attention towards non-volatile *N*-nitrosamines does not mean that these compounds are completely ignored, but several studies reported in the literature have shown that non-volatile *N*-nitrosamines are biologically inactive [18–20].

In the literature, reports have been published pointing out to proline as an important NPYR precursor [16,17]. These conclusions have been drawn upon analytical results referring to both cooked and unprocessed bacon, as well as to certain commercially distributed meat products. Free proline is present in pork bellies at a concentration level of ca. 20 mg kg^{-1} [16]. To the authors, it seemed quite obvious that free proline was the most probable precursor of NPYR [16].

Although an exact reaction scheme is rather obscure, two possible pathways of proline transformation to NPYR were suggested. NPYR can either be formed out of proline by nitrosation and subsequent decarboxylation at elevated temperatures, or by proline decarboxylation in the first step, followed by pyrrolidine formation and final nitrosation [21–23]. Based on this preliminary knowledge, further investigations have been carried out on different experimental models, making use of variable proportions of proline or pyrrolidine, and also $NaNO_2$, in various solvents [24]. However, in none of the experiments described in the literature, meat models have been tested with proline or pyrrolidine directly added to the meat. No similar study has ever been carried out with hydroxyproline either, which together with proline is an amino acid present in collagen, and no investigation has ever focused on its role as an NPYR precursor.

In a primary study [25], the impact of proline and hydroxyproline on the formation of *N*-nitrosamines, when heating meat at different temperatures with an addition of different amounts of sodium nitrite, was researched. The aim of the present study was to investigate also the influence of pyrrolidine on *N*-nitrosamine formation in meat samples, in the presence or absence of sodium nitrite and at different meat-processing temperatures. Experimental evidence was produced under the same conditions as described in the primary study [25], i.e. using gas chromatography in combination with Thermal Energy Analysis (GC-TEA). The concentrations of *N*-nitrosamines (resulting from the tests in the primary and the secondary experimental setup) were modelled as a function of the temperature and the nitrite concentration for different situations (i.e. either with or without adding proline, hydroxyproline or pyrrolidine). The significance of the influence of the changing parameters was evaluated by Analysis of Variance (ANOVA) [26].

2. Experimental

2.1. Preparation of meat samples

2.1.1. Apparatus and materials

For the meat processing procedure, the following devices were used: a balance, a homogenisator, a canning machine, an autoclave, an oven and a demineralization plant (Eurowater, Silex 11, Skanderborg, Denmark). Cans (Ø = 7.30 cm, height = 5.35 cm) were purchased at Crown Brand-Building Packaging (Philadelphia, USA).

2.1.2. Ingredients to process the meat products

Lean meat (pork *m. Longissimus*) was purchased at the slaughterhouse on the day after slaughtering. Sodium chloride and pentasodium triphosphate (Deraphos BC TRI) were provided by Dera Food Technology (Bornem, Belgium). Proline (99%) and trans-4-hydroxy-L-proline (99+%) were purchased from Sigma–Aldrich (St Louis, MO, USA), and pyrrolidine (≥99.5%) was obtained from Fluka (St. Louis, MO, USA). Sodium nitrite was obtained from VWR International (West Chester, PA, USA).

2.1.3. Meat model processing procedure

The cooked ham model was prepared as follows: 300-g aliquot of minced meat was mixed with 2% NaCl and 0.3% pentasodium triphosphate. For that purpose, 30 g brine, containing 6.6 g NaCl

and 0.99 g pentasodium triphosphate in 22.4 g of ultrapure water, was injected into the meat sample.

Depending on the particular case, $NaNO_2$ was added in final amounts of 0, 120 and 480 mg kg^{-1} meat sample, respectively. In addition, 1000 mg kg^{-1} proline or hydroxyproline, or 10 mg kg^{-1} pyrrolidine, was added to the meat sample. The selection of these different added amounts was based upon results of preliminary experiments. After canning, the meat samples were heated for 30 min at different temperatures, thereby imitating the industrial processes (pasteurization (85 °C), sterilization (120 °C), and baking and roasting (120–220 °C)). A given set of conditions was replicated at least three times.

2.2. Analytical procedure

2.2.1. Apparatus and materials

For extraction and clean-up, a homogenisator/mixer, a balance, a mini-shaker, a rotary vacuum evaporator (Heidolph Laborota 4010-digital), a Kuderna-Danish apparatus (Sigma–Aldrich, Schnelldorf, Germany) and a water bath were needed. Glassware and other materials were selectively chosen to be suitable in each step of the procedure.

2.2.2. Reagents and reference standards

NDMA, NDEA, *N*-nitrosodipropylamine (NDPA), NDBA, NPIP and NPYR were purchased from Sigma–Aldrich (St. Louis, MO, USA). Other chemicals were obtained from VWR International (West Chester, PA, USA), except for dichloromethane (Merck, Darmstadt, Germany). All reagents were of analytical purity grade, except when indicated differently. The reference standard solution (ST) was composed of five *N*-nitrosamines most frequently present in the processed meat, i.e. NDMA, NDEA, NDBA, NPIP and NPYR. Quantification was carried out using NDPA as an internal standard (IS). In order to prepare the ST solution, the five aforementioned liquid *N*-nitrosamines (250 mg) were introduced to a 10.0 mL calibrated flask, and subsequently diluted with dichloromethane to obtain final concentrations of each individual *N*-nitrosamine equal to 0.25 μg mL^{-1}. The IS solution consisted of NDPA at 1 μg mL^{-1} in dichloromethane. The IS and ST solutions could be stored in a freezer at −16 °C or lower, for at least 3 months.

In order to prepare the 1N or 3N KOH and the 6N HCl solution, suitable aliquots of potassium hydroxide (KOH) or hydrochloric acid (HCl) (VWR International, West Chester, PA, USA) were dissolved in deionised water.

2.2.3. Extraction of N-nitrosamines

50 g aliquots of processed meat samples, to which 10 μg kg^{-1} NDPA had been added, were mixed with a 200 mL 3N KOH solution (schematic representation of the analytical procedure is shown in Fig. 2). After vacuum distillation, primary extraction was carried out according to Gasarasi's method [27]. The distillate (150 mL) was mixed with 4 mL 37% HCl and extracted three times with 50 mL of dichloromethane (DCM) (twice with DCM being recuperated after vacuum distillation in order to collect any remaining *N*-nitrosamines, followed by the analytical purity grade DCM). Secondary liquid–liquid extraction was performed with 50 mL 6N HCl, and 2× 50 mL DCM. A purification step was carried out with 50 mL 1N KOH and 50 mL DCM. After drying the extract with anhydrous sodium sulfate, the filtrate was concentrated to 100 μL in a Kuderna-Danish apparatus.

2.3. Chromatographic separation and detection

The meat sample extracts were analyzed by Gas Chromatography (GC) (Thermo Electron Corporation, Rodano, Italy) coupled to a Thermal Energy Analyzer (TEA) (Model 610, Thermo Electron Corporation, Breda, The Netherlands) for the detection and quantification of *N*-nitrosamines. After injection of 5 μL into the GC-TEA system, chromatographic separation was carried out on a packed column (10% Carbowax 20M + 2% KOH on Chromosorb WAW, 80/100 mesh, 1.8 m × 2 mm i.d. Varian, Middelburg, The Netherlands). Argon was used as carrier gas at a flow rate of 25 mL min^{-1}. The injection port was set at 175 °C. The temperature of the column port was ramped at 110 °C for 1 min and then increased to 180 °C at 5 °C min^{-1} and held for five additional minutes.

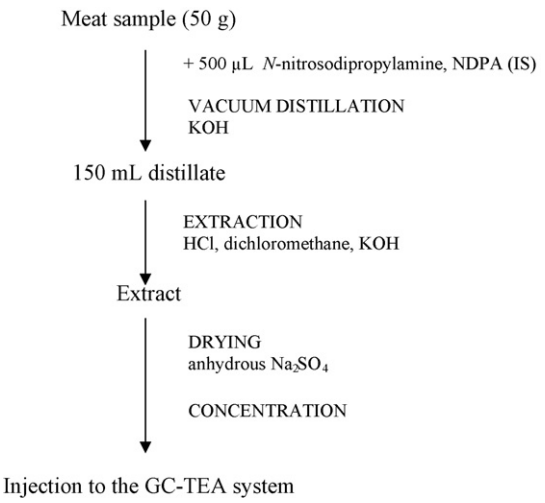


Fig. 2. Schematic representation of the extraction procedure.

The parameters for optimal TEA conditions were: interface temperature: 250 °C, pyrolizer temperature: 500 °C, GC operating mode pressure: 1.2 mmHg, attenuation: 4, vent mode pressure: 0.6 mmHg.

2.4. Validation elements

The NAs could be selectively identified by gas chromatography using the chromatographic method mentioned above. All components could be baseline-separated (Fig. 3 shows the chromatogram of the investigated *N*-nitrosamines).

Based upon a Signal-to-Noise ratio (S/N) equal to 3, representing the limit of detection (LOD), a 0.125 μg mL^{-1} standard solution containing the NAs was found to be the lowest detectable concentration. The limit of quantification (LOQ) was calculated as 3 × LOD.

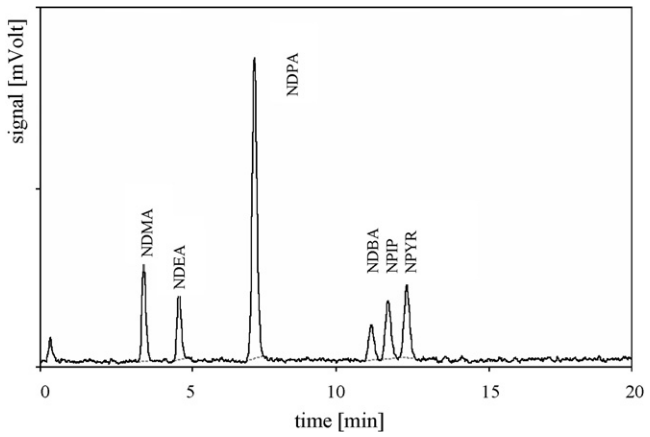


Fig. 3. The GC-TEA chromatogram showing separation of *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosodipropylamine (NDPA) (used as internal standard), *N*-nitrosodibutylamine (NDBA), *N*-nitrosopiperidine (NPIP) and *N*-nitrosopyrrolidine (NPYR).

Table 1
Range of the detected NDMA (A) and NPYR (B) concentrations ($\mu g\,mL^{-1}$) in relation to addition of amino acids, nitrite concentration and temperature; nd: not detected.

	Nitrite concentration ($mg\,mL^{-1}$)	Temperature (°C)			
		85	120	160	220
(A)					
Blank	0	nd-0.199	nd	nd	nd-0.125
	120	nd-0.175	nd-0.188	nd-0.262	nd-0.375
	480	nd-0.382	nd-0.294	0.148–0.283	0.170–0.441
Proline	0	nd-0.260	nd	nd-0.603	nd
	120	nd-0.189	nd-0.220	nd-0.349	nd-0.314
	480	nd-0.320	0.127–0.408	0.140–0.641	nd-0.313
Hydroxyproline	0	nd-0.125	nd-0.125	nd-0.207	nd-0.515
	120	nd	nd-0.125	nd-0.312	nd-0.334
	480	nd-0.188	nd-0.174	0.152–0.466	0.236–0.654
Pyrrolidine	0	nd-0.125	nd-0.215	nd-0.407	nd-0.212
	120	nd	nd-0.354	nd-0.318	0.125–0.379
	480	nd-0.309	nd-0.751	0.158–0.455	0.178–0.620
(B)					
Blank	0	nd	nd	nd	nd-0.429
	120	nd	nd	nd	nd
	480	nd	nd-0.128	nd-0.157	nd-0.523
Proline	0	nd-0.169	nd	nd	nd-0.613
	120	nd	nd	nd-0.163	0.210–1.322
	480	nd-0.230	nd-0.269	nd-0.422	0.570–1.560
Hydroxyproline	0	nd-0.210	nd	nd-0.154	nd-0.154
	120	nd	nd	nd	nd-0.127
	480	nd	nd	nd-0.149	nd-0.194
Pyrrolidine	0	nd-1.830	nd-0.366	0.151-0.825	nd-0.589
	120	0.244–0.337	0.342–0.642	0.262–0.627	0.452–1.009
	480	0.420–1.535	0.909–2.004	0.850–1.745	1.836–2.472

Table 2
Numbers of samples with measured *N*-nitrosamine concentrations in the different data sets.

Data set	Total number of samples	Number of samples					
		NDMA			NPYR		
		<LOD	<LOQ and >LOD	≥LOQ	<LOD	<LOQ and >LOD	≥LOQ
(1) Blank	68	28	37	3	60	5	3
(2) 1000 ppm proline	74	32	39	3	48	9	17
(3) 1000 ppm hydroxyproline	83	32	45	6	76	7	0
(4) 10 ppm pyrrolidine	91	40	37	14	11	23	57

Table 3
Results of the ANOVA analyses (F and p values for the factors and interactions) on the NDMA concentration observed in the different data sets ((A) individual data sets and (B) combined data sets); x_1 (temperature), x_2 (sodium nitrite level), x_3 (absence/presence of precursor); bold: significant terms at $\alpha = 0.05$ level.

Factor	Data set							
	(1) Blank		(2) Proline		(3) Hydroxyproline		(4) Pyrrolidine	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
(A)								
x_1	**2.94**	**0.015**	1.29	0.274	**5.91**	**0.000**	**3.98**	**0.002**
x_2	**12.68**	**0.000**	**6.64**	**0.003**	**8.64**	**0.000**	**20.51**	**0.000**
x_1x_2	0.81	0.568	1.33	0.260	1.34	0.252	**2.68**	**0.021**

Factor	Data set					
	(1)+(2)		(1)+(3)		(1)+(4)	
	F	*p*	*F*	*p*	*F*	*p*
(B)						
x_1	2.08	0.061	**6.44**	**0.000**	**4.22**	**0.001**
x_2	**17.20**	**0.000**	**19.16**	**0.000**	**29.75**	**0.000**
x_3	0.03	0.855	3.21	0.076	**5.15**	**0.025**
x_1x_2	1.63	0.146	1.74	0.118	**2.45**	**0.028**
x_1x_3	1.66	0.137	**2.27**	**0.041**	2.03	0.066
x_2x_3	0.51	0.600	1.34	0.266	1.61	0.204

Number of values used: see Table 2.

Table 4
Results of the ANOVA analyses (F and p values for the factors and interactions) on the NPYR concentrations observed in the different data sets ((A)individual data sets and (B) combined data sets); x_1 (temperature), x_2 (sodium nitrite level), x_3 (absence/presence of precursor); bold: significant terms at $\alpha = 0.05$ level.

Factor	Data set							
	(1) Blank		(2) Proline		(3) Hydroxyproline		(4) Pyrrolidine	
	F	p	F	p	F	p	F	p
(A)								
x_1	1.86	0.105	**28.78**	**0.000**	0.965	0.455	**4.97**	**0.000**
x_2	1.68	0.196	**9.71**	**0.000**	0.628	0.537	**69.49**	**0.000**
x_1x_2	0.83	0.554	**5.01**	**0.000**	0.581	0.744	**7.29**	**0.000**

Factor	Data set					
	(1)+(2)		(1)+(3)		(1)+(4)	
	F	p	F	p	F	p
(B)						
x_1	**27.60**	**0.000**	**2.67**	**0.018**	**5.27**	**0.000**
x_2	**9.51**	**0.000**	2.36	0.099	**52.66**	**0.000**
x_3	**42.20**	**0.000**	2.49	0.118	**179.99**	**0.000**
x_1x_2	**4.41**	**0.000**	1.19	0.318	**5.33**	**0.000**
x_1x_3	**16.94**	**0.000**	1.37	0.232	**2.41**	**0.031**
x_2x_3	**5.27**	**0.006**	1.30	0.276	**43.93**	**0.000**

Number of values used: see Table 2.

In order to quantify the concentration of the detected NAs, calibration curves for each target NA were build using NDPA as internal standard and with NA concentrations of 0.125, 0.2, 0.5, 1.0, 1.5, 2.0, 2.5 μg mL^{-1}, respectively. The calibration curves sample results (areas NA/area NDPA) were modelled using linear regression. The concentration level of the particular NAs in a given sample was then estimated based upon its area response (area NA/area NDPA).

2.5. Data handling

In the global data set, the following four subsets were considered: (1) blank meat samples processed as such, and samples fortified with (2) proline, (3) hydroxyproline, or (4) pyrrolidine. Within each subset, two variable factors, i.e. temperature and concentration of $NaNO_2$, were studied. Prior to model-

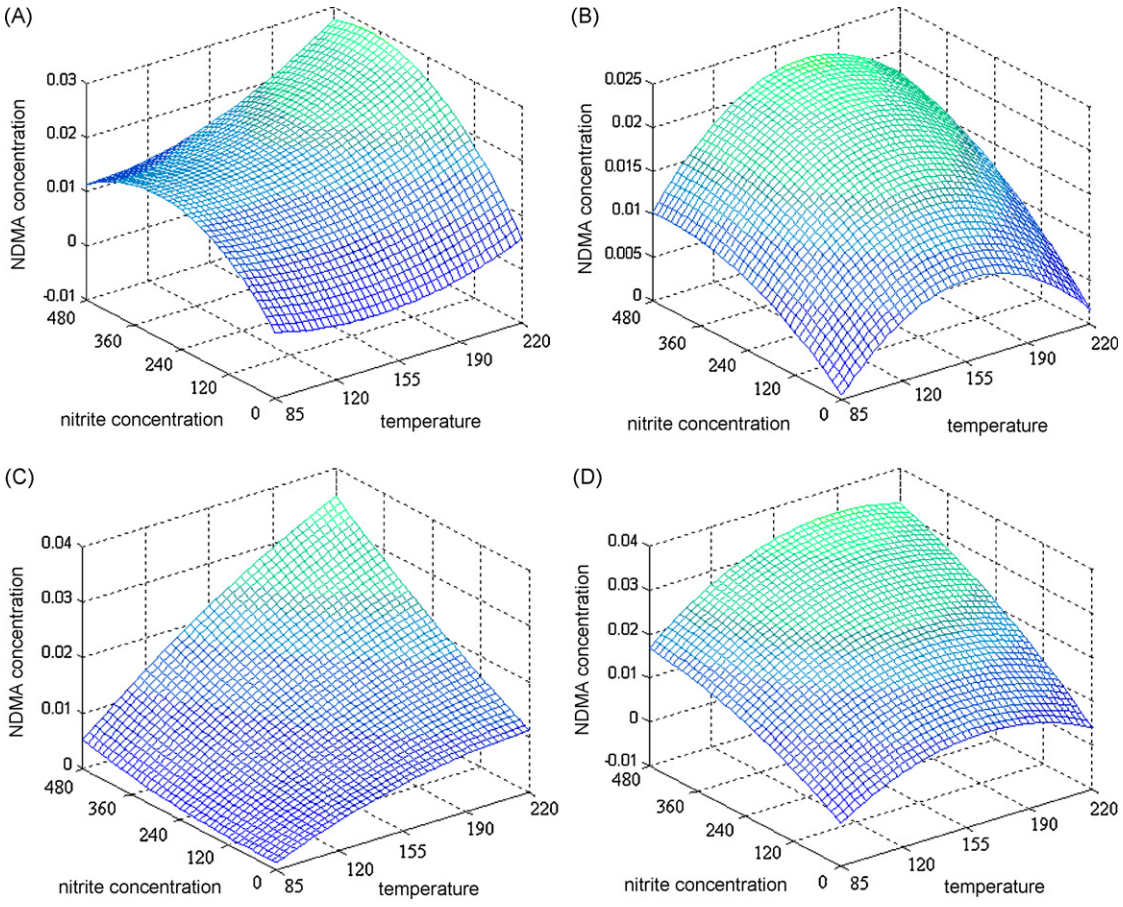


Fig. 4. 3D response surface plots of the NDMA concentration as a function of temperature and nitrite concentration for blank samples (A) and samples fortified with proline (B), hydroxyproline (C), and pyrrolidine (D).

ing, the factor levels were scaled in the interval [−1,1] in order to obtain comparable coefficients in a given model and between different models. Models were built for the four subsets and subjected to ANOVA. The *N*-nitrosamines' models were visualized both as 3D response surface plots and 2D contour plots.

3. Results and discussion

In order to study the presence of NAs in the processed meat products, the cooked ham models fortified either proline, hydroxyproline, or pyrrolidine were compared with the reference models processed out of blank meat samples. Only two *N*-nitrosamines, i.e. NDMA and NPYR, could be detected in a number of samples (Tables 1 and 2) in concentration levels between LOD (i.e. 0.125 μg mL^{-1} for NDMA and NPYR) and the maximal concentrations found for NDMA and NPYR, i.e. 0.751 and 2.472 μg mL^{-1}, respectively. Only a low number of the samples were established with a measured NA concentration above LOQ (Table 2). In this study, the estimated concentrations above LOD but lower than LOQ were therefore included in the data sets as well. Values below LOD were reported as "zero" meaning that each data set still contained a relatively high number of the zero values. Thus, each data set contained a high number of values with a rather high uncertainty. However, the analysis of the data allowed at least to detect tendencies, i.e. to indicate the influential factors and to point out to the most important.

A comparison of data sets (2), (3) and (4) (samples fortified with proline, hydroxyproline, or pyrrolidine, resp.) with data set (1) (blank samples) allowed exposing the importance of meat fortification, temperature and the sodium nitrite concentration in the meat processing. ANOVA tests on these data sets allowed evaluating the impact of the addition of (hydroxy)proline or pyrrolidine on the *N*-nitrosamine formation, and also of the temperature and the nitrite concentration (Tables 3 and 4). The response surfaces illustrate the impact of the temperature and the concentration of $NaNO_2$ on the formation of NDMA and NPYR (Figs. 4 and 5). These surfaces represent the quadratic polynomial model of Eq. (1) fitted to the experimental data:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2 \quad (1)$$

where x_1 and x_2 denote temperature (°C) and concentration of $NaNO_2$ (mg kg^{-1}), respectively, and y holds for the *N*-nitrosamine concentration.

In the blank samples, NDMA was detected more frequently than NPYR, i.e. 59% vs. 12% (Tables 1 and 2). Addition of proline clearly increased the number of detections of NPYR, from 12% to 35%, while addition of pyrrolidine largely increased the number of samples in which NPYR was detected (from 12% to 88%). Addition of (hydroxy)proline or pyrrolidine did not largely increase the number of positive results for NDMA, but when the $NaNO_2$ concentration increases, concentrations of the NA tend to be higher.

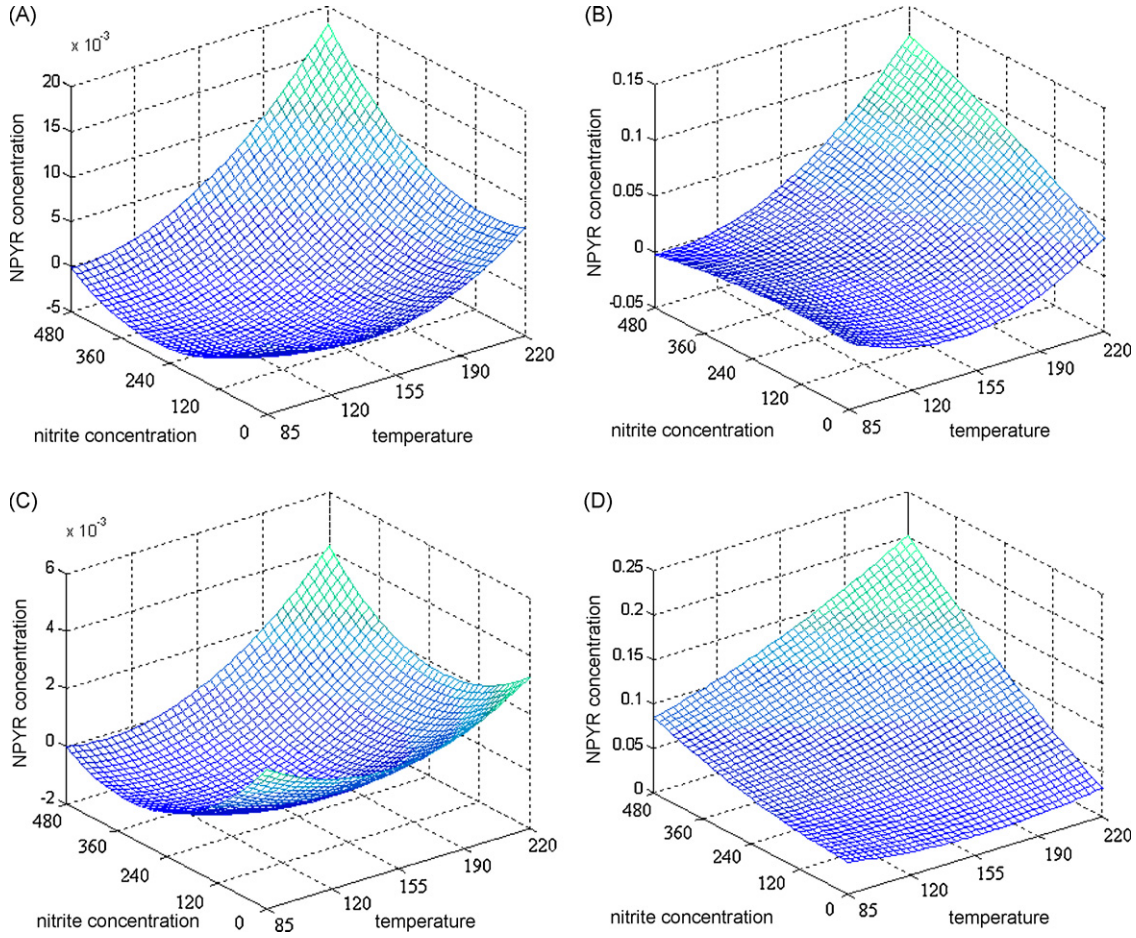


Fig. 5. 3D response surface plots of the NPYR concentration as a function of temperature and nitrite concentration for blank samples (A), and samples fortified with proline (B), hydroxyproline (C), and pyrrolidine (D).

3.1. Evaluating the NDMA formation

The results presented in Fig. 4 and Table 3 indicated that NDMA formation is mainly due to the added $NaNO_2$ concentration and, to a lesser extent, to an elevated temperature, i.e. addition of $NaNO_2$ and an elevated temperature resulted in slightly increased yields of NDMA (Fig. 4). As a consequence, the highest yields of NDMA (between 0.170 and 0.654 $\mu g\,mL^{-1}$) were established, when the meat sample was fortified with 480 $mg\,kg^{-1}$ $NaNO_2$ and processed at highest temperature (220 °C) (Table 1). For temperatures ranging from 85 °C to 120 °C and addition of 120 $mg\,kg^{-1}$ $NaNO_2$, NDMA was detected in a concentration level below LOQ (between 0.125 and 0.354 $\mu g\,mL^{-1}$) (Table 1). From a practical point of view, the NDMA formation is limited when $NaNO_2$ is added in concentration levels within the legally prescribed limits (150 $mg\,kg^{-1}$) and when extreme heating temperatures are avoided.

Addition of pyrrolidine tends to increase the NDMA concentration (borderline significance) (Fig. 4 and Table 3). Addition of hydroxyproline tends to increase the NDMA concentration (borderline non-significance). In comparison with the blank sample, a slight increase of NDMA yield could be observed. In this case, when the amino acids were added, the concentrations of NDMA increased maximally to 0.654 $\mu g\,mL^{-1}$ for hydroxyproline and to 0.751 $\mu g\,mL^{-1}$ for pyrrolidine.

Addition of proline to the meat sample had a different effect (see Fig. 4B and Table 3), as it apparently did not influence the formation of NDMA.

3.2. Evaluating the NPYR formation

For the NPYR formation, only the results of data set (4) and to a lesser extent, of data set (2), could be evaluated. For a discussion of the influence of the factors in the other data sets (1 and 3), the number of the samples with NPYR > LOD was too small (Table 2). For the former data sets, the influence of the $NaNO_2$ concentration and the temperature was analogous to these earlier discussed for NDMA (Fig. 5 and Table 4).

In the blanks and the samples fortified with 1000 $mg\,kg^{-1}$ hydroxyproline, yields below LOD of the NPYR formation were detected. Thus, neither NPYR was formed in the process, nor was it formed in a concentration below the LOD level of the analytical method applied. To make conclusions, this issue should be explored further by supplementary experiments, such as optimization of the analytical procedure towards lower NPYR LOD levels.

The results obtained for the meat samples being fortified with 1000 $mg\,kg^{-1}$ proline or 10 $mg\,kg^{-1}$ pyrrolidine, respectively, demonstrated an impact of these two compounds on the NPYR formation (Fig. 5 and Table 4). Addition of pyrrolidine had a major impact on the NPYR formation. For this *N*-nitrosamine, many more results were detected above LOD and LOQ, and the obtained amounts ranged from 0.151 to 2.472 $\mu g\,mL^{-1}$ (Tables 1 and 2). Contrary to the NDMA formation, the addition of pyrrolidine had a considerably larger influence on the NPYR formation than the $NaNO_2$ concentration (Table 4B). The addition of proline also seemed to affect the NPYR formation, although considerably less than pyrrolidine (Fig. 5 and Table 4B) (maximum established concentration was 1.560 $\mu g\,mL^{-1}$). NPYR was detected in the highest concentrations after addition of 480 $mg\,kg^{-1}$ $NaNO_2$ or when heating at the highest temperature investigated (220 °C).

In Table 4B, all two-factor interactions are shown as significant. For the cases when x_3 (addition or not of pyrrolidine or proline) was involved, it was well perceptible that the values on the *y*-axis (i.e. response) were contained within the range from 0.02 to 0.25 (Fig. 5D), whereas for the blank samples, the *y* values tended to appear very close to zero (Fig. 5A). It is thus evident that an effect of x_1 or x_2 observed in the first situation (addition of amine) is different from that in the second case (blank meat), being, by definition, expressed by the interactions x_1x_3 and x_2x_3.

4. Conclusions

Upon the performed statistical evaluation of the experimental results presented in this study, it could be concluded that higher concentrations of $NaNO_2$ and higher processing temperatures resulted in higher yields of NDMA and NPYR in the processed meat samples. In most cases, the effect of $NaNO_2$ was larger. However, NDMA and NPYR formation does not seem problematic when the concentration level of added $NaNO_2$ is within legally prescribed limits (150 $mg\,kg^{-1}$).

Addition of proline did not affect formation of NDMA, while it had a significant influence on formation of NPYR. Addition of hydroxyproline induced a slight increase of the NDMA yields (although with a borderline non-significance). No influence of hydroxyproline on NPYR formation could be detected. Addition of pyrrolidine induced minor increase of the NDMA yields (also with a borderline significance only), yet it very significantly affected the NPYR yields.

In this study, the importance of proline and pyrrolidine as precursors of the selected *N*-nitrosamines was confirmed. To the contrary, the importance of hydroxyproline, the amino acid which – similar to proline – is one of major constituents of collagen and incorporates a pyrrolidine ring in its structure, was shown as marginal.

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