Determination of volatile *N*-nitrosamines by gas chromatography–mass spectrometry with positive-ion chemical ionization

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Abstract. A gas chromatography–mass spectrometry method was developed and optimized for the determination of volatile *N*-nitrosamines in smoked meat products. Five volatile *N*-nitrosamines (*N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosopiperidine, *N*-nitrosopyrrolidine, and *N*-nitrosodibutylamine) were separated by gas chromatography and differentiated by positive-ion chemical ionization using two different reagent gases – methane and ammonia. The HP 6890 Plus GC/HP 5973 MSD with positive-ion chemical ionization option was used in the selected ion-monitoring mode. For cleaning the sample solid-phase extraction was used. The detection limit for *N*-nitrosamines using positive-ion chemical ionization was 0.01 ppb with about 70–80% recovery.

Key words: volatile *N*-nitrosamines, positive-ion chemical ionization, gas chromatography, mass spectrometry, food.

INTRODUCTION

N-nitrosamines (NA) are known as a widely acting group of animal carcinogens and are found, usually at trace levels, in diverse places in the environment. Volatile NA may be also formed in certain foods from naturally occurring amines present in them. These amines are nitrosated by agents derived from added nitrite or nitrogen oxides [1]. Nitrosation reactions can be influenced by the presence of nitrosation inhibitors (redox compounds such as ascorbate, vitamin E, and alcohols) and catalysts (metal ions, phenols, nucleophilic anions such as Cl^-, J^-, Br^-, SCN^-, carbonyl compounds, and surface-active agents) [2].

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NA can be formed in food, water, air, and human organism. They constitute a large group of genotoxic chemical carcinogens, which occur in human diet and other environmental media, and can be formed endogenously in the human body.

Table 1 presents data on the toxicity and carcinogenicity of five volatile NA (NDMA = *N*-nitrosodimethylamine, NDEA = *N*-nitrosodiethylamine, NPYR = *N*-nitrosopyrrolidine, NPIP = *N*-nitrosopiperidine, NDBA = *N*-nitrosodibuthylamine) from the literature [2, 3].

Limit concentrations for two NA have been established in Estonia: the maximum allowed content of summed NDMA and NDEA in meat and meat products is 2 µg/kg; in smoked meat products 4 µg/kg [4].

NA may be formed from nitrate or nitrite used as preservatives in food, or from nitrogen oxides in combustion gases when direct heating, smoking, or drying processes are used. The concentration of NA depends on the cooking method, frying temperature and time, concentration of NA precursor, possibly smoking procedures, and ascorbate concentration [5].

Various methods have been used for the determination of volatile NA in diverse food matrixes: beer [6, 7], groundwaters and drinking waters [8, 9], fried bacon [10], cheeses [11], and nonfat dry milk [12]. Sample preparation methods used are based on vacuum distillation [13], steam distillation [14, 15], mineral oil distillation [16], solid-phase extraction (SPE) [17, 18], and liquid–liquid extraction [19]. Fidder & Pensabene [20] used a supercritical fluid extraction (SFE) method to analyse 10 different NA in fried bacon. The results were compared with mineral oil distillation, low-temperature vacuum distillation, and SPE methods. Analysis of NPYR data for different sample preparation methods used (SFE, SPE, low-temperature vacuum distillation, and mineral oil distillation) showed significant variation [20].

Analytical methods to detect volatile NA are based on gas chromatography (GC) with a thermal energy analyser (TEA) [16, 21, 22], with mass spectrometry (MS) by electron impact ionization (EI) [23–25], and with mass spectrometry by chemical ionization [26–29].

Planar chromatography [30], high pressure liquid chromatography (HPLC), flame thermionic detection, colorimetric detection [31], fluorescence determination [32], and fluorescence and chemiluminescence [15] are methods used less often to detect volatile NA in food.

Table 1. Half lethal dose (LD_{50}) and tumorgenic dose rate 50 (TD_{50}) values of volatile *N*-nitrosamines [2, 3]

Compound	LD_{50}, µg/kg	TD_{50}, mg/kg body weight/day
NDMA	40	0.01
NDEA	280	0.11
NPYR	900	2.10
NPIP	200	1.30
NDBA	1200	0.69

The task of this study was to work out a rapid and efficient GC–MS method for the determination of volatile NA in food products. Sample cleaning optimization and selection of the most suitable reagent gas for positive-ion chemical ionization (PCI) were dealt with.

METHODS
Sample preparation

For sample cleaning SPE was used. SPE sorbents are available in a wide range of different surfaces, pore size, particle size, and base support. Which sorbent is optimal for extraction depends upon the properties of the NA, the sample/matrix composition, and other factors that are discussed below. To get the proper SPE product one has to take into account the mode of NA retention, sorbent chemistry, mass, and physical configuration.

Two SPE steps with Extrelut (Kieselguhr) and Florisil (85% SiO_2, 15.5% MgO, 0.5% Na_2SO_4) sorbents were used. The food sample (6.0 ± 1.0 g in 100 mL glass beaker) 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 95:5 (v:v) dichloromethane/methanol solution, and eluted with 6 mL dichloromethane/methanol solution. The solution was evaporated at 60°C to 1 mL. Then 200 μL of internal standard (NDPA, 1 μg/mL) was added. The prepared solution was transferred to the GC injector vial. Extractions were performed in duplicate [17].

Chemicals

Methanol (J. T. Baker, Holland), dichloromethane (Sigma-Aldrich, USA), hexane (Rathburn, Scotland), and 0.1 N NaOH solution (Chemapo, Czechoslovakia) were used. As internal standard NDPA solution 100 μg/mL in methanol from Ultra Scientific was used. NA mixtures in methanol were commercial products from Sigma except NDMA, which was from Aldrich. Extrelut was from Merck, Germany; Florisil 100/200 from Alltech, Belgium.

The following volatile NA were analysed: NDMA, NDEA, NDBA, NPYR, and NPIP.

1. $R^1 = R^2 = CH_3$ — NDMA
2. $R^1 = R^2 = CH_2CH_3$ — NDEA
3. $R^1 = R^2 = (CH_2)_2CH_3$ — NDPA, internal standard
4. $R^1 = R^2 = (CH_2)_3CH_3$ — NDBA

5. NPYR

6. NPIP
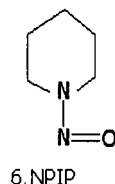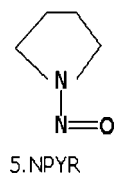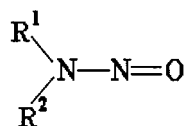
Gas chromatography–mass spectrometry determination

Analyses were carried out using a HP (Hewlett-Packard) 6890 Plus GC gas chromatograph with (MSD) – HP 5973 MSD mass selective detector.

Sample portions (2 µL) were injected into a chromatograph column (30 m HP-1701 MS; 0.25 mm i.d., 0.25 µm film thickness) containing 14% cyano-propylphenyl and 86% methyl polysiloxane. GC oven programme was set at 55°C for 2 min and then raised to 260°C at a rate of 10°C/min and held at the final temperature for 2 min; the velocity of He carrier gas (99.99%) was 1 mL/min. Oven conditions for GC and mass selective detector settings are given in Table 2.

Table 2. Positive-ion chemical ionization with ammonia as reagent gas

Injector	
Injection volume	2.0 µL
Columns	
Detector	MSD
Outlet pressure	54.8 kPa
Flow (He carrier gas)	1.0 mL/min
Average velocity	37 cm/s
Inlets	
Heater	220 °C
Pressure	54.7 kPa
Total flow	33.8 mL/min
Injection pulse	Pressure 90.0 kPa, 1.40 min
Purge flow to split vent	30.0 mL/min, 1.20 min
Gas saver	15.0 mL/min, 2.00 min
Oven	
Heating time at intial temperature 55 °C	2.00 min
Heating time at 260 °C	3.00 min
Solvent washes	Heptane
MSD parameters	
Tune file	PCINH3.U
Acquistion mode	SIM
Run time	25.50 min

PCI MS option was used in the selected ion-monitoring mode (SIM) with two reagent gases – methane and ammonia.

Mass spectra for NA were registered in SCAN and SIM modes. (SCAN refers to the sequence of control over operating parameters of a mass spectrometer that results in a spectrum of masses, velocities, momenta, or energies; SIM refers to the use of the instrument to record the ion current at selected masses characteristic of the compound of interest in an expected retention time window [33]). For sensitivity optimization the temperature of the ion source and the reagent gas flow rate were varied. The method may be further optimized for even more rapid analysis.

Calculation method

Recovery is the proportion of the amount of analyte present in or added to the analytical portion of the test material that is extracted and presented for measurement. In this work recoveries of five volatile NA were investigated by a standard addition experiment. The meat product was fortified with appropriate volumes of standard solutions in methanol to get recoveries at the levels 0.1–1 ppb. We used two SPE steps – Extrelut and Florisil. The sample was analysed by GC–MS and recovery was calculated by Eq. 1 [34].

$$Rec, \% = [(C_1 - C_2)/C_3] \times 100, \tag{1}$$

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.

The concentration of NA, C_{NA} (μg/mL), detected by GC, was calculated using the following equation [35]:

$$C_{NA} = C_{NDPA} \, (A_{NA}/A_{NDPA}), \tag{2}$$

where C_{NDPA} is the concentration of the internal standard (NDPA) solution in the sample, A_{NA} designates the area of NA peak, and A_{NDPA} is the area of the internal standard peak.

The content of NA, X (μg/kg), in a sample was calculated as follows [14]:

$$X = 10^5 C_{NA} \, V/m \, Rec, \tag{3}$$

where C_{NA} stands for NA concentration by GC detection, m is the weight of the sample (g), V is the volume of the final concentrated extract (mL), and Rec is the recovery of NA (%). The peaks were integrated by a Hewlett-Packard 6890 Gas Chromatograph.

Calibration

The ratio of the chromatogram peak area of the NA (A_{NA}) to the peak area of the internal standard (A_{NDPA}) in the spiked bacon sample was calculated. The linear relationship of this ratio to the concentration ratio is shown in Fig. 1. This
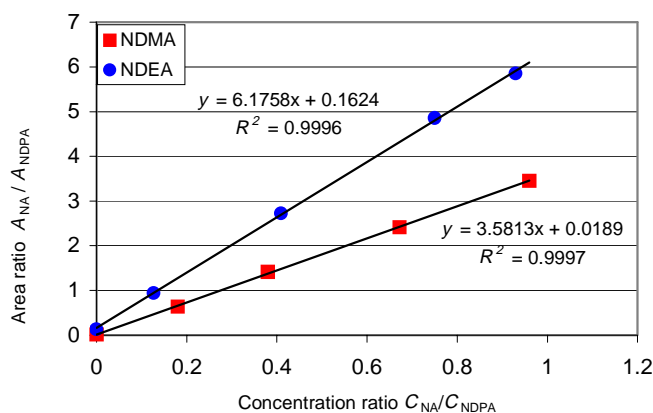
Fig. 1. Calibration curve for NDEA and NDMA in bacon with ammonia used as reagent gas.

calibration curve enables to calculate the NA concentration using the GC–MS peak area measurements. The correlation coefficient $R^2 = 0.9998$ for NDMA and $R^2 = 0.9986$ for NDEA.

Limit of detection of NA

Limit of detection (LOD) is the threshold concentration below which identification is unreliable [36].

The value of LOD is given by the equation:

$$LOD = X_{bl} + K\,S_{bl}, \tag{4}$$

where X_{bl} is the mean of the blank measures, S_{bl} is the standard deviation of the blank measures, and K is a numerical factor chosen according to the confidence level desired [34].

The most widely used method for the determination of volatile NA in meat products has been GC with different detectors. These detectors include a flame ionization detector [16], Coulson electrolytic detector [14], thermal energy analyser (TEA) [17, 19], and mass selective detector (MSD) [26, 28].

Prest & Hermann [29] developed a GC–MS method with a MSD by PCI using ammonia reagent gas and large volume injection (50 μL) for the determination of NDMA in water. This method provides the lowest values for NA detection limit reported in the literature (1–3 ng/L). In this work the same temperature program was used as in the optimized method in [29] except the initial temperature, which was 55 °C in our study to minimize the changes of analyte concentrations through possible solvent and matrix effects.

Thermal Energy Analyzer as a selective detector for *N*-nitroso compounds has been succesfully interfaced to gas [17] and liquid [19] chromatographs. In these publications the authors evaluated LOD for GC as 0.3 ppb and for LC, 1 ppb.

Table 3. Limit of detection (ppb) reached for volatile *N*-nitrosamines with positive-ion chemical ionization (PCI) and electron impact ionization (EI)

Compound*	PCI with methane	PCI with ammonia	EI
NDMA	0.5	0.1	10.0
NDEA	0.02	0.01	2.0
NPYR	0.2	0.2	2.0
NPIP	0.01	0.01	1.0
NDBA	0.01	0.01	1.0

* See Table 1 for abbreviations.

Unfortunately, the authors do not mention what kind of NA they studied. With a Coulson electrolytic detector LOD of 0.1–5 ppb was reached [14].

In this work five volatile NA were separated by GC with MSD and recognized by PCI using two different reagent gases, ammonia and methane. The sensitivity for NA increased and LOD was better when ammonia was used as reagent gas in the PCI experiment (Table 3). LOD was determined as the mean value of six independent measurements for each volatile NA. PCI with ammonia as reagent gas was superior to EI ionization for volatile NA in most cases giving up to 10^3 times lower LOD, except for NPYR and NDMA, where the LOD values are almost equal.

RESULTS
Electron impact ionization

Ionization is achieved through the interaction of an analyte with an energetic electron beam. This results in the loss of an electron from the analyte and production of a radical cation. As samples are thermally desorbed to the gas phase and subjected to the high energy of EI, analytes must be both thermally and energetically stable. EI ionizing energy used is 70 eV to promote greatest sensitivity and to produce molecular and fragment ions used for chemical characterization and identification.

A summary of the mass spectra of the NA obtained by EI in this work is given in Table 1 in the Appendix and shown in Figs. 1 and 2 in the Appendix. The spectra of NDMA, NDEA, NDPA, NPYR, and NPIP are compared with those published in NIST Standard Reference Database [37] (see Table 1 in the Appendix). The mass spectrum of NDBA is not available in NIST and is compared to the spectrum published elsewhere [38].

Positive-ion chemical ionization

PCI is a soft ionization method developed to ionize compounds. The probability of proton exchange is based on the relative gas phase basicities

(proton affinities) of the analyte and the reagent gas. Although PCI is capable of generating molecular weight information from labile species, it still requires the sample to be volatile, which could hinder the detection of thermally unstable analytes.

A summary of the mass spectra of NA studied by SIM–PCI using different reagent gases (methane, PA = 129.9 kcal/mol, and ammonia, PA = 204.0 kcal/mol [37]), is presented in Table 2 in the Appendix and shown in Figs. 3–6 in the Appendix.

PCI with methane as reagent gas

Table 2 in the Appendix gives simple PCI mass spectra for all NA measured, with the $(M+H)^+$ ion accounting for almost all the ion current. Volatile NA molecules M for which proton affinity is greater than that of methane (i.e. $PA(M) > PA(CH_4)$) give mass spectra with $(M+H)^+$ as the base peak, owing to protonation of the molecule, and a much smaller peak for the $(M+C_2H_5)^+$ adduct. Unfortunately, the proton affinities of these NA are not available in the literature.

PCI with ammonia as reagent gas

As expected, the use of ammonia as reagent gas led to lower fragmentation than observed with methane. The best sensitivity was achieved at ammonia flow of 1 mL/min. Usually, molecules where proton affinity is lower than the value for ammonia (i.e. $PA(M) < PA(NH_3)$) give mass spectra with $(M+NH_4)^+$ as the base peak and a much smaller peak for the $(M+H)^+$ adduct.

Recovery and content of volatile NA in some meat products

The recovery measurements of volatile NA in smoked meat samples are given in Table 4. Recoveries were calculated by Eq. 1. The differences in the recovery between NA can be explained most likely by different polarities, which affect their elution from extraction columns. Figure 2 presents as an example a chromatogram of a fried meat sample spiked with a standard solution containing five NA.

A combination of Extrelut–Florisil gave the highest recovery of NA compared to other cartridges that were investigated, such as Cyano, Silica, Amino and Alumino (Fig. 3).

Table 4. Recovery (%) of *N*-nitrosamines added in different concentrations to a spiked smoked meat sample (extraction by Extrelut–Florisil SPE)

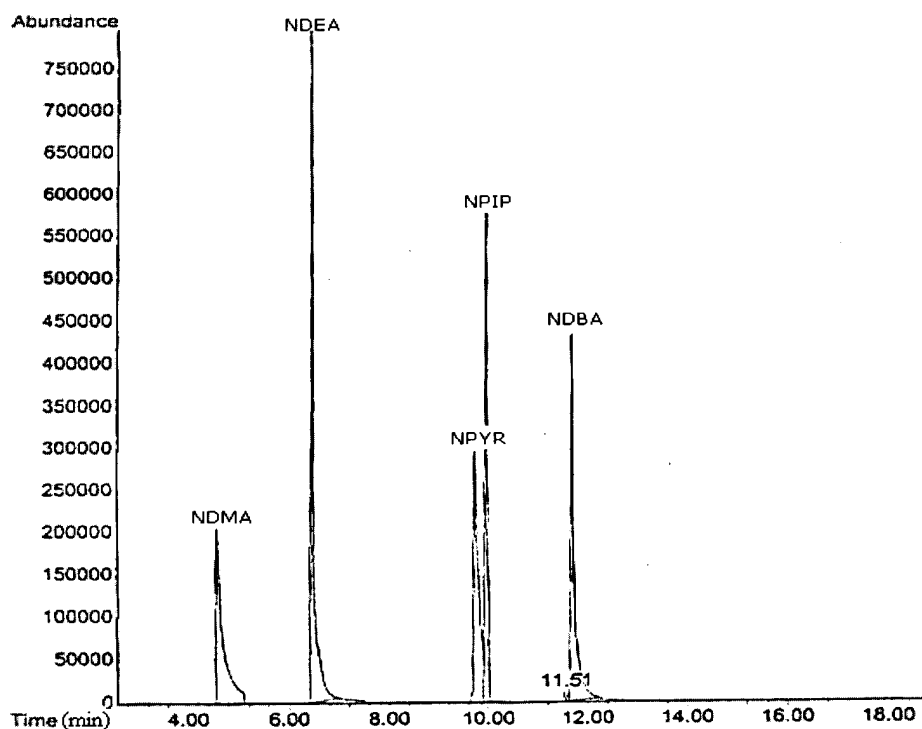| Concentration of NA added, ppb	NDMA	NDEA	NPIP	NPYR	NDBA
0.1	61	65	73	64	60
0.5	59	68	74	66	59
1.0	68	74	78	76	67

Fig. 2. Chromatogram of five *N*-nitrosamine standards in a spiked fried meat sample.
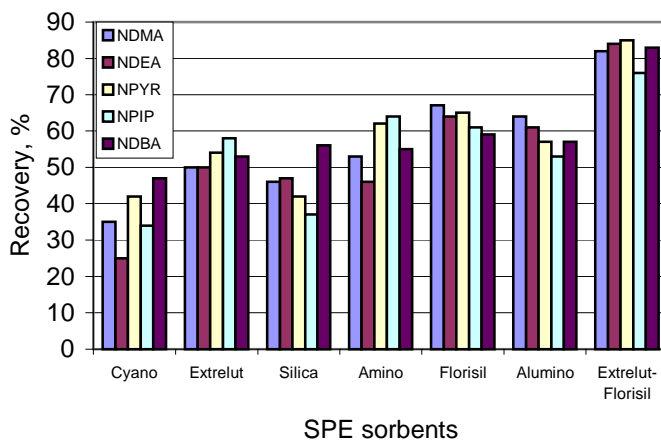

Fig. 3. Recovery of volatile *N*-nitrosamines with different solid-phase extraction sorbents used.

Data on the content of volatile NA in some Estonian meat products measured in this work are presented in Table 5. All meat products studied were purchased from local market and stored at $-20\,^\circ$C until analysis.

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Table 5. Mean level (μg/kg) of volatile *N*-nitrosamines in some Estonian meat products

Product	No. of samples	NDMA	NDEA	NPIP	NPYR	NDBA	Total
Fried meat	3	2.37	0.54	0.6	3.54	0.16	**7.21**
Ham	21	1.80	0.42	0.53	2.32	0.24	**5.31**
Smoked chicken	7	1.90	0.33	1.31	8.94	0.19	**12.67**

The highest NA levels were found in smoked and fried foodstuffs. The obtained results showed that the formation of NA in food is influenced by NO_2^- and NO_3^- concentrations, smoking and frying temperature regime and time, and storage of food. Addition of sodium nitrite to smoked chicken increased the exposure of NPYR. The phenols and nitrous gas in smoke and addition of nitrites may increase NA levels in smoked products. Nitrous gas in smoke accelerates nitrosation reactions [2]. The highest level of NPYR was detected in pork fat, most likely due to its lipophilic properties.

CONCLUSIONS

In this work a method for separation and identification of volatile *N*-nitrosamines was developed. The advantages of this method are rapidity, effectiveness, and simplicity. The recovery of NA in food samples with different extraction methods is in the range 50–70%. The SPE method was found to be superior to other extraction methods used for high recovery and rapidity of analysis. The advantages of the proposed method are low time consumption for sample preparation and decreased possibility of pollution of the GC–MS system by additives.

Two different reagent gases, methane and ammonia, were tested. Methane causes higher fragmentation and can be used for NA with high molecular weight only. The required sensitivity cannot be reached for low-molecular weight NA. Ammonia as a reagent gas of relatively "soft" nature shows milder fragmentation but is a very specific reagent gas for NA. The limit of detection using positive-ion chemical ionization with ammonia was 0.01 ppb and the recovery was about 70–80%. As a conclusion, PCI with ammonia as reagent gas was the most selective technique distinguishing volatile NA on the basis of relative abundances of their adduct ions.

Five volatile NA of different molecular structures were determined in meat products. Comparison of the results of our analyses with the results obtained by other authors encourages us to use this highly sensitive method for identification and quantitative analysis of NA in addition to meat products also for other matrixes.

ACKNOWLEDGEMENT

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Table 1. Intensities (%) of the *N*-nitrosamine ions observed with electron impact ionization in the present work and in NIST Database (relative to the most intensive peak)

N-nitrosamine	Present work, m/z, Rel. intensity (in parentheses)	NIST Database [37], m/z, Rel. intensity (in parentheses)
NDMA	74(100), 42(98), 43(44)	74(100), 42(72), 43(45)
NDEA	102(100), 44(25), 42(20), 56(21)	102(100), 44(87), 42(83), 56(54)
NDPA	130(28), 42(65), 43(100), 70(98), 101(13)	130(22), 42(66), 43(100), 70(66), 101(13)
NPYR	100 (100), 41(80), 42(61), 68(18)	100 (100), 41(69), 42(45), 68(14)
NPIP	114(90), 41(25), 42(100), 55(54)	114(100), 41(28), 42(94), 55(83)
NDBA	158(38), 84(100), 116(43), 99(36), 57(54)	158(18), 84(100), 116(35), 99(19), 57(70)

Table 2. Intensities (%) of the *N*-nitrosamine ions observed with positive-ion chemical ionization with methane and ammonia used as reagent gases (relative to the most intensive peak)

| *N*-nitros-amines	M, g/mol	m/z, Rel. intensity (in parentheses)					
		Methane			Ammonia		
		$(M+H)^+$	$(M+C_2H_5)^+$	$(M+C_3H_5)^+$	$(M+H)^+$	$(M+NH_4)^+$	$(M+N_2H_7)^+$
NDMA	74	75(100)	103(0)	115(4)	75(16.6)	92(100)	109(1.6)
NDEA	102	103(100)	131(10)	143(8)	103(27)	120(100)	137(0.6)
NPYR	100	101(100)	129(0)	141(0)	101(30.9)	118(100)	135(0.6)
NPIP	114	115(100)	143(7.2)	155(4.7)	115(32.6)	132(100)	149(0.6)
NDPA	130	131(100)	159(8)	171(0)	131(44)	148(100)	165(4.5)
NDBA	158	159(100)	187(0)	199(0)	159(86)	176(100)	193(1.3)
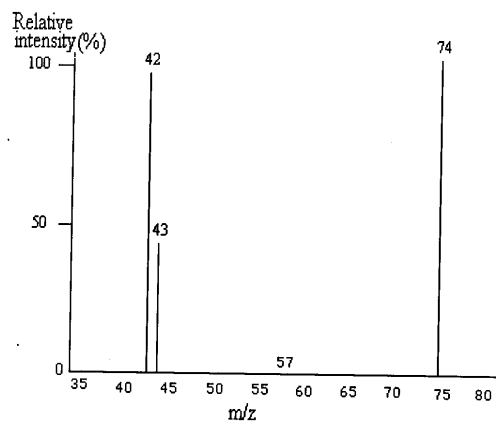
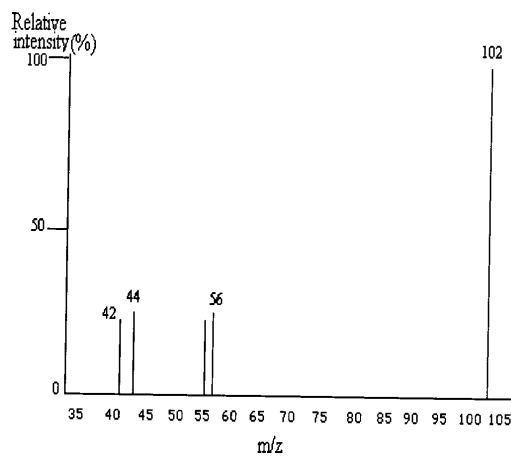**Fig. 1.** Electron impact ionization mass spectrum of NDMA.

Fig. 2. Electron impact ionization mass spectrum of NDEA.
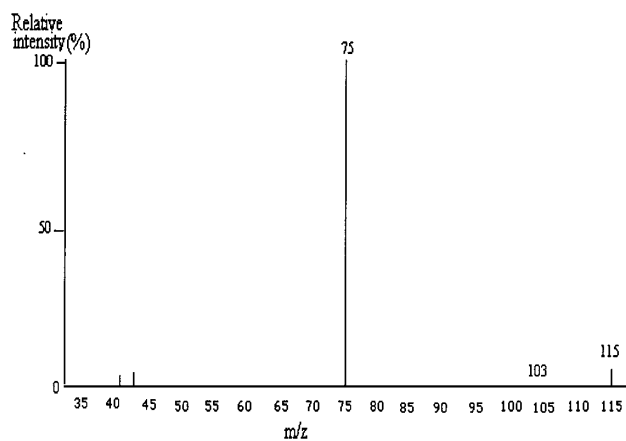

Fig. 3. Positive-ion chemical ionization mass spectrum of NDMA with methane reagent gas.
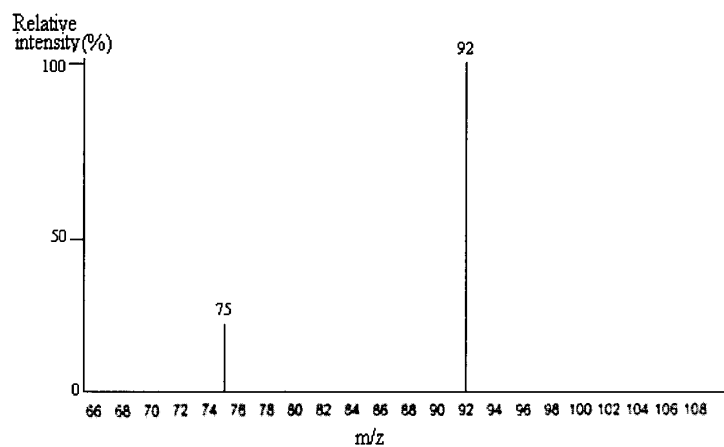
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Fig. 4. Positive-ion chemical ionization mass spectrum of NDMA with ammonia reagent gas.
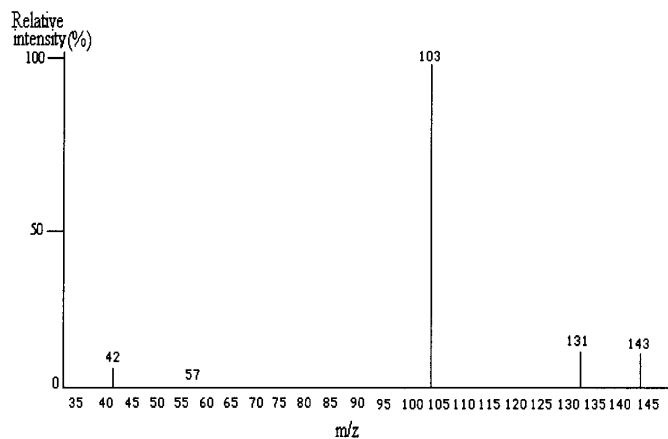

Fig. 5. Positive-ion chemical ionization mass spectrum of NDEA with methane reagent gas.
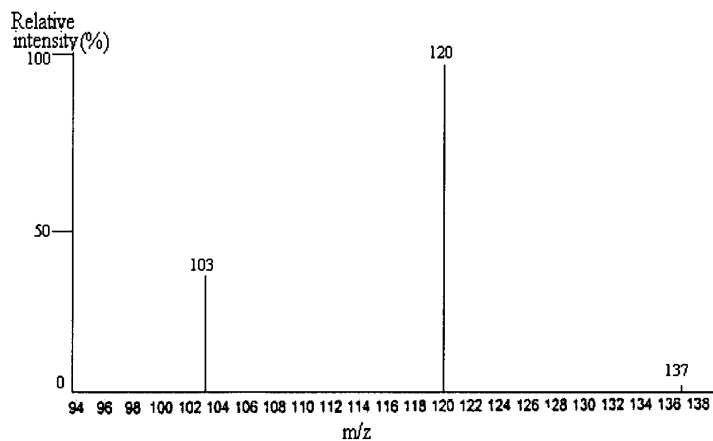

Fig. 6. Positive-ion chemical ionization mass spectrum of NDEA with ammonia reagent gas.

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Gaasikromatograafiline massispektromeetria meetod *N*-nitrosoamiinide määramiseks positiivse iooni keemilise ionisatsiooni abil

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Gaasikromatograafiline massispektromeetria meetod on välja töötatud ja optimeeritud lenduvate *N*-nitrosoamiinide määramiseks suitsutatud lihaproduktides. Meetodi puhul on kasutatud massiselektiivset detektorit HP 5973 MSD ja gaasikromatograafi HP (Hewlett-Packard) 6890 Plus GC.

Viis lenduvat *N*-nitrosoamiini – NDMA (*N*-nitrosodimetüülamiin), NDEA (*N*-nitrosodietüülamiin), NPIP (*N*-nitrosopipperidiin), NPYR (*N*-nitrosopürrolidiin) ja NDBA (*N*-nitrosodibutüülamiin) – eraldati gaasikromatograafiliselt ja identifitseeriti positiivse iooni keemilisel ionisatsioonil kahe reagentgaasiga – metaani ja ammoniaagiga. Metaani puhul esineb rohkem fragmentatsiooni ja see sobib ainult kõrgema molekulmassiga *N*-nitrosoamiinide puhul. Ammoonium annab "pehmes" ionisatsioonis vähem fragmente oma kõrgema prootonafiinsuse tõttu ning on seepärast väga spetsiifiline ja sobilik reagentgaas lenduvate *N*-nitrosoamiinide määramiseks.

Proov puhastati tahke faasi ekstraktsiooniga. Positiivse iooni keemilise ionisatsiooni, SIM režiimi ja ammoniaagi kasutamise korral reagentgaasina on avastamispiir 0,01 ppb ja saagis 70–80%.