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Volatile *N*-Nitrosamines in various fish products

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Abstract

N-Nitrosamines (NAs) are a group of carcinogens which have been detected in various fish products. In this study the level of five NAs (*N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosodibutylamine, *N*-nitrosopiperidine, and *N*-nitrosopyrrolidine) was determined in 294 various samples of fish, and in 77 samples of oil during 2001–2005. For the sample cleaning the two-step solid-phase extraction with Extrelut and Florisil sorbents was used. NAs were separated by gas chromatography and detected by positive-ion chemical ionization using ammonia as reagent gas. The HP 6890 Plus GC/HP 5973 MSD was used in the selected ion monitoring mode with pulsed splitless injection. In this work, the limit of detection and the limit of quantitation of NA were approximately 0.10 and 0.35 μg/kg, respectively with about 85%. The sum of the average of five NAs content in cold-smoked fish was found to be 1.92 μg/kg, in hot-smoked fish – 4.36 μg/kg, in fried fish – 8.29 μg/kg, in pickled fish – 5.37 μg/kg, in salted fish – 3.16 μg/kg, in salted/dried fish – 3.81 μg/kg, and in the fresh fish it was not detected.

Keywords: *N*-Nitrosamines; Positive-ion chemical ionization; Gas chromatography; Mass spectrometry; Fish products

1. Introduction

N-Nitrosamines (NAs) are potent carcinogens that can induce tumours in various animal species. Humans are exposed to NAs both from diet and other environmental sources as well as from endogenous synthesis within the body. *N*-nitrosodimethylamine (NDMA) is the most commonly encountered volatile NA in food samples (Rubenchik, 1990). In 1978 the International Agency for Research on Cancer (IARC) classified a number of NAs with respect to the cancer risk for humans. The IARC considers NDMA and *N*-nitrosodiethylamine (NDEA) into the group of probably carcinogenic to human, and *N*-nitrosodibutylamine (NDBA), *N*-nitrosopiperidine (NPIP) and *N*-nitrosopyrrolidine (NPYR) into the group of possibly carcinogenic to human (IARC, 1978).

The main precursors of NDMA in the fish are believed to be dimethylamine, diethylamine, trimethylamine, and trimethylamine oxide all of which are abundant in various fish, especially marine fish. The presence of other secondary amines has not yet been established. However, since dimethylamine is a strongly basic amine, its rate of nitrosation is very low, and that for the trimethylamine and trimethylamine oxide are even slower (Groenen et al., 1982; Sen, Tessier, Seaman, & Baddoo, 1985). NAs are formed by the reaction of nitrogen oxides with, mainly, secondary amines present in the fish. The nitrogen oxides are generated from nitrites, and they are also present in wood smoke. The concentration of amines in the fish products depends on various factors such as species, age, environment, bacterial flora, and storage conditions. Amines are usually present in larger quantities in marine fish than in

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fresh-water fish. It is, therefore, difficult to predict whether a particular sample of fish, especially a marine fish, will be free of these amines, and be safe to treat with nitrite (Sen, Smith, Schwinghamer, & Howsam, 1970; Sen et al., 1985; Tricker & Preussmann, 1991).

NAs occur as contaminants in different food categories and beverages including cured meat (Biaudet, Mavelle, & Debry, 1994; Cornee, Lairon., Velema, Guyader, & Berthezene, 1992; Sen & Seaman, 1981a, 1981b), vegetable oil (Fiddler, Pensabene, & Kimoto, 1981; Hedler, Schurp, & Marquardt, 1979; Sen & Seaman, 1981a, 1981b), cheese (Dellisanti, Cerutti, & Airoldi, 1996), drinking water (Jenkins et al., 1995), and beer (Sen, Seaman, Begeson, & Brousseau, 1996; Yurchenko & Mölder, 2005). Also the fish has been studied and NAs have been detected in smoked fish (Fazio, Damico, Howard, White, & Watts, 1971; Gadbois, Ravesi, Lundstrom, & Maney, 1975; Howard, Fazio, & Watts, 1970), in fish meal (Sen, Schwinghamer, Donaldson, & Miles, 1972), in the fish cooked by natural gas and electricity (Key, Bayly, Massey, & McWeeny, 1982), and in marine salted fish (Fong & Chan, 1973; Huang, Ho, Webb, Wood, & Gough, 1981; Zou, Lu, & Liu, 1994).

In the literature, methods for sample preparation are based on vacuum distillation and steam distillation (Sen et al., 1970), mineral oil distillation (Pedersen & Meyland, 1981), celite column extraction method (Österdahl, 1988), and solid-phase extraction (Raoul, Gremaud, Biaudet, & Tureski, 1997).

The determination of volatile NAs in the fish has been carried out by different analytical methods, including gas chromatography (Fazio et al., 1971) and gas–liquid chromatography with Thermal Energy Analyzer (Howard et al., 1970; Pensabene, Fiddler, & Phillips, 1990; Sen et al., 1985; Zou et al., 1994) or with a Coulson electrolytic conductivity detector (Sen et al., 1972), and gas chromatography high resolution mass spectrometry (Huang et al., 1981). Positive-ion chemical ionization mass spectrometry with methane (Fish, Holmstead, & Gaffield, 1976; Fong, Ding, & Liu, 1984; Gadbois et al., 1975) or ammonia (Prest & Hermann, 1999) as reagent gases has been used to differentiate between volatile NAs.

Allowed content of NAs in fish and fish products is limited in Estonia: maximum permitted concentration of the sum of two NAs (NDMA and NDEA) in fresh and smoked fish is 3 μg/kg (The Decision of the Government of the Republic of Estonia, 2000). The purpose of the present study was to measure the content of volatile NAs in Estonian fish products using the method of sample preparation performed in our previous work (Jurtchenko, Tenno, Mölder, & Reinik, 2002), applying mass spectrometry for determination of NAs in the fish, and to investigate influence of cooking temperature on the growth of volatile NAs in the fish.

2. Method

2.1. Samples

All samples of fish and oil were purchased from supermarkets in Estonia. Information on the smoking conditions, the species and kind of the fish, was collected for each sample and are presented in Table 1. The skin and the bone of fish were removed, and the sample was crushed and mixed with no addition of fluid. As an exception, sprats in vegetable oil, sprats and herring in tomato sauce were used skin- and bone-on. The samples of fish and oil were stored at −20 and 4 °C before analysis, respectively.

2.2. Chemicals

For the sample preparation were purchased methanol from J.T. Baker (Holland), dichloromethane from Sigma–Aldrich (USA), hexane from Rathburn (Scotland), 0.1 N NaOH solution from Chemapo (Czechoslovakia), Extrelut from Merck (Germany), and Florisil 100/200 from Alltech (Belgium). NDMA, NDEA, NDBA, NPIP and NPYR in methanol were commercial products from Aldrich. Mixtures were stored at −20 °C and analyzed at room temperature. Helium (99. 9996%) was used for gas chromatographic (GC) analysis.

2.3. Sample preparation

For the fish and oil samples preparation the method described in our previous work, was used (Jurtchenko et al., 2002). Two-step solid-phase extraction with Extrelut and Florisil sorbent was used for the sample cleaning. The fish sample (6.0 ± 1.0 g in 100 mL glass beaker) or the oil sample (10 ± 1.0 mL) was mixed with 0.1 N NaOH (6 mL).

As the first step, about 6 g of Extrelut was placed at the bottom of the glass column (30 cm × 1.5 cm) and wetted with 20 mL hexane/dichloromethane 40:60 (v:v). After that the sample was eluted with two 20 mL portions of hexane/dichloromethane solution. The eluate was collected in a 50-mL concentrator flask and evaporated in water bath at 60 °C. As the second step, about 1 g Florisil was placed at the bottom of the Florisil cartridge (6.5 cm × 1.3 cm), wetted with 6 mL dichloromethane/methanol 95:5 (v:v), and eluted with 6 mL dichloromethane/methanol solution. The solution was evaporated at 60 °C to 1 mL. The prepared solution was transferred to the GC injector vial. Extractions were performed in duplicate.

2.4. Gas chromatography with mass selective detector (MSD)

GC analysis was carried out using Hewlett–Packard Model 6890 gas chromatograph equipped with a split/

Table 1
Results of NAs levels in various fish products and vegetable oils analyzed during 2001–2005

Product	No. of samples	Mean concentrations ($n = 3$) of NAs, μg/kg					Sum of five NAs (μg/kg)
		NDMA	NDEA	NPYR	NPIP	NDBA	
Fresh fish							
Herring	10	n.d.[a]	n.d.	n.d.	n.d.	n.d.	n.d.
Silver hake	10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mackerel	10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sea perch	10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sardine	10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cold-smoked fish							
Mackerel	13	0.54	n.d.	0.88	0.50	n.d.	1.92
Salmon	10	0.52	n.d.	0.82	0.36	n.d.	1.70
Herring	12	0.58	n.d.	0.85	0.62	0.10	2.15
Hot-smoked fish							
Mackerel	12	1.10	0.13	1.40	0.84	0.10	3.57
Herring	10	1.20	0.11	1.84	0.98	0.12	4.25
Trout	10	1.26	0.14	2.33	0.88	0.13	4.74
Bream	10	1.17	0.17	2.12	0.89	0.14	4.49
Cod	10	1.16	0.13	2.16	1.07	0.16	4.68
Sprat	10	1.09	0.14	2.23	1.17	0.12	4.75
Salmon	10	1.13	0.16	2.18	0.91	0.17	4.55
Burbot	10	0.96	0.11	1.67	0.98	0.14	3.86
Silver hake	10	1.02	0.15	1.87	1.17	0.12	4.33
Sea perch	10	1.11	0.13	2.15	1.35	0.11	4.85
Pike	10	1.15	0.17	2.19	1.14	0.13	4.78
Sprats in oil	13	1.76	0.40	1.80	0.37	0.15	4.48
Sprats in tomato sauce	10	1.26	0.28	1.19	0.42	0.15	3.30
Fried fish							
Herring in tomato sauce	10	1.82	0.21	3.37	1.24	0.32	6.96
Sprats in tomato sauce	10	1.99	0.22	4.19	2.90	0.32	9.62
Salted fish							
Herring	12	1.20	0.17	0.91	0.42	0.24	2.94
Salmon	10	0.92	0.13	0.73	0.71	0.34	2.83
Trout	12	0.84	0.18	1.60	0.89	0.19	3.70
Pickled fish							
Herring	10	1.12	0.50	2.10	1.30	0.35	5.37
Salted/dried fish							
Roach	10	0.83	0.22	1.62	0.92	0.22	3.81
Fractions of smoked sprats in oil							
Smoked[b] sprat	13	1.50	0.20	1.10	0.36	0.10	3.26
Oil	13	0.30	0.20	0.65	n.d.	n.d.	1.15
Vegetable oil							
Olive oil	12	0.51	0.45	n.d.	n.d.	n.d.	0.96
Rape oil	11	0.36	0.19	n.d.	n.d.	n.d.	0.55
Sunflower oil (refined)	12	0.64	0.41	n.d.	n.d.	n.d.	1.05
Sunflower oil (not refined)	10	0.71	0.47	n.d.	n.d.	n.d.	1.18
Soybean	12	0.24	0.18	n.d.	n.d.	n.d.	0.42
Corn oil	10	0.50	0.24	n.d.	n.d.	n.d.	0.74
Peanut oil	10	0.43	0.22	n.d.	n.d.	n.d.	0.65

[a] n.d., not detected.
[b] The sprats fraction without oil was crushed, mixed and analyzed.

splitless injector. Five microlitre of the sample solution were injected into the gas chromatograph using pulsed splitless injection in the selected ion monitoring mode. Detection was done by a Hewlett–Packard MSD 5973 MSD mass spectrometer using a positive-ion chemical ionization.

Positive-ion chemical ionization mass spectrometry was performed with ammonia as reagent gas. Usually, NA molecules (M), for which the proton affinity is lower than that of ammonia (i.e. PA (M) < PA (NH_3), where PA (NH_3) = 853.6 kJ/mol (Hunter & Lias, 1998)) give mass spectra with $[M + NH_4]^+$ as

Table 2
Ammonia positive-ion chemical ionization mass spectra of NA

Compounds	M (g/mol)	Relative intensities (%)		
		$(M + 1)^+$	$(M + 18)^+$	$(M + 35)^+$
NDMA	74	16.6	100	1.6
NDEA	102	27	100	0.6
NPYR	100	31	100	0.6
NPIP	114	32.6	100	0.6
NDBA	158	86	100	1.3

the base peak. The details of NA ammonia positive-ion chemical ionization mass spectra are summarized in Table 2.

Sample portions were injected into a chromatograph column (30 m HP-1701 MS; 0.25 mm i.d., 0.25 μm film thickness) containing 14% cyanopropylphenyl and 86% methyl polysiloxane. For the gas chromatography separation of NAs oven programme started at 35 °C (held 1 min), set at 50 °C/min from 35 to 240 °C and held isothermally at 240 °C for 1 min; the velocity of He carrier gas (99.9996%) was 1 mL/min.

Fig. 1(a) shows the mass spectrum acquired from the injection of 20 ng/mL concentration of NDMA standard solution. Fig. 1(b) shows the spectrum of NDMA obtained when 5 μL of a cleaned-up hot-smoked fish sample was injected. No additional ions of significant relative abundance were detected in the spectrum of the extract, indicating that there was no interference from compounds having the same or similar retention time. Comparison of the two spectra shows the good agreement between the relative abundance of the ions in the sample spectrum and those obtained in the spectrum of the standard.

To calibrate the GC–MSD spectra six different standard solutions were prepared which cover the concentration range from 0.1 to 60 ng/mL. In this study the squared correlation coefficient for different calibration curves of NAs was found to be 0.9994–0.9998. This calibration curve enables to calculate NA concentration using the GC–MS peak area measurements.

2.5. Validation of method

To demonstrate the method was under analytical control the limit of detection (LOD), the limit of quantitation (LOQ), and recovery experiments were performed. The LOD and the LOQ have been established using spiked samples. Fish was fortified with appropriate volumes of standard solutions in methanol to get recovery at the level 0.44 ppb. The LOD is the threshold concentration below which identification is unreliable.

The value of the LOD was calculated as follows:

$$\mathrm{LOD} = X_{\mathrm{bl}} + K \cdot \mathrm{SD}_{\mathrm{bl}},$$

where X_{bl} is the mean of the blank measures and $\mathrm{SD}_{\mathrm{bl}}$ is the standard deviation of the blank measures, and K is a numerical factor chosen according to the confidence level desired. If confidence level is 95%, the K is 3.36. The LOQ is then 3.3 times the LOD (The Nordic Committee of Food Analysis, 1996). The values of the LOD and the LOQ for this method are shown in Table 3.

For the recovery experiment a sample with a low content (cold-smoked mackerel) of NAs was chosen, and fortified with two different amounts of NAs standard mixture. The sample was analyzed by GC–MS and recovery of NAs was calculated as follows (Eurachem Guide, 1998):

$$\mathrm{Rec}\ (\%) = [(C_1 - C_2)/C_3] \times 100,$$

where C_1 is the concentration determined in the fortified sample, C_2 is the concentration determined in the unfortified sample, and C_3 is the concentration of fortification.

Table 4 lists for the two fortification levels, the NAs amounts (μg/kg) found in the fish sample after fortification, the percentage recovery (mean of three replicates), the standard- and relative standard deviation of the replicates. The relative standard deviation is expressed in percent and is obtained by multiplying the standard deviation by 100 and dividing this product by the average concentration. The selectivity of the method was assessed evaluating the purity of NAs chromatographic peaks, and comparing the exication and emission

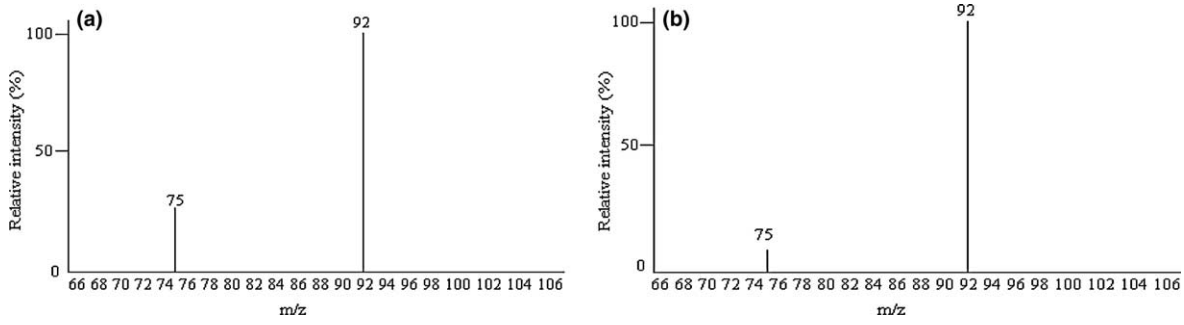

Fig. 1. Positive-ion chemical ionization mass spectra of NDMA standard (a) and NDMA isolated from hot-smoked fish sample (b).

Table 3
The LOD and the LOQ data (μg/kg) of volatile NAs in cold-smoked mackerel ($n = 12$)

Compound	Unfortified sample[a]	Fortified samples[b]					
		Found[c]	Rec (%)[d]	SD[e]	RSD (%)[f]	LOD	LOQ
NDMA	<0.10	0.35	79	0.033	9.43	0.11	0.37
NDEA	<0.10	0.36	82	0.030	8.43	0.10	0.33
NPYR	<0.10	0.38	87	0.032	8.42	0.11	0.35
NPIP	<0.10	0.38	86	0.031	8.16	0.10	0.34
NDBA	<0.10	0.39	88	0.030	7.69	0.10	0.33

[a] Amount of NA (μg/kg) found in the unfortified sample.
[b] 0.44 μg/kg of NA added in the fortified sample.
[c] Amount of NA (μg/kg) found in the fortified sample.
[d] Average recovery of NA in percent.
[e] Sample standard deviation.
[f] Relative standard deviation in percent.

Table 4
Validation data of five NAs spiked at two levels in cold-smoked mackerel obtained with GC–MS method

Compound	Unfortified sample[a]	Fortified samples							
		Level I[b]				Level II[c]			
		Found[d]	Rec (%)[e]	SD[f]	RSD (%)[g]	Found	Rec (%)	SD	RSD (%)
NDMA	<0.10	1.52	76	0.151	9.93	3.70	74	0.351	9.49
NDEA	<0.10	1.56	78	0.180	11.54	3.80	76	0.312	8.21
NPYR	<0.10	1.70	85	0.152	8.94	4.20	84	0.231	5.50
NPIP	<0.10	1.66	83	0.191	11.51	4.10	82	0.370	9.02
NDBA	<0.10	1.70	85	0.182	10.71	4.15	83	0.341	8.22

[a] Amount of NA (μg/kg) found in the unfortified sample.
[b] 2 μg/kg of NA added in the fortified sample.
[c] 5 μg/kg of NA added in the fortified sample.
[d] Amount of NA (μg/kg) found in the fortified sample.
[e] Average recovery of NA in percent.
[f] Sample standard deviation.
[g] Relative standard deviation in percent.

spectra of each NA peak in the chromatogram of the different smoked fish samples assayed with those obtained with standard solutions. Fig. 2 shows the chromatogram of five NAs extracted from cold-smoked mackerel spiked with 10 μg/kg of each single NA. The repeatability data, resulting from six replicate analyses of the same smoked samples (sprats in oil) are reported in Table 5. The results characterize the suitability of the method for determination the NAs in various samples of fish products.

3. Results and discussion

In this work, the level of five NAs, namely NDMA, NDEA, NDBA, NPYR and NPIP, in 135 samples of hot-smoked fish, 35 samples of cold-smoked fish, 50 samples of fresh fish, 20 samples of fried fish, 34 samples of salted fish, 10 samples of pickled fish, 10 samples of salted/dried fish, and in 77 samples of vegetable oil purchased from Estonian market was determined. The samples with the high NAs content were repeatedly analyzed.

Data on NAs levels in various samples of fish products, and in the oil samples are given in Table 1. In samples of fresh fish the level of NAs was not detected. In 117 of 294 samples of various fish products the concentration of NDMA exceeded 1 μg/kg. The level of NAs in cold-smoked fish are lower than the level in hot-smoked and fried fish samples. The formation of significant amounts of volatile NAs in the fish is probably caused by the interaction of nitrites and amines in the fish. In 8 of 294 samples of various fish products the concentration of the sum of NDMA and NDEA exceeded the tolerance limit of 3 μg/kg. The samples with the high level of NAs have the potential risk to human health. The highest levels of NAs were found in samples of fried- and pickled fish. Relatively high level was found in hot-smoked fish, in sprats in oil, in salted- and in salted/dried fish. In the opposite, low level of NAs was detected in cold-smoked fish.

Volatile NAs could not detected in the all analyzed samples of fish. This may be due to the variation in amine levels in various fish. The concentration of amines in the fish depends on various factors such as species, age, environment, bacterial flora, and strorage

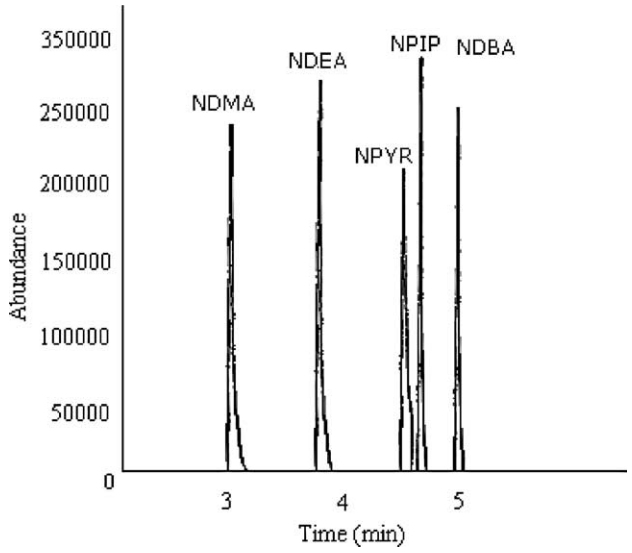

Fig. 2. Total ion chromatogram of five volatile NAs extracted from cold-smoked mackerel sample after analytical cleanup (spiked with 10 μg/kg of each single NA).

Table 5
Analytical repeatability of the proposed method (sample of smoked sprats in oil)

Compounds	Mean[a]	SD	RSD (%)
NDMA	1.70	0.122	7.15
NDEA	0.40	0.033	8.35
NPYR	1.80	0.122	6.80
NPIP	0.37	0.031	8.38
NDBA	0.15	0.012	8.27

[a] Mean (μg/kg) of replicate analyses ($n = 6$).

conditions (Fiddler, Doerr, Ertel, & Wasserman, 1971). Pyrolysis of protein and cooking of protein food can be another source of secondary amines. It can release amino acids like proline, hydroxy proline and arginine as well as nitrosable amines, like pyrrolidine and piperidine. Assuming the presence of nitrate and nitrite, high temperature accelerates NAs synthesis in the fish (Iyengar, Panalaks, Miles, & Sen, 1976).

In this study, the levels of five NAs in 13 samples of smoked sprats in oil were investigated. In these samples, the ratio of smoked sprats to vegetable oil was 7:3. In 11 of 13 samples of smoked sprats in oil contained NDMA at levels exceeding 1 μg/kg. Results of analysis show the great differences in NAs concentration in samples of the same type. Probably, the effect is due to differences in smoking technology. Data on NAs levels in samples of smoked sprats in oil (the oil and the fish fractions) and in samples of vegetable oil (rape and olive oils) are presented in Table 1. The obtained results show the influence of the formation of NAs in samples of smoked sprats on concentration of NAs in oil. It is possible, that the NAs migrate into oil. The extent of migration being dependent upon the lipophilic character of the product and its storage time. Moreover, 77 samples of edible vegetable oil were analyzed. These oil samples contain sum of NAs at levels not exceeding to 1.18 μg/kg, and oil of sprats contain on an average 1.15 μg/kg of NAs.

Ten samples of pickled fish, 34 samples of salted fish, and 10 samples of salted/dried fish from the Estonian markets were analyzed for volatile NAs. High level of the sum of the average of five volatile NAs content was found in pickled fish to be 5.37 μg/kg, in salted fish – 3.16 μg/kg, and in salted/dried fish – 3.81 μg/kg. In fish itself there are rich sources of secondary and tertiary amines, while in the crude salt used to pickle the fish, there is nitrate and possibly nitrite. All these factors suggest the possibility that NAs might be formed in the salted fish (Zou et al., 1994). The possible reason of variation of NDMA content in salted fish was related in part to the degree of contamination by nitrate-reducing *Staphylococcus aureus*. This organism isolated from fish has been shown to increase the NDMA content in salted fish broth. The level of NDMA also depends on species of fish. It is possible that the contents of secondary and tertiary amines as precursors of NA, may vary widely in different species of the fish. The above observations suggest that the prerequisites for NDMA production in salted fish may be the availability of secondary amines in the fish, the presence of nitrate in the crude salt used for pickling and the intervention of nitrate-reducing *S. aureus* or other bacteria. The source of *S. aureus* is probably contamination during preparation of fish. The nitrate present as an impurity in the pickling salt is a precursor of NDMA (Fong & Chan, 1973).

Increased levels of NAs were observed after baking and frying indicating formation of these compounds during cooking. In 20 samples of fried fish from Estonian market, the average of NDMA concentration was 1.91 μg/kg, NDEA was 0.22 μg/kg, NPYR was 3.78 μg/kg, NPIP was 2.07 μg/kg, and NDBA – 0.32 μg/kg.

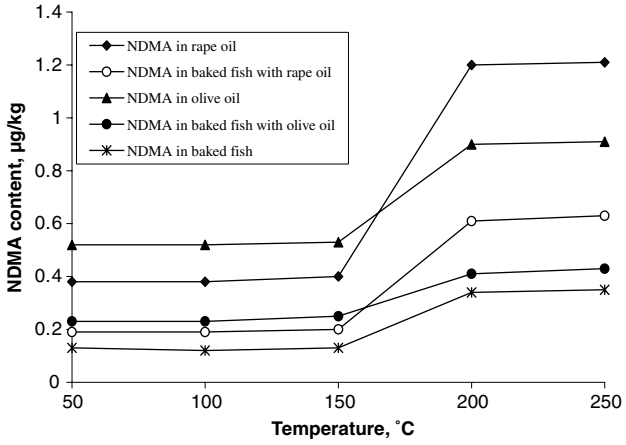

Fig. 3. The graphs showing the temperature versus NDMA content in rape oil and in baked fish (silver hake) with rape oil, in olive oil and in baked fish with olive oil, and in baked fish.

Table 6
Influence of baking temperature on the content of volatile NAs in samples of oil and fish (silver hake)

Temperature (°C)	Mean concentrations ($n = 3$) of NAs (μg/kg)					Sum of NAs (μg/kg)
	NDMA	NDEA	NPYR	NPIP	NDBA	
Baked fish with rape oil						
50	0.19	0.10	0.11	0.10	[a]n.d.	0.50
100	0.19	0.10	0.12	0.11	n.d.	0.52
150	0.20	0.11	0.14	0.11	n.d.	1.56
200	0.61	0.48	0.93	0.72	0.12	2.86
250	0.63	0.48	0.94	0.74	0.12	2.91
Excess fat of baked fish with rape oil						
50	0.45	0.30	0.23	0.20	n.d.	1.18
100	0.45	0.32	0.24	0.20	n.d.	1.21
150	0.48	0.32	0.24	0.21	n.d.	1.25
200	1.31	0.90	1.64	1.31	0.32	5.48
250	1.31	0.91	1.66	1.30	0.34	5.52
Rape oil						
50	0.38	0.19	0.11	0.10	n.d.	0.78
100	0.38	0.21	0.11	0.12	n.d.	0.82
150	0.40	0.21	0.12	0.10	n.d.	0.83
200	1.20	0.81	1.52	1.15	0.20	4.88
250	1.21	0.82	1.52	1.16	0.20	4.91
Repeatedly baked rape oil						
50	0.41	0.22	0.15	0.12	n.d.	0.90
100	0.42	0.24	0.17	0.14	n.d.	0.97
150	0.42	0.24	0.17	0.15	n.d.	0.98
200	1.24	0.85	1.60	1.25	0.26	5.20
250	1.24	0.86	1.60	1.25	0.26	5.21
Baked fish with olive oil						
50	0.23	0.16	n.d.	n.d.	n.d.	0.39
100	0.23	0.16	n.d.	n.d.	n.d.	0.39
150	0.25	0.18	n.d.	n.d.	n.d.	0.43
200	0.41	0.37	0.81	0.63	n.d.	2.22
250	0.43	0.38	0.81	0.62	n.d.	2.24
Excess fat of baked fish with olive oil						
50	0.60	0.50	0.17	0.15	n.d.	1.42
100	0.61	0.50	0.18	0.17	n.d.	1.46
150	0.61	0.52	0.19	0.17	n.d.	1.49
200	0.99	0.81	1.21	0.84	0.21	4.06
250	1.00	0.81	1.23	0.85	0.21	4.10
Olive oil						
50	0.52	0.46	0.12	0.11	n.d.	1.21
100	0.52	0.48	0.13	0.11	n.d.	1.24
150	0.53	0.48	0.12	0.12	n.d.	1.25
200	0.90	0.71	1.10	0.75	0.11	3.57
250	0.91	0.71	1.09	0.76	0.11	3.58
Fish baked without oil						
50	0.13	0.10	0.15	0.10	n.d.	0.48
100	0.12	0.10	0.15	0.11	n.d.	0.48
150	0.13	0.11	0.20	0.11	n.d.	0.55
200	0.34	0.24	0.71	0.53	0.10	1.92
250	0.34	0.25	0.72	0.53	0.11	1.95

[a] n.d., not detected.

The concentration of NAs in the fish after baking in pre-heated electrical oven depends on the fish cooking temperature. This was demonstrated in the next experiment. Approximately 300 g of silver hake (*Merluccius bilinearis*) was divided into six roughly equal parts. One portion of fish was reserved for analysis in a fresh

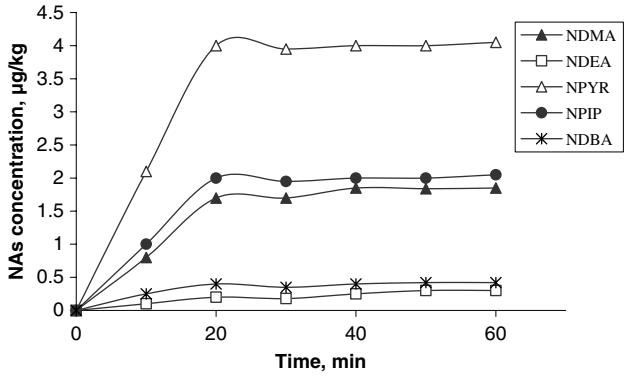

Fig. 4. Effect of frying time on NAs concentration in fish.

state. The other five portions were baking separately in pre-heated electrical oven in open oven-proof glass dishes with 50 mL oil at regulo 50, 100, 150, 200 and 250 °C, during 30 min. The test was repeated for rape and olive oils. The cooked portions were allowed to cool but before becoming cold the excess fat and oil were separated from the sample. Since the aim was to measure NAs in the fish and in the oil as normally consumed this excess fat was analysed. Then rape and olive oils has been analysed after baking in electrical oven at different temperatures. The levels of NAs were determined also baked fish without oil. Fig. 3 shows the graphs the temperature versus NDMA content in rape oil and in baked fish with rape oil, in baked fish with olive oil, and in baked fish, respectively. Data on NAs levels in silver hake and in oil after baking with electrical oven at different temperatures are presented in Table 6. There was significant increase in NAs levels in the fish and in the oil after baking with electrical oven at temperature around 150 °C. In repeatedly baked rape oil samples was found insignificant increase in NAs concentrations.

In the next experiment, the fish was fried in a pan with oil (rape and olive) using natural gas. After frying for 30 min per side, NAs were found in five of five samples of fish. The mean values of NDMA, NDEA, NPYR, NPIP and NDBA concentration in fried silver hake varied from 0.8 to 1.90 µg/kg, not detectable to 0.30, 2.35–4.50, 1.40–2.70 and 0.20–0.40 µg/kg, respectively. The amounts detected in the fried oil in the present work were similar with the amount present in the fried fish. In most cases was the level of NDMA in gas-frying sample higher than that in the electric-oven-baking fish. As shown in Fig. 4, the concentration of NAs increases rapidly in first 20 min and then stay stabile.

For the purpose of comparison, the content of NAs found in various fish products was confronted with data from the literature. The levels of NAs in Estonian fish products in the present study are lower than those found in various fish products from Russia (Dikun, Romanova, Shedrikova, Reim, & Yunosova, 1980), Japan (Kawabata et al., 1980) and China (Zou et al., 1994), but show good agreement with results from France (Biaudet et al., 1994), Sweden (Österdahl, 1988), and Denmark (Pedersen & Meyland, 1981; Thomsen & Gotfred, 1983).

4. Conclusions

In the present study, five NAs were separated by gas chromatography and differentiated by positive-ion chemical ionization using ammonia as a reagent gas. The limit of detection and the limit of quantitation for this method were approximately 0.10 and 0.35 µg/kg, respectively. The recovery of NAs in smoked fishery products varied from 79% to 88%.

Total concentrations of NAs in 294 studied samples of fish products ranged from non-detectable to 10.34 µg/kg. Apparently, cooking temperature and time have a significant effect on the concentration of NAs.

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