

EFFECTS OF TEMPERATURE, LENGTH OF STORAGE, AND TECHNOLOGICAL PROCESSES ON THE FORMATION OF N-NITROSAMINES IN LIVER PÂTÉ

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The aim of the study was to determine the possible presence of *N*-nitrosamines in liver pâtés available on the Croatian market and to compare them with a liver pâté from the EU market. In addition, the effects of temperature and length of storage on *N*-nitrosamine concentrations were assessed. A total of 630 samples were randomly chosen. Thirty samples of each were analysed immediately upon sampling, whereas another 30 samples were stored for 5 and 10 days at 4 °C, 22 °C and 37 °C, respectively. In the samples stored at 4 °C, the mean total *N*-nitrosamine level was 1.3–6.8 µg kg⁻¹ on day 5 and 1.0–5.0 µg kg⁻¹ on day 10. In the samples stored at 22 °C, the mean total *N*-nitrosamine level was 3.6–9.3 µg kg⁻¹ (day 0), 11.9–24.5 µg kg⁻¹ on day 5, and 22.7–32.3 µg kg⁻¹ on day 10. In the samples stored at 37 °C, the mean total *N*-nitrosamine level was 104.9–231.1 µg kg⁻¹ (day 5) and 801.3–1329.0 µg kg⁻¹ (day 10). Temperature and length of storage were found to be associated with the formation of *N*-nitrosamines in meat products, but carcinogenic diEt in particular, accidentally or not, was not present at all in the products originating from the EU.

Keywords: *N*-nitrosamines, diEt, meat products, liver pâté, temperature, storage

Meat has a central place in most menus. Irrespective of the original species, meat is a rich source of animal protein, and thus of essential amino acids. Beside protein, dietary meat provides iron, vitamins B₆ and B₁₂, folic acid, etc. Along with the usual methods of preparation, such as boiling and roasting, meat is also found in sausages, air-dried and/or smoked meat, pâté, and other products (EC, 1995).

Since 1920, nitrites and nitrite salts are used in food industry as preservatives in numerous foodstuffs. They are added to meat and meat products to preserve the characteristic red color of meat and specific taste of processed meat, smoked and cured meat products (EC, 1995; BOŠNIR et al., 2003; REPUBLIC OF CROATIA, 2007). On meat or meat product processing, meat proteins (amines) react with the nitrites added, whereby *N*-nitrosamines, demonstrated to be harmful compounds, may develop as a product of nitrosation (LIJINSKY, 1992; EC, 1995; BOŠNIR et al., 2003; REPUBLIC OF CROATIA, 2007). Toxic and potential carcinogenic effects of nitrosamines have been investigated in many studies all over the world (LIJINSKY, 1992; 1999;

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SEEL et al., 1994; GIRARD, 1996; CAMMACK et al., 1999; PIERSON, 2005; HONIKEL, 2008). Nitrosation reactions including the formation of nitrosamines depend on an array of factors, among others, the amount of amines, the amount of nitrites added, pH, temperature, redox potential, and presence of other chemical compounds or agents. Beside their use as preservatives, nitrites may be produced by enzymatic reduction from nitrates (generally present in foodstuffs of plant origin), during processing or with the addition of nitrates to foodstuffs (MASSEY et al., 1991; LIJINSKY, 1992; SCHUDEBOOM, 1993; KNAPP, 2005; EC, 2005; SABRANEK & BACUS, 2007). In addition, cigarette smoking, working and living environment, drinking water, consumer goods (rubber), and even drugs imply exposure to *N*-nitrosamines (TRICKER et al., 1991; LIJINSKY, 1992).

Although the entire process of *N*-nitrosamine development in meat products has not yet been fully elucidated, dried, smoked, salted, and pickled products are considered to be at risk (SEEL et al., 1994; GLORIA et al., 1997; LIJINSKY, 1999). Yet, temperature to which foodstuffs are exposed on preparation by frying, roasting, etc. (GLORIA et al., 1997), or on storage, with special reference to meat products (REPUBLIC OF CROATIA, 2007; DOMANSKA-BLICARZ et al., 2004), is believed to play a major role in the formation of *N*-nitrosamines.

Although studies conducted all over the world have definitely demonstrated the occurrence of nitrosamines in certain foodstuffs, defined the mechanism of their formation to a certain extent and described their toxicity and probable carcinogenicity, there is no consensus (or legislative) in the European Union (EU) on the necessity of systematic monitoring of their concentration in foodstuffs (with a note that it has been used in some EU member countries). Similar situation is found in Croatia. This is even more surprising knowing that both the EU and Croatian legislative include compulsory control of certain common goods, in particular those intended for children (bottle nipples, dummies, nibblers, balloons, etc.) for the presence of *N*-nitrosamines (EN, 2002).

In previous studies, we investigated liver pâté samples from the Croatian market for the presence of *N*-nitrosamines immediately upon sampling and on days 5 and 10 of product storage at temperatures of 4 °C, 22 °C (room temperature), and 37 °C. The measured concentrations of *N*-nitrosamines ranged from 0.8 µg kg⁻¹ to 2997 µg kg⁻¹, and were found to increase with the rising storage temperature as well as with the length of storage at room and higher temperatures. However, this correlation could not be demonstrated due to the small number of study samples (BOŠNIR et al., 2003).

The aim of the present study was to analyse a greater number of canned meat product samples (N=630) and to determine whether temperature and length of storage influence the formation of *N*-nitrosamines in these products. The aim was also to assess the possible differences in *N*-nitrosamine concentrations among various manufacturers, i.e. different manufacture technologies employed in Croatian and EU products, with special reference to *N*-diethylnitrosamine (diEt), classified by IARC in the group of agents probably carcinogenic to humans (group 2A).

1. Material and methods

A total of 630 liver pâté samples from three different manufacturers, two Croatian and one from the EU (Austria), were randomly sampled in the stores. The samples were transferred for analysis to the Laboratory of Food Quality and Safety, Division for Food and Objects of Common Use Analysis, Department of Environmental Health, Dr Andrija Štampar Institute of Public Health, Zagreb, Croatia. Thirty samples from each manufacturer taken from store

shelves (room temperature) were analysed immediately upon receipt to determine the concentrations of nitrites, nitrates, and *N*-nitrosamines, whereas the rest of samples were divided into three groups and stored at different temperatures (4 °C, 22 °C and 37 °C) for 5 and 10 days (Table 1).

Table 1. Sample distribution according to origin, temperature, and length of storage

Origin (N)	4 °C		22 °C		37 °C		
	Day 5	Day 10	Day 0	Day 5	Day 10	Day 5	Day 10
Manufacturer 1 Croatia (210)	30	30	30	30	30	30	30
Manufacturer 2 Croatia (210)	30	30	30	30	30	30	30
Manufacturer 3 EU (Austria) (210)	30	30	30	30	30	30	30

The samples were tins with declared composition:

- manufacturer 1 (Croatia): pork meat tissue, water, pork liver 15%, pork, milk protein, spices, salt, flavor enhancer, preservative
- manufacturer 2 (Croatia): pork meat tissue, water, pork liver 15%, pork, emulsifier (soy protein, lecithin, carrageenan, xanthan gum), salt, spices, preservative
- manufacturer 3 (Austria): pork 36%, bacon, pork liver 25%, water, salt, spices, dextrose, sucrose, emulsifier, flavor enhancer, preservative

Preparation of samples for the determination of nitrite and nitrate: upon weighing the given amount of liver pâté (5 ± 0.01 g), the sample was minced with the addition of 50 ml water and stirred for 15 min. Prepared sample was filtered through filter paper. Then 1 ml is transferred to SPE columns and eluted with 2 ml NaCl solution. Identification and quantification of nitrites and nitrates were done by High Performance Liquid Chromatography-HPLC with DAD detector series 1200 (Agilent).

Preparation of samples for determination of *N*-Nitrosamines: upon weighing the given amount of liver pâté (25 ± 0.01 g), the sample was minced and transferred to a distillation flask with the addition of sodium chloride (NaCl). The sample was distilled by the procedure of classic distillation with water vapor and a certain amount of distillate was collected. Upon distillation, 6N sulfate acid (H_2SO_4) and anhydrous sodium sulfate (Na_2SO_4) were added to the collected distillate and a certain amount of redistillate was collected during the procedure of redistillation. Then, potassium hydroxide (KOH) and sodium chloride (NaCl) were added to the redistillate. Thus prepared, the redistillate was further extracted by the organic solvent dichloromethane in a separating funnel and the collected amount of the extracted sample was evaporated to dryness on a rotavapor. For better identification of compounds and further analytical procedures, the samples were purified by use of SPE columns, whereas the combined gas chromatography/mass spectrometry (GC-MS SIM) method (GC-MS QP 2010 Plus Shimadzu, Tokyo, Japan) was used on quantification of volatile *N*-nitrosamines in study samples. The so-called SIM analytical technique was employed to identify the selected and known analytes. The basic validation parameters included linearity, precision, accuracy, selectivity, limit of detection, and limit of quantification. The level of detection was set at $0.1 \mu\text{g kg}^{-1}$ and the level of quantification at $0.3 \mu\text{g kg}^{-1}$. Total and individual *N*-nitrosamines

were determined, as follows: *N*-diethylnitrosamine (diEt), *N*-ethylmethylnitrosamine (EtMet), *N*-nitrosomorpholine (morpholine), *N*-dipropylnitrosamine (diProp), *N*-nitrosopiperidine (piperidine), and *N*-dibuthylnitrosamine (diBut).

For statistical analysis, descriptive statistics and nonparametric Wilcoxon analysis were used. The level of significance was set at $P < 0.01$. Principal component analysis (PCA) was performed to assess possible differences among the three different manufacturers according to the presence of carcinogenic diEt ($P < 0.05$ was considered statistically significant).

2. Results and discussion

2.1. Results

The results are shown in Tables 2–10. Total nitrite and nitrate concentrations determined in liver pâté samples ranged from 30.7 mg kg^{-1} in the sample originating from Croatia (manufacturer 2) through 68.28 mg kg^{-1} in the samples from Croatia (manufacturer 1) to 90.13 mg kg^{-1} in the sample from the EU (manufacturer 3)

Table 2. Nitrite and nitrate concentrations (mg kg^{-1}) in liver pâté samples

Origin	Average	\pm SD	Range
Manufacturer 1 (N=30)			
NaNO ₂	3.03	0.18	2.73–3.22
NaNO ₃	65.25	4.05	58.7–72.1
Total	68.28	2.11	2.73–72.1
Manufacturer 2 (N=30)			
NaNO ₂	4.92	0.32	4.21–5.39
NaNO ₃	25.78	1.79	23.8–27.6
Total	30.7	1.05	4.21–27.6
Manufacturer 3 (N=30)			
NaNO ₂	37.16	0.62	36.5–38.4
NaNO ₃	52.97	0.71	52.2–54.1
Total	90.13	0.66	36.5–54.1

Total nitrosamine concentrations determined in liver pâté samples stored at 4°C ranged from $1.3 \text{ }\mu\text{g kg}^{-1}$ in the sample originating from the EU (manufacturer 3) to $6.4 \text{ }\mu\text{g kg}^{-1}$ and $6.8 \text{ }\mu\text{g kg}^{-1}$ in the samples from Croatian manufacturers on day 5 of storage, and from $1.0 \text{ }\mu\text{g kg}^{-1}$ in the sample from EU manufacturer to $3.8 \text{ }\mu\text{g kg}^{-1}$ and $5.0 \text{ }\mu\text{g kg}^{-1}$ in the samples from Croatian manufacturers on day 10 of storage (Tables 3 and 6). Nitrosamine concentrations did not rise with storage at 4°C .

Total nitrosamine concentrations determined in liver pâté samples stored at 22°C ranged from $3.6 \text{ }\mu\text{g kg}^{-1}$ in the sample from manufacturer 2 to $5.0\text{--}8.3 \text{ }\mu\text{g kg}^{-1}$ on day 0, from $11.9 \text{ }\mu\text{g kg}^{-1}$ through $15.7 \text{ }\mu\text{g kg}^{-1}$ to $24.5 \text{ }\mu\text{g kg}^{-1}$ on day 5, and from $25.2 \text{ }\mu\text{g kg}^{-1}$ to $32.3 \text{ }\mu\text{g kg}^{-1}$ on day 10. Obviously, nitrosamine concentrations exceeded those recorded in samples stored at 4°C , having gradually increased at room temperature (Tables 4 and 6).

Table 3. N-nitrosamine concentrations ($\mu\text{g kg}^{-1}$) in liver pâté samples according to origin and storage at 4 °C

Origin	Length of storage (days)					
	Day 5			Day 10		
	X	Range	\pm SD	X	Range	\pm SD
Manufacturer 1 (N=30)						
diEt	1.1	0.2–1.1	0.9	1.1	0.2–2.3	0.7
EtMet	0.9	0.2–1.6	0.4	0.8	0.6–1.1	0.2
Morpholine	0.9	0.2–1.9	0.5	0.8	0.5–1.2	0.2
diProp	1.9	0.2–4.8	1.4	1.4	0.2–5.5	1.3
Piperidine	1.0	0.2–3.3	0.9	0.9	0.2–3.0	0.8
diBut	0.6	0.5–0.7	0.1	ND	–	–
Total	6.4	0.2–13.4	0.7	5.0	ND–13.1	0.6
Manufacturer 2 (N=30)						
diEt	1.5	0.2–5.0	1.5	1.5	0.2–2.9	0.9
EtMet	1.4	0.2–4.9	1.0	ND	–	–
Morpholine	1.8	0.4–5.1	1.5	0.7	0.2–2.7	0.6
diProp	1.9	0.3–4.9	1.3	1.3	0.2–3.8	1.1
Piperidine	0.2	0.2–0.4	0.1	0.3	0.2–1.0	0.2
diBut	ND	–	–	ND	–	–
Total	6.8	ND–20.3	1.1	3.8	ND–10.4	0.7
Manufacturer 3 (N=30)						
diEt	ND	–	–	ND	–	–
EtMet	0.5	0.2–2.9	0.2	0.3	0.2–0.6	0.1
Morpholine	0.2	0.2–0.2	0.0	0.2	0.2–0.2	0.0
diProp	ND	–	–	ND	–	–
Piperidine	0.2	0.2–0.3	0.0	0.2	0.2–0.2	0.0
diBut	0.4	0.2–0.7	0.1	0.3	0.2–0.6	0.1
Total	1.3	ND–2.1	0.1	1.0	ND–1.6	0.05

Total nitrosamine concentrations determined in liver pâté samples stored at 37 °C ranged from 104.9 $\mu\text{g kg}^{-1}$ (manufacturer 3 from the EU) through 231.1 $\mu\text{g kg}^{-1}$ to 304.4 $\mu\text{g kg}^{-1}$ on day 5, and from 801.3 $\mu\text{g kg}^{-1}$ through 1207.2 $\mu\text{g kg}^{-1}$ to 1329.0 $\mu\text{g kg}^{-1}$ on day 10, having obviously increased with the length of exposure to this temperature level (Tables 5 and 6).

Statistical significance of differences among nitrosamine concentrations measured at different temperatures and length of storage was assessed by nonparametric Wilcoxon analysis. The level of significance was set at $P < 0.01$.

The effect of temperature with constant length of storage is illustrated in Tables 7 and 8. Analysis of results on the measured concentrations of diEt, EtMet, morpholine, diProp, piperidine, and diBut in liver pâté samples from three different manufacturers showed differences in their concentrations according to different temperature levels at the same length of storage to be statistically significant for diEt, EtMet and morpholine ($P < 0.01$), with

the exception of carcinogenic diEt concentrations in the samples from the EU manufacturer (Austria), which were below the limit of detection. In case of diProp, piperidine, and diBut, differences were less pronounced, especially with shorter (day 5) length of storage.

Table 4. N-nitrosamine concentrations ($\mu\text{g kg}^{-1}$) in liver pâté samples according to origin and storage at 22 °C

Origin	Length of storage (days)								
	Day 0			Day 5			Day 10		
	X	Range	$\pm\text{SD}$	X	Range	$\pm\text{SD}$	X	Range	$\pm\text{SD}$
Manufacturer 1 (N=30)									
diEt	ND	–	–	0.4	0.1–1.9	0.5	0.8	0.2–2.9	0.8
EtMet	3.7	0.5–5.8	1.1	5.4	0.8–8.5	2.1	9.2	1.1–39.1	7.2
Morpholine	1.0	0.4–2.8	0.6	2.7	0.7–5.8	1.6	4.9	0.8–9.6	2.5
diProp	2.2	0.8–4.9	1.5	4.2	0.9–8.1	2.1	5.6	1.1–9.9	2.4
Piperidine	1.4	0.6–8.2	1.6	3.0	0.4–8.9	2.0	4.7	0.9–9.5	2.3
diBut	ND	–	–	ND	–	–	ND	–	–
Total	8.3	ND–21.7	1.2	15.7	ND–33.2	1.7	25.2	ND–71.0	3.0
Manufacturer 2 (N=30)									
diEt	0.6	0.3–0.8	0.2	1.4	0.6–7.8	1.4	3.0	0.8–9.4	1.7
EtMet	1.1	0.5–2.0	0.4	3.0	0.9–7.7	1.8	5.9	1.9–11.8	3.0
Morpholine	0.7	0.4–1.4	0.2	4.0	0.5–8.9	2.6	6.7	0.9–11.2	2.7
diProp	0.6	0.3–0.8	0.1	2.0	0.6–3.9	1.1	3.7	1.1–6.1	1.6
Piperidine	0.6	0.3–1.3	0.2	1.5	0.5–4.4	1.1	3.4	1.2–11.8	2.0
diBut	ND	–	–	ND	–	–	ND	–	–
Total	3.6	ND–6.3	0.2	11.9	ND–32.7	1.6	22.7	ND–50.3	2.2
Manufacturer 3 (N=30)									
diEt	ND	–	–	ND	–	–	ND	–	–
EtMet	1.6	0.8–3.9	1.0	12.2	8.0–20.2	3.7	17.5	9.8–24.5	4.3
Morpholine	0.2	0.1–0.9	0.2	0.6	0.1–2.7	0.6	0.8	0.1–1.6	0.4
diProp	ND	–	–	ND	–	–	0.7	0.2–4.0	0.7
Piperidine	0.2	0.1–0.3	0.1	0.2	0.1–0.6	0.1	0.4	0.2–1.0	0.2
diBut	3.0	0.8–9.9	2.8	11.5	3.6–34.3	6.3	12.9	8.1–21.2	3.7
Total	5.0	ND–15	1.0	24.5	ND–57.8	2.7	32.3	ND–52.3	1.9

The effect of the length of storage at constant temperature is shown in Tables 9–10. Result analysis revealed statistically significant differences in nitrosamine concentrations according to different length of storage at 4 °C, 22 °C, and 37 °C, with the exception of diBut, which showed no storage time dependence or the measured concentrations were below the level of detection (Tables 9–10). It should be noted again that measurable concentrations of the carcinogenic diEt could not be determined in the samples originating from the EU.

Table 5. N-nitrosamine concentrations ($\mu\text{g kg}^{-1}$) in liver pâté samples according to origin and storage at 37 °C

Origin	Length of storage (days)					
	Day 5			Day 10		
	X	Range	\pm SD	X	Range	\pm SD
Manufacturer 1 (N=30)						
diEt	106.7	61.2–283.7	49.6	488.9	98.9–774.8	175.6
EtMet	43.2	15.7–80.8	20.1	107.9	28.2–959.1	178.2
Morpholine	143.1	69.3–303.4	64.6	665.5	235.8–996.4	234.5
diProp	5.2	0.5–10.7	2.7	43.1	4.9–328.1	77.1
Piperidine	6.2	1.3–10.2	2.3	23.6	4.9–199.9	39.6
diBut	ND	–	–	ND	–	–
Total	304.4	ND–769.6	27.9	1.329.0	ND–5.540.8	141.0
Manufacturer 2 (N=30)						
diEt	41.0	18.9–89.5	18.4	383.6	2.4–887.8	250.6
EtMet	56.2	1.2–108.3	23.6	291.6	18.4–789.1	197.3
Morpholine	57.1	1.8–109.3	26.9	221.1	11.8–585.1	140.7
diProp	44.1	2.1–113.0	30.9	158.1	67.1–343.9	59.2
Piperidine	26.7	3.6–80.8	22.6	126.6	47.1–323.3	78.8
diBut	6.0	2.4–8.6	2.3	26.2	2.9–77.0	30.0
Total	231.1	1.2–509.5	21.0	1.207.2	2.4–2.682.9	126.1
Manufacturer 3 (N=30)						
diEt	ND	–	–	ND	–	–
EtMet	68.7	16.2–117.6	24.6	567.1	103.9–876.6	219.4
Morpholine	9.5	2.8–41.3	6.9	161.4	0.8–899.0	178.5
diProp	5.3	1.6–9.0	1.7	32.6	1.2–289.8	49.9
Piperidine	1.8	0.8–5.8	1.2	7.6	1.9–20.1	3.6
diBut	19.6	9.0–31.0	3.6	32.6	20.3–39.9	6.1
Total	104.9	ND–204.7	7.6	801.3	ND–2.125.4	91.5

Table 6. Mean concentrations of total N-nitrosamines ($\mu\text{g kg}^{-1}$) in liver pâté samples according to origin, temperature, and length of storage

Origin	Length of storage (days)								
	Day 0			Day 5			Day 10		
	Temperature (°C)			Temperature (°C)			Temperature (°C)		
	4	22	37	4	22	37	4	22	37
Manufacturer 1 (N=30)	–	8.3 \pm 1.2	–	6.4 \pm 0.7	15.7 \pm 1.7	304.4 \pm 27.9	5.0 \pm 0.6	25.2 \pm 3.0	1.329.0 \pm 141.0
Manufacturer 2 (N=30)	–	3.6 \pm 0.2	–	6.8 \pm 1.1	11.9 \pm 1.6	231.1 \pm 21.0	3.8 \pm 0.7	22.7 \pm 2.2	1.207.2 \pm 126.1
Manufacturer 3 (N=30)	–	5.0 \pm 1.0	–	1.3 \pm 0.1	24.5 \pm 2.7	104.9 \pm 7.6	1.0 \pm 0.05	32.3 \pm 1.9	801.3 \pm 91.5

Table 7. Wilcoxon analysis of the effect of storage temperature on individual N-nitrosamine concentrations in Manufacturers 1-2 products (origin: Croatia)

Length of storage	Day 5			Day 10		
	4 °C	22 °C	37 °C	4 °C	22 °C	37 °C
Manufacturer 1						
diEt	Yes P=0.000024*	No	No P=0.000011*	No P=0.45	Yes	Yes P=0.000007*
EtMet	Yes P=0.000002*	Yes	Yes P=0.000002*	Yes P=0.000002*	Yes	Yes P=0.000002*
Morpholine	Yes P=0.000009*	Yes	Yes P=0.000002*	Yes P=0.000002*	Yes	Yes P=0.000002*
diProp	Yes P=0.00020*	No	No P=0.198	Yes P=0.000003*	Yes	Yes P=0.00008*
Piperidine	Yes P=0.00009*	No	No P=0.038	Yes P=0.000005*	Yes	Yes P=0.0006*
diBut	No P=0.043	No	No P=0.043	Null values		
Manufacturer 2						
diEt	No P=0.936	Yes	Yes P=0.000005*	Yes P=0.000018*	Yes	Yes P=0.000003*
EtMet	Yes P=0.00074*	Yes	Yes P=0.000004*	Yes P=0.000002*	Yes	Yes P=0.000002*
Morpholine	Yes P=0.00107*	Yes	Yes P=0.000002*	Yes P=0.000002*	Yes	Yes P=0.000002*
diProp	No p=0.370	Yes	Yes P=0.000004*	Yes P=0.000024*	Yes	Yes P=0.000004*

*Statistically significant difference

Table 8. Wilcoxon analysis of the effect of storage temperature on individual N-nitrosamine concentrations in Manufacturer 3 products (origin: EU)

Length of storage	Day 5			Day 10		
	4 °C	22 °C	37 °C	4 °C	22 °C	37 °C
diEt	Null values		Null values	Null values		Null values
EtMet	Yes P=0.000002	Yes	Yes P=0.000002*	Yes P=0.000002*	Yes	Yes P=0.000002*
Morpholine	Yes P=0.00005	Yes	Yes P=0.00003*	Yes P=0.000012*	Yes	Yes P=0.000002*
diProp	Null values		Yes P=0.000002*	Yes P=0.000003*	Yes	Yes P=0.000002*
Piperidine	No P=0.021	Yes	Yes P=0.000002*	Yes P=0.000002*	Yes	Yes P=0.000002*
diBut	Yes P=0.000005*	No	No P=0.097	Yes P=0.00005*	Yes	Yes P=0.0024*

*Statistically significant difference

Table 9. Wilcoxon analysis of the effect of the length of storage on individual N-nitrosamine concentrations in Manufacturers 1-2 products (origin: Croatia)

Temperature	4 °C		22 °C		37 °C		
Day	5	10	0	5	10	5	10
Manufacturer 1							
diEt	Yes P=0.0001*		Yes P=0.0017*	Yes P=0.00019*		Yes P=0.000012*	
EtMet	Yes P=0.000003*		Yes P=0.000002*	Yes P=0.000031*		Yes P=0.000003*	
Morpholine	Yes P=0.000005*		Yes P=0.000004*	Yes P=0.000003*		Yes P=0.000002*	
diProp	Yes P=0.0019*		Yes P=0.000006*	Yes P=0.000002*		Yes P=0.000004*	
Piperidine	No P=0.148		Yes P=0.000006*	Yes P=0.00004*		Yes P=0.000018*	
diBut	No P=0.043		Null values			Null values	
Manufacturer 2							
diEt	Yes P=0.000044*		Yes P=0.000005*	Yes P=0.000004*		Yes P=0.000006*	
EtMet	Yes P=0.000002*		Yes P=0.000003*	Yes P=0.000002*		Yes P=0.000002*	
Morpholine	Yes P=0.00018*		Yes P=0.000005*	Yes P=0.000003*		Yes P=0.000002*	
diProp	Yes P=0.00041*		Yes P=0.00002*	Yes P=0.000004*		Yes P=0.000004*	
diBut	Null values		Null values			No P=0.011	

*Statistically significant difference

Table 10. Wilcoxon analysis of the effect of the length of storage on individual N-nitrosamine concentrations in Manufacturer 3 products (origin: EU)

Temperature	4 °C		22 °C		37 °C			
Day	5	10	0	5	10	5	10	
diEt	Null values		Null values		Null values		Null values	
EtMet	Yes P=0.00008*		Yes P=0.000002*	Yes P=0.000002*		Yes P=0.000002*		
Morpholine	No P=0.108		Yes P=0.000004*	No p=0.027		Yes P=0.000003*		
diProp	Null values		Null values		Yes P=0.000003*	Yes P=0.000003*		
Piperidine	Yes P=0.00029*		Yes P=0.0012*	Yes P=0.000024*		Yes P=0.000002*		
diBut	No P=0.031		Yes P=0.000008*	No P=0.058		No P=0.0135		

*Statistically significant difference

PCA was performed to identify similarities or differences in the occurrence of carcinogenic diEt among the three manufacturers. Results of PCA with Varimax rotation yielded three groups of data on diEt concentrations (Fig. 1). Storage temperature of 37 °C and 22°C had the greatest impact on pâté samples from Croatian manufacturer 1 (HR-1) and Croatian manufacturer 2 (HR-2), respectively, whereas carcinogenic diEt was not detected in any pâté sample from the EU manufacturer 3 (A) at any temperature or length of storage. Beside data grouping into three groups according to temperature and length of storage, PCA enabled detection of outlier samples, e.g., HR1-24 with highest diEt concentration among HR-1 samples (774.8 µg kg⁻¹) and HR2-19 with very high diEt concentration among HR-2 samples (887.8 µg kg⁻¹).

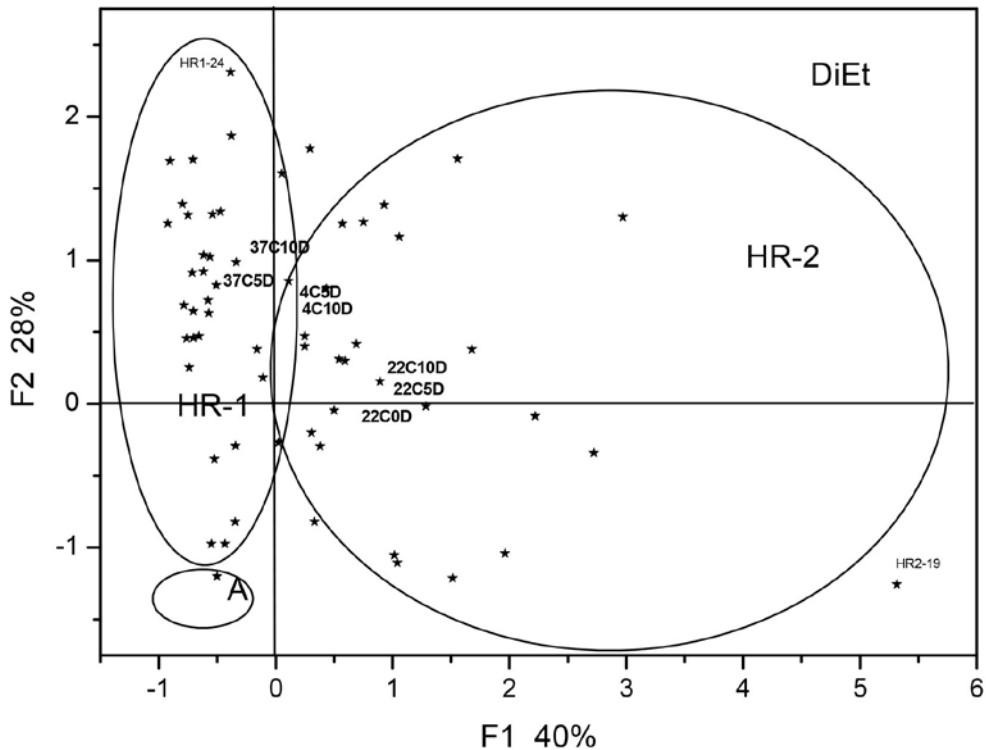


Fig. 1. Principal component analysis (PCA) of carcinogenic diEt in pâté samples from three manufacturers. HR-1: Manufacturer 1, origin: Croatia; HR-2: Manufacturer 2, origin: Croatia; A: Manufacturer 3, origin: EU; ($p < 0.05$ was considered statistically significant)

2.2. Discussion

The aim of the present study that included a large number of liver pâté samples from two Croatian manufacturers and one EU (Austria) manufacturer was to determine the possible occurrence and concentrations of *N*-nitrosamines, in particular carcinogenic diEt, and to assess differences in nitrosamine concentrations according to different manufacture

technology, temperature and length of storage. In addition, our intention was to point to the need of legal regulations in this field and of continuous monitoring of these products because user exposure to carcinogenic products is by no means an issue that could be neglected. This aspect is additionally emphasized by the results of our study performed nearly ten years ago, which revealed that these products are generally kept on store shelves at room or even higher temperature. Therefore, it is necessary at least to modify public opinion and awareness of the manufacturers, salesmen, and users of the storage conditions required for meat products to which nitrites and nitrates have been added as preservatives (BOŠNIR et al., 2003).

In Croatia, it is a widely accepted opinion that a meat product remains safe irrespective of ambient temperature if canned in metal or aluminum can, which was convincingly denied by our 2003 study (BOŠNIR et al., 2003). According to the widely accepted public opinion, only pâté products packed in artificial or natural casing are considered unsafe if not stored in a refrigerator. Unfortunately, nothing has changed since then.

The European legislation prescribes the maximum residue levels (0.002 mg kg^{-1}) while in Croatia there is no MRL levels for concentrations of *N*-nitrosamines or legal obligation to control such products for the presence of *N*-nitrosamines, there is no continuous monitoring on the market, not even fundamental temperature conditions for storage of these products at warehouses and stores are respected; the more so, we found the same products from different manufacturers to vary considerably in their response to these conditions.

Thirty of these samples were analysed immediately after sampling and delivery to the laboratory. The results showed that none of the analysed sample was identified by presence of selected nitrosamines in quantities greater than MRL levels (0.002 mg kg^{-1}).

The product from one Croatian manufacturer was highly susceptible to room temperature ($22 \text{ }^{\circ}\text{C}$) and therefore should not be stored and exposed to this temperature because of the *N*-nitrosamine concentrations increasing with time, with special reference to the statistically significant rise in the concentration of the carcinogenic diEt. For example, this may refer to some stores cooling the ambient temperature down to $20\text{--}22 \text{ }^{\circ}\text{C}$ in summer. The product from the other Croatian manufacturer was highly sensitive to the temperature of $37 \text{ }^{\circ}\text{C}$, suggesting a high risk if kept in small stores without any air conditioning in summer or in stores with low turnover of such products, thus the products being stored for weeks at temperatures as high as $30 \text{ }^{\circ}\text{C}$. That is why samples with very high diEt levels were now found in pâté products from both of these manufacturers ($774.8 \text{ } \mu\text{g kg}^{-1}$ and $887.8 \text{ } \mu\text{g kg}^{-1}$).

Accidentally or not, diEt was not detected in any of the pâté samples from the EU at any temperature or length of storage even with higher amount of nitrites added, probably indicating better manufacture technology, quality input material, quality of the additive, which are considered to influence the formation of *N*-nitrosamines. As high amounts of nitrites are known to result in high *N*-nitrosamine concentrations, the amount of nitrites added as preservative is strictly limited (SCHUDEBOOM, 1993; DOMANSKA-BLICHARZ et al., 2004; PEGG & SHAHIDI, 2004; KNAPP, 2005). However, our study revealed inverse findings, as the mean nitrite concentrations were higher in the samples originating from EU (range $36.5\text{--}38.4 \text{ mg kg}^{-1}$) as compared with all Croatian samples (range $2.73\text{--}5.39 \text{ mg kg}^{-1}$). This can be explained by the fact that Austrian samples were stored at $4 \text{ }^{\circ}\text{C}$ on the shelves before sampling while Croatian samples were stored at room temperature which obviously affects the results in the formation of *N*-nitrosamines.

Two studies similar to our present and previous ones were conducted in Poland (DOMANSKA & KOWALSKI, 2002; DOMANSKA-BLICHARZ et al., 2004). Both studies found positive correlation between the amount of nitrites added and the development of *N*-nitrosamines in

some traditional Polish dishes and, comparable to our study, increased nitrite and *N*-nitrosamine concentrations with prolonged storage, especially at room temperature (DOMANSKA & KOWALSKI, 2002; DOMANSKA-BLICHARZ et al., 2004). It should be noted that variation in nitrite and *N*-nitrosamine concentrations in particular foods were ascribed to different recipes, in particular traditional products, where various plant spices, such as red pepper, onion, garlic, hot pepper, etc., are added, which contain certain nitrate levels known to be a potential precursor for the formation of nitrites and consequently *N*-nitrosamines.

Studies of safety and risk of *N*-nitrosamine development in various foodstuffs were carried out in some EU member countries (PENTTILÄ et al., 1990; TRICKER et al., 1991; REINIK et al., 2005;). In Estonia, two studies investigated the intake of nitrites, nitrates, and *N*-nitrosamines in more than 300 samples of commercial smoked/cured meat products from 21 and then again from 16 Estonian manufacturers. The levels of nitrites and nitrates did not exceed maximal allowable concentration in any of the study samples, while elevated concentrations of individual *N*-nitrosamines were recorded in 9% of the study samples. The concentrations of *N*-nitrosamines, nitrites, and nitrates have been on a decrease over years owing to the systematic control of the presence of additives and contaminants performed in Estonia since 1998 (REINIK et al., 2005).

Similar analyses were carried out in two projects in Germany, which included more than 3000 food samples and 38 drink samples. Individual *N*-nitrosamine concentrations greater than $5 \mu\text{g kg}^{-1}$ were detected in 6% of the study products. Interestingly, almost one-third of *N*-nitrosamine intake for men derived from beer drinking (TRICKER et al., 1991).

The impact of nitrite, nitrate, and *N*-dimethylnitrosamine (diMet) intake on the risk of gastrointestinal tumor development was investigated in Finland. There was no significant association between nitrite and nitrate intake and tumor development, or between diMet intake and gastric tumor; however, *N*-nitroso compounds were generally found to have a potential of inducing gastrointestinal tumors. It is known since the 1970s that *N*-nitroso compounds can be synthesized in the body by *in vivo* digestion in the acidic gastric medium and/or by bacterial activity while passing through the gastrointestinal tract (PENTTILÄ et al., 1990).

Some studies tried to point to the possible manufacture of natural, 'organic' meat products, in which the nitrites and nitrates added as preservatives would be substituted by the additives, such as lemon juice, black cherry powder, celery powder, rosemary, etc. (SABRANEK & BACUS, 2007). However, apart from initially promising results, this concept suffered from at least two shortcomings. First one was the need to ensure microbiological safety of meat products, in particular inhibition of the development of the *Clostridium botulinum* bacterium as a causative agent of life-threatening poisoning with preserved foodstuffs in humans, which could not be warranted and controlled by adding 'natural' nitrites and nitrates from the above 'ecologic' additives. The process of nitrate transformation to nitrites depends on a number of factors, such as pH, thermal conditions, unwanted and unexpected chemical reactions, etc.; therefore, it would be hard to determine the optimal yet adequate amount of such additives. The other problem referred to the required (minimal) amount, i.e. dosage, of natural additives relative to temperature and length of product storage, which is very difficult or even impossible to identify (SABRANEK & BACUS, 2007).

On the other hand, nitrites and nitrates are potent, inexpensive, and widely available preservatives, only care should be taken that the amounts used be balanced against the known risks, along with continuous education of the manufacturers, salesmen, and users.

3. Conclusion

Study results confirmed the hypothesis on the causal relationship between the increase in *N*-nitrosamine concentration and the temperature and length of storage in the majority of liver pâté samples analysed. Our findings imply the need of legal regulations in the field and systematic control of the processes of manufacture, processing, sale, and storage of these products. It is of utmost importance to modify the manufacture technology of meat products in order to reduce the use of nitrites and nitrates, thus reducing the possible formation of *N*-nitrosamines and their presence in meat products. Storage conditions should also be modified because proper storage was demonstrated to contribute most to gradual reduction of nitrite and nitrate usage as well as to the consequential reduction of *N*-nitrosamine concentrations in meat products.

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