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Rapid and Simple Extraction Method for Volatile N-Nitrosamines in Meat Products

Mohammad Al-Kaseem[*], Zaid Al-Assaf, Franswa Karabet

Department of Analytical and Food Chemistry, Faculty of Pharmacy, Damascus University, Damascus, Syria.
Email: [*]mkaseem74@gmail.com

ABSTRACT

A new methodology for extraction, pre-concentration and analysis of volatile nitrosamines in meat-derived products was developed and compared with conventional methods (Distillation and two-step solid-phase extraction). The samples (canned sausages, cured meat, luncheon and smoked meat) were treated with an aqueous sodium hydroxide (NaOH) by autoclaving at 121°C for 10 min and extracted by liquid-liquid extraction with dichloromethane, then the nitrosamines were pre-concentrated using activated silica. Then, gas chromatography coupled with flame ionization detector was used for the separation and determination of the different nitrosamines contained in a real sample and gas chromatography with mass spectrometry detection was used as the confirmation technique. The newly invented autoclaving method allowed the determination of nitrosamine compounds at trace levels with limit of detection ranged from 0.077 to 0.18 ppb and quantitation limits were from 0.26 to 0.6 ppb for all nitrosamines, and found to be superior to the conventional ones, yielding approximately about 10% - 20% increasing in the recovery compared with the mean recovery obtained when applying conventional methods.

1. Introduction

N-Nitrosamines (NAs), mainly N-nitrosodimethylamine (NDMA), and N-nitrosodiethylamine (NDEA) are receiving special attention, because they present high mutagenic and carcinogenic potential that can induce tumors in variety of organs, including the liver, lung, kidney, bladder, pancreas, esophagus and tongue depending on the species, but not in the skin, brain, colon or bone. For example, N-nitrosodimethylamine at the levels of 20 ppm can induce liver cancer in a human [1]. The International Agency for Research on Cancer (IARC) classified NDMA and NDEA as most carcinogenic to humans, and N-nitrosodi-n-butylamine (NDBA), N-nitrosopiperidine (NPIP) and N-nitrosopyrrolidine (NPYR) as possibly carcinogenic to humans [2,3]. In the diet, they occur as unintentional byproducts of food preparation, preservation and processing [4], although they can also occur in the environmental tobacco smoke [5], and may be formed endogenously within the human body [6]. These compounds are formed by the reaction of secondary amines with nitrosating agents, such as nitrates or nitrites, which are commonly used in the manufacture of meat products [4-7]. For this reason, the use of these nitrosating agents for curing meat is strictly controlled in some countries [8]. Moreover, some known inhibitors of the nitrosation reaction, such as ascorbic acid, are used in the processing of several foods [9]. The determination of nitrosamines in food and water samples has been carried out by different analytical methods, including colorimetry [10,11], capillary electro-chromatography [12], micellar electro-kinetic capillary chromatography [13], gas chromatography with flame ionization detection, nitrogen phosphorous detection, thermal energy detection, nitrogen chemiluminescence detection [14-17], and mass spectrometry detection [18-20], high-performance liquid chromatography with thermal energy analyzer, mass spectrometry and fluorescence detection [21,22]. The extraction of the nitrosamine from the complex food matrices and the cleanup of the extract have been the critical points of the sample preparation step, and several approaches are documented in the literatures, including distillation (steam or vacuum) [23-25], solvent extraction, [26] solid-phase

[*]Corresponding author.

extraction [27], solid-phase micro-extraction (SPME) [28], and supercritical fluid extraction [29].

This paper describes the development of a simple method for the extraction of volatile nitrosamines from meat products using sodium hydroxide to enhance the stability under autoclaving conditions and comparing the new method with the conventional procedures (vacuum distillation and two-step solid-phase extraction with Extrelut and Florisil sorbents).

2. Methods

2.1. Samples

All meat samples (10 samples of each of cured meat, sausage, luncheon, and smoked meat) were purchased from supermarkets in Damascus. The samples were crushed and mix with no addition of the fluid and then stored in −20°C before analysis.

2.2. Materials

EPA 521 nitrosamine mix standard was purchased from Supleco (USA), this solution contained seven analytes at 2000 μg/mL of each: N-nitrosodimethylamine (NDMA), N-nitrosomethylethylamine (NMEA), N-nitrosodiethylamine (NDEA), N nitrosopyrrolidine (NPYR), N-nitroso-din-propylamine (NDPA), N-nitrosopiperidine (NPIP) and N-nitrosodi-n-butylamine (NDBA). For sample preparation, sodium hydroxide, ethanol, Methanol, hexane, octane and dichloromethane (DCM) and Florisil were purchased from Sigma-Aldrich (USA). Florisil water sep-pak cartridges from Waters (Milford, MA, USA). Extrelut and Activated Silica Gel (silica gel 60 extra pure. 70 - 230 mesh) were obtained from Merck (Germany). All other chemicals (ascorbic acid, sulfanilic acid, sulfuric acid, anhydrous sodium sulfate and sodium chloride) used in this research were of analytical laboratory grade.

2.3. Apparatus

Pyrex tubes (20 ml) with heat-stable Teflon-lined caps and glass column (30 cm × 1.5 cm) were used. Kuderna-Danish (KD) concentrator was used for the concentration of organic solvents. Autoclave (Selecta, Spain, 4001757) and gas chromatograph equipped with a flame ionization detector controlled by computer running GC-Solution software (GC-2014, Shimadzu, Japan) and an optima-XLB Capillary Columns (0.25 mm i.d. 30 m, 0.25 μm) from (MACHEREY-NAGEL GmbH & Co) were used. Agilent gas chromatography 7890A equipped with mass selective detector MS 5975C controlled by computer running Agilent Chemstation software (Agilent Technologies, Madrid, Spain) for confirmation of the results. Local steam distillation device and glassware were used

for sample preparation.

2.4. Preparation of Nitrosamine Mix Standard

A series of working standard solutions were prepared by appropriate dilution of the EPA 521 nitrosamine mix with dichloromethane and stored at −20°C before use. From the primary stock solution 2000 μg/mL of each nitrosamine, a 1/200 dilution was done to get 100 μg/mL secondary stock solution of each nitrosamine. Sequentially dilute secondary stock solution was performed to get standards titrating at 4, 10, 50, 100, 500, 1000 μg/L in by taking the following volumes from secondary stock solution, 40 μl, 100 μl, 500 μl, 1000 μl, 5 mL, 10 mL in series of 100 mL volumetric flasks respectively, these solution kept in the absence of light.

2.5. Analytical Conditions

GC analysis was carried out using GC-2014 gas chromatograph equipped with flame ionization. (GC-FID) (Shimadzo Technologies, Japan). One microliter of the extracted solution sample was injected into optima-XLB Capillary Column (30 m × 0.25 mm I.D. × 0.25 film thickness (df)). For the gas chromatograph separation of N-nitrosamines, the injection port and detector temperature were kept at 250°C and 300°C. The oven program was as follows: 40°C, held for 3 min; ramp to 100°C at 10°C/min, held isothermally for 1 min; ramp to 250°C at 15°C/min, held isothermally at 250°C for 2 min. The sample was injected in splitless mode; using an injection volume of 1 μl. Nitrogen was used as make-up gas. The purity of all gases used was greater than 99.999%. The velocity of the helium carrier was 1 mL/min. GC-MS were carried out to confirmation of the results using an optima XLB column (30 m × 0.25 mm I.D. × 0.25 film thickness (df) column with same conditions used in GC-FID and the injector and transfer line temperature in GC-MS were 240°C and 275°C, respectively. The ion source temperature was 240°C and operated in positive electron ionization (EI) 70 eV mode.

2.6. Conditions for Autoclave Treatment

In this study, half ml of the N-nitrosamine Mix standard containing 50 μg/L of each nitrosamine was diluted with 10 mL of 0, 0.5, 1 N, 2 N aqueous sodium hydroxide and 10 mg of ascorbic acid (Inhibitor of endogenous nitrosation) in 20 mL Pyrex tubes tightly capped and autoclaved at 121°C for 5, 10, 30 min. After being allowed to stand at room temperature, the autoclaved solution was transferred to 50 mL separatory funnel. The tubes was rinsed twice with 5 mL of ethanol and then 10 mL of dichloromethane, and the rinsing solutions and 10 mL of 10% aqueous sodium chloride were combined with the origi-

nal extract in the separatory funnel. After being shaken, the dichloromethane layer was collected, and the water layer was re-extracted with 10 ml of dichloromethane. The dichloromethane extracts were combined, dried over anhydrous sodium sulfate and concentrated to approximately 0.5 mL using KD concentrator and nitrogen gas flow. The concentrate was loaded onto a silica gel column (30 cm × 1.5 cm) (equilibrated with dichloromethane) and the column was eluted with 10 ml of dichloromethane. After the addition of 100 μL of octane (to prevent exsiccation of the solvent), the elute was concentrated to 1 mL using KD concentrator and nitrogen gas flow and analyzed by GC-FID using 1 μL injection volume, and confirmatory analyses were performed on GC-MS.

2.7. Extraction of N-Nitrosamine from Meat

2.7.1. Autoclave Treatment
Approximately one gram of meat sample was placed in the Pyrex tube into which 10 mL of sodium hydroxide 1 N was poured. The tube was capped tightly and autoclaved at 121°C for 5, 10 and 30 min; dichloromethane extract was obtained by the procedure described for the standard solution in (Section 2.6). The individual extracts were analyzed by GC-FID, GC-MS as in the case of the standard solution (Section 2.5).

2.7.2. Steam Distillation
Extraction was done as described by Komarova and Velikanov (2000) [22]. A weighed portion (80 - 100 g) of a meat reduced in a homogenizer was placed in a 500-mL round-bottom flask connected with a steam generator and a condenser. Distilled water (100 - 150 mL depending on the humidity of the foodstuff) and 0.1 mL of 50 ppb solution of each nitrosamine were added to the foodstuff and stirred. Next, 10 g of sodium chloride, 10 g of sodium or magnesium sulfate, 5 mL of a 2% sulfanilic acid solution (to inhibit endogenous nitrosation), and 10 mL of a 1 N sulfuric acid solution were added, and N-nitrosamines were steam distilled off collecting 250 mL of the distillate. The distillate was transferred into a separatory funnel, and N-nitrosamines were extracted with dichloromethane five times (with portions of 10 mL each). Each portion of the extract was passed through a funnel with a red-ribbon paper filter filled with 5 g of anhydrous sodium sulfate. The filter was washed with 10 mL of the extractant. All portions of the extract were combined and condensate using KD to 1 ml and analyzed using GC-FID.

2.7.3. Two-Step Solid-Phase Extraction
Extraction of nitrosamines was performed using the same method as Yurchenko and Molder (2007) [31]. A two-step solid-phase extraction using Extrelut and Florisil

sorbents was used for sample cleaning. Each sample (6.0 ± 1.0 g) was minced and mixed with 0.1 mol·L^{-1} NaOH (6 mL). For the first step, 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). Then, the sample was eluted with two 20 mL portions of hexane/dichloromethane solution 40:60 (v: v). the eluate was collected in a 50 mL concentrator flask and evaporated under a nitrogen stream. During the second step, 1 g of Florisil was placed at the bottom of the Florisil cartridge (6.5 cm × 1.3 cm) and wetted with 6 mL dichloromethane/methanol 95:5 (v:v). Then the sample solution was eluted with 6 mL dichloromethane/methanol solution 95:5 (v: v). the solution was evaporated under a nitrogen stream to about 1 mL. The prepared solution was transferred to the GC auto sampler vial. Extractions were performed in triplicate [30].

3. Results and Discussion

3.1. Optimization of Conditions for Autoclave Treatment

Figure 1 shows a chromatogram of the N-nitrosamines standard solution obtained by GC-FID. The total recovery of nitrosamine for the autoclave treatment in water ranged from 61.1% to 98% depending on the heating period as shown in **Table 1**. This indicates that N-nitrosamines remained almost stable during the autoclave treatment because of the stability of N-nitrosamines under the alkaline conditions and difficulty to destroy them under these conditions [31], however, the total recovery of N-nitrosamines slightly decreased as the NaOH concentration increased or the heating period elongated. To clarify the reason for the decrease in the total N-nitrosamine recovery, the volatile nitrosamines in **Figure 1** were analyzed and the results are shown in **Table 2**.

The recoveries of some N-nitrosamines with rather short retention times such NDMA and NDEA were satisfactory under any conditions. In contrast, the decrease in the recovery was notable for the peaks with much longer retention times as the NaOH concentration increased or the heating period became longer. Since NPYR and NPIP giving longer retention times that, the decrease in the total N-nitrosamines recovery would result from the instability under the conditions of high NaOH concentration and lasted heating at 121°C. Consequently, it was proposed that samples for N-nitrosamines analysis should be treated with 1 N NaOH for 10 min.

3.2. Efficacy of Autoclave Treatment for Extraction of N-Nitrosamines from Meat

Table 3 shows the peak areas and total peak areas of N-nitrosamines for all extraction procedures, the peak areas
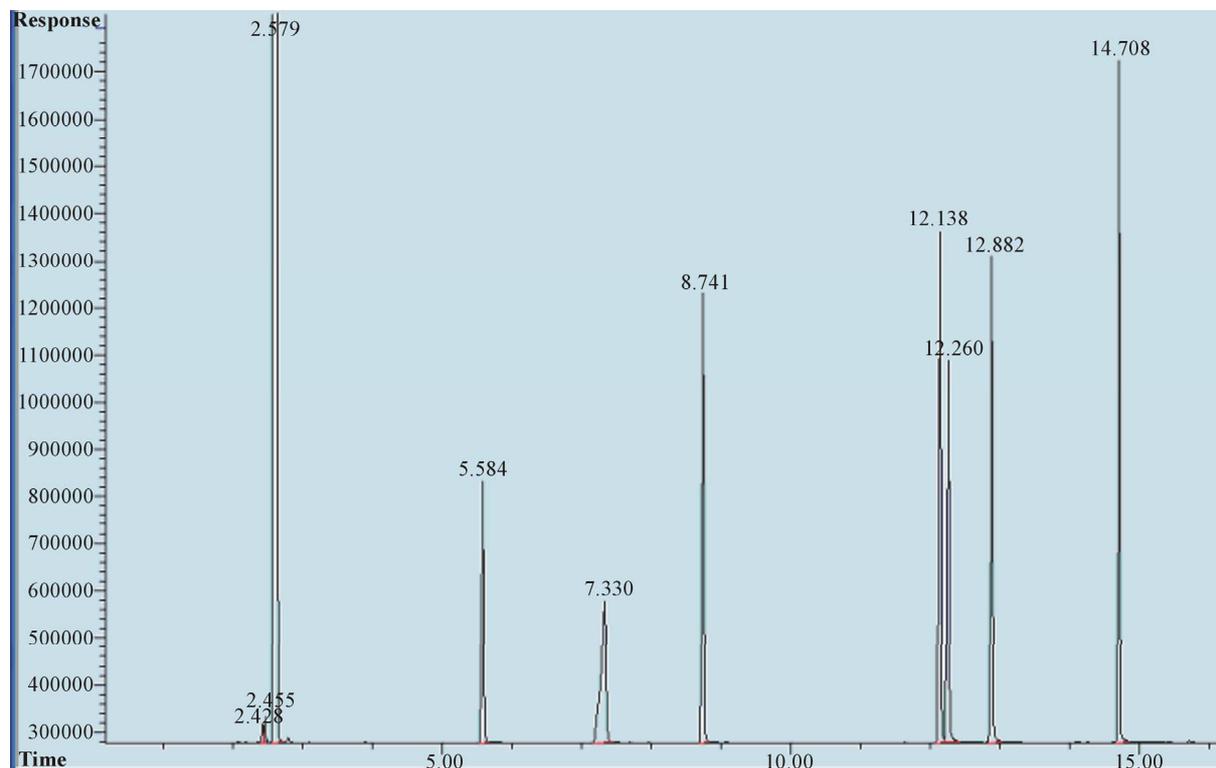
Figure 1. Chromatogram of 50 µg/L of N-nitrosamine mix (GC-FID).

Table 1. Effect of alkali concentration and autoclaving times on total recovery of N-nitrosamine.

Alkali concentration	Autoclaving time (min)					
	5		10		30	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
0 N NaOH (water)	61.1	6.8	76	8.9	62.7	9.24
0.5 N NaOH	85.9	2.7	97.8	4.3	87.9	8.5
1 N NaOH	88.4	2.8	98.1	1.05	88.4	3.1
2 N NaOH	87.2	5.6	87.6	4.6	88.5	6.4

Each value represents mean (S) and relative standard deviation (RSD) in three experiments.

Table 2. Effect of alkali concentration and autoclaving times on recovery of N-nitrosamines.

NA	Autoclaving time (min)								
	5 min	10 min	15 min	5 min	10 min	15 min	5 min	10 min	15 min
	With NaOH 0.5 N (Recovery %)			With NaOH 1 N (Recovery %)			With NaOH 2 N (Recovery %)		
NDMA	80.0	89.7	88.9	90.2	105.5	93.7	88.4	80.7	80.6
NMEA	88	89	76	67	89	89	76	73	89
NDEA	85.6	87.8	85.6	80.1	102.3	91.4	88.5	79.3	80.5
NPYR	77.1	79.0	77.1	80.2	99.1	90.2	86.9	75.3	70.3
NDPA	80.6	86.3	76.4	80.3	98.5	93.8	86.4	77.1	83.6
NPIP	73.2	80.2	75.3	86.3	99.3	88.9	83.4	77.3	86.1
NDBA	69.9	73.7	69.3	80.3	92.1	88.3	85.3	82.9	71.6

Each value represent mean of three experiments, N; normality, N-nitrosodimethylamine (NDMA), N-nitrosomethylethylamine (NMEA), N-nitrosodiethylamine (NDEA), N nitrosopyrrolidine (NPYR), N-nitrosodin-propylamine (NDPA), N-nitrosopiperidine (NPIP) and N-nitrosodi-n-butylamine (NDBA).

Table 3. Determination of N-nitrosamines with and without autoclave treatment, and with conventional methods.

NAs	RT (min)	With autoclave treatment		Without autoclave treatment		Distillation method		Two step solid phase extraction	
		Area	Relative ratio*	Area	RV*	Area	RV*	Area	RV*
NDMA	5.617	203348	0.08	103456	0.51	120087	0.59	180323	0.88
NMEA	7.344	287513	0.113	123456	0.43	160000	0.55	247909	0.86
NDEA	8.733	353106	0.139	201345	0.57	98000	0.27	314876	0.89
NPYR	12.104	442116	0.175	286776	0.65	121564	0.27	399887	0.90
NDPA	12.224	355971	0.14	173456	0.49	123546	0.34	291006	0.81
NPIP	12.853	400387	0.158	127999	0.32	259099	0.64	328987	0.82
NDBA	14.681	483347	0.191	254888	0.53	178988	0.37	393000	0.81
total		2525788	1	1271376		1061284		2155988	

Relative ratio*: area/total area, RV*: peak area as the percentages of that of the autoclave treatment, NAs: nitrosamines, RT: retention time.

after the conventional treatments were shown as the percentages of the peak areas corresponding of autoclave treatment. All N-nitrosamines tested were detected after autoclaving. The peaks of N-nitrosamines were sharp and completely separated, and there is an increasing of the recovery (expressed as peak area) after autoclave treatment compared with the same sample when extracted without an autoclaving, or by conventional methods. Obviously, the relative percentage of peak area for each nitrosamine (**Table 3**) under autoclaving conditions was as twice as that of extraction without autoclaving or by distillation, and it was about 10% - 20% more than that of the two-step solid-phase extraction.

3.3. Method Validation

The new method was validated to demonstrate that it is suitable for its intended purpose by the standard procedure to evaluate adequate validation characteristics [30, 32]. Retention times of selected N-nitrosamines was determined by using standard solutions of each nitrosamine, and the confirmation was performed using GC-MS. **Figure 1** shows the chromatogram obtained by GC-FID of 50 ppb (part per billion) standard solution of each N-nitrosamines. **Figure 2** shows the chromatogram obtained by unspiked meat sample. **Table 4** shows the confirmation results of seven nitrosamines obtained when analyzing the same solution and identifying the retention times using GC-MS. In order to examine the linearity a series of working standard solutions at the concentrations of 4, 10, 50, 100, 500, 1000 µg/L of each N-nitrosamine were prepared and analyzed under the selected chromatographic and flame ionization detector conditions. The linear equations, linear ranges and correlation coefficients for the seven nitrosamines are shown in **Table 4**. The limits of quantification with a signal-to-noise ratio of 10 for the seven nitrosamines were from 0.26 to 0.6 ppb with limits of detection with a signal-to-noise ratio of 3 for the seven nitrosamines were from 0.077 to 0.18 ppb.

The recovery and precision experiments were conducted by spiking different blank samples at three spiking levels of 1, 5, 10 µg/L, six replicates at each level, the mean recoveries were from 89% - 105.5% with the relative standard deviations (RSDs) from 2.8% to 4.4%.

3.4. Sample Results

Meat samples were treated with 1 N NaOH for 10 min in autoclaved conditions and then analyzed by GC-FID and the results are shown in **Table 5**. The results of 40 samples of different meat products which examined for the test of the extraction procedure shows the presence of some volatile nitrosamine in all different products, and the absence of NDEA, NDPA, and NDBA in all the sample tested which may related to the presence of these three nitrosamines in low amounts which may below under the quantitation limit of our procedure, or the real absence of these nitrosamine in the sample tested. Statistically when applying (PASW Statistics 18) one way ANOVA test for statistical comparisons between the means of sum of seven nitrosamines in meat samples, there was significant difference ($p > 0.05$) which may related to the low number of different samples, or because that some of tested samples are commonly preserved by nitrite salts (sausages and luncheon) which contributing in nitrosamine formation and the effect of processing (smoking) in the formation of these carcinogenic compounds in smoked meat.

4. Conclusion

It was included in this paper that the extent of recovery of N-nitrosamines from the meat products was highly dependent on the extraction conditions, and it was concluded that extraction recovery could be increased when adopting the autoclave conditions and sodium hydroxide to increase the stability of volatile nitrosamine, and adding ascorbic acid to inhabit endogenous nitrosation which may occur during extraction. We recommend the newly
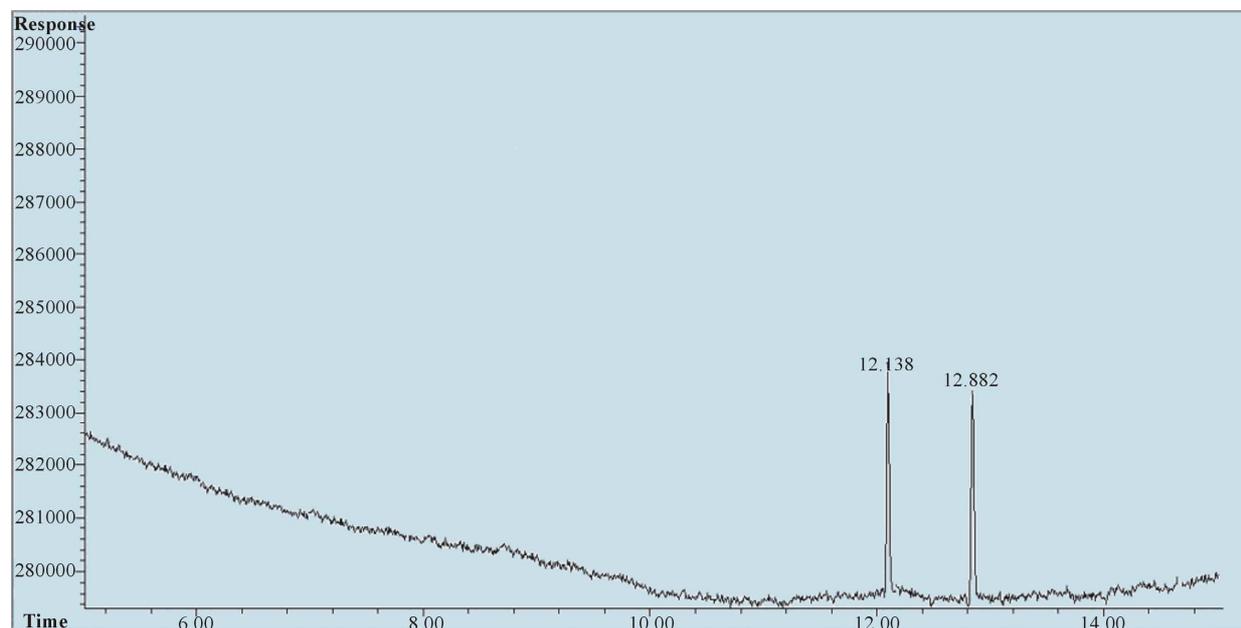
Figure 2. Chromatogram of unspiked meat sample.

Table 4. Linear equations, linear ranges and correlation coefficients of seven volatile nitrosamines.

N-nitrosamine	RT[*] GC-FID	Confirmation RT[*] GC-MS	Linear equation	Linear range µg/L	Correlation coefficients	LOQ µg/kg	LOD µg/kg
NDMA	5.617	5.617	$y = 4161.7x - 9274.8$	0.6 - 500	0.9997	0.6	0.18
NMEA	7.344	7.344	$y = 5816.1x - 14015$	0.43 - 500	0.9994	0.43	0.13
NDEA	8.733	8.733	$y = 7095.3x - 13214$	0.33 - 500	0.9996	0.33	0.1
NPYR	12.104	12.104	$y = 8699.3x - 8839.9$	0.26 - 500	0.9995	0.26	0.08
NDPA	12.224	12.224	$y = 6674.6x - 3555.2$	0.25 - 500	0.9981	0.25	0.077
NPIP	12.853	12.853	$y = 7992.3x - 141000$	0.34 - 500	0.9995	0.34	0.105
NDBA	14.681	14.681	$y = 9805.1x - 16361$	0.30 - 500	0.9998	0.309	0.093

RT[*]: retention time (minutes), GC-FID: gas chromatography-flame ionization detection, LOQ: limit of quantitation, LOD: limit of detection.

Table 5. Results on nitrosamine results in meat samples.

products	No. of samples	Mean concentration (n = 3) of N-nitrosamine µg/kg						Sum of seven N-nitrosamine µg/kg
		NDMA	NDEA	NDPA	NDBA	NPYR	NPIP	
Sausage	10	nd[*]	nd[*]	nd[*]	nd[*]	2.78	2.98	4.76
Cured Meat	10	nd[*]	nd[*]	nd[*]	nd[*]	3.2	nd	3.20
Luncheon	10	nd[*]	nd[*]	nd[*]	nd[*]	2.60	nd	2.60
Smoked meat	10	nd[*]	nd[*]	nd[*]	nd[*]	3.70	2.60	6.60

nd[*]: not detected.

invented method which employs an autoclave for the estimation of volatile N-nitrosamines contents in meat products.

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