

Determination of Seven Volatile N-Nitrosamines in Fast Food

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Received December 13th, 2013; revised January 14th, 2014; accepted January 29th, 2014

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ABSTRACT

In this study, various fast food samples, including raw meat, cured meat, grilled meat, fried meat, smoked meat and canned meat products, were analyzed for the determination of the seven volatile N-nitrosamines. The samples were treated with an aqueous sodium hydroxide (NaOH) by autoclaving at 121°C for 10 min and extracted by liquid-liquid extraction with dichloromethane, and then the nitrosamines were pre-concentrated by 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. The applied method allowed the determination of nitrosamine compounds at trace levels with limit of detection ranging from 0.077 to 0.18 ppb, and quantitation limits were from 0.26 to 0.60 ppb for all nitrosamines. The total concentrations of the seven nitrosamines in the studied meat samples ranged from 2.60 to 13.28 µg/kg.

KEYWORDS

GC-FID; N-Nitrosamines; Fast Food; Meat; Autoclave

1. Introduction

Nitrite is one of the very important additives in the meat production process in terms of desirable color, texture, lipid oxidation, and especially for preventing a toxin formation by *Clostridium botulinum*. Recent evidence has suggested that the nitrite is bactericidal for gastrointestinal, oral and skin pathogenic bacteria when ingested and mixed with gastric acid. But significant concerns exist because nitrite may react with amines and amino acids to produce N-nitrosamines, which are known to be carcinogenic, mutagenic and teratogenic [1]. 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 [2]. Investigations carried out at various research centers, and food controlling institutions have shown that many food products are contaminated with nitroso compounds, as the result of various technological processes in which they form in slightly acidic environments by

reaction of sodium nitrite and nitrogen oxide with such precursors as proteins, peptides, amino acids and amines present in food [3]. For this reason, the use of these nitrosating agents for curing meat is strictly controlled in some countries [4]. Moreover, some known inhibitors of the nitrosation reaction, such as ascorbic acid, are used in the processing of several foods [5,6]. N-nitrosamines are formed after cooking of meat in a complex process that is influenced by a large variety of substances [7]. The main precursors of N-nitrosamines are believed to be formed by reaction of nitrogen oxides (that are generated from nitrites and are also present in wood smoke) with mainly secondary amines present in the meat [8], although they can also occur in the environmental tobacco smoke [9] and may be formed endogenously within the human body [10]. The International Agency for Research on Cancer (IARC) classified N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) as most carcinogenic to humans, and N-nitrosodi-n-butylamine (NDBA), N-ni-

trospiperidine (NPIP) and N-nitrosopyrrolidine (NPYR) as possibly carcinogenic to humans [11]. The determination of nitrosamines in food and water samples has been carried out by different analytical methods [12], including colorimetry [13], capillary electro-chromatography [14], micellar electro-kinetic capillary chromatography [15], gas chromatography with flame ionization detection, nitrogen phosphorous detection, thermal energy detection, nitrogen chemiluminescence detection [16-19], mass spectrometry detection [20-22], high-performance liquid chromatography with thermal energy analyzer, mass spectrometry and fluorescence detection [23,24]. 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) [25-27], solvent extraction [28], solid-phase extraction [29], solid-phase micro-extraction (SPME) [30] and supercritical fluid extraction [31].

The purpose of the present study was to measure the content of seven volatile N-nitrosamines in meat products (fast food) in Syria by using the method of sample preparation performed in our previous work [32], and gas chromatography with flame ionization detector (GC-FID) for the separation and determination of nitrosamines in samples which are collected from fast food restaurants in Damascus and Damascus countryside.

2. Methods

2.1. Samples

All samples of meat products used in this research were purchased from fast food restaurants (cooked or as provided to consumers) in Damascus and Damascus countryside. Information about the kind of meat and the

numbers of each sample was collected and presented in **Table 1**. 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 $2000\ \mu\text{g/mL}$ of each: 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). For sample preparation, sodium hydroxide, ethanol, octane and dichloromethane (DCM) were purchased from Sigma-Aldrich (USA). All other chemicals (ascorbic 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 were used. Glass column ($30\ \text{cm} \times 1.5\ \text{cm}$). 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 ($0.25\ \text{mm i.d. } 30\ \text{m}, 0.25\ \mu\text{m}$) from (MACHEREY-NAGEL GmbH & Co) were used. CAMAG UV Lamp dual wavelength, $254/366\ \text{nm}$, 2×8 watt in combination (Switzerland).

2.4. Preparation of Nitrosamine Mix Standard

A series of working standard solutions were prepared by

Table 1. Information about samples collected from fast food restaurants.

Products		No. Of Samples	notes
Raw meat	Beef	10	Uncooked- raw meat for fast food
	Poultry	10	Uncooked-raw meat for fast food
Cured meat	Beef	10	Preserved with sodium nitrite
	Sausage	30	Preserved with sodium nitrite
Smoked meat	Luncheon	30	Preserved with sodium nitrite
	Sausage	20	With paprika pepper – no preservatives
Grilled meat	Poultry	20	With paprika pepper - no preservatives
	Poultry	20	With paprika pepper -no preservatives
Canned meat	Beef	20	Preserved with sodium nitrite
	Luncheon	10	Preserved with sodium nitrite

appropriate dilution of the EPA 521 nitrosamine mix with dichloromethane and stored at -20°C before use. From the primary stock solution $2000\ \mu\text{g/mL}$ of each nitrosamine, a 1/200 dilution was done to get $100\ \mu\text{g/mL}$ secondary stock solution of each nitrosamine. Sequentially dilute secondary stock solution was performed to get standards titrating at 4, 10, 50, 100, 500, 1000 $\mu\text{g/L}$ 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) (Shimadzu Technologies, Japan). One micro liter of the extracted sample solution was injected into optima-XLB Capillary Column (30 m \times 0.25 mm I.D. \times 0.25 film thickness (df)). For the gas chromatograph separation of N-nitrosamines, the injection port and detector temperature were kept at 250 and 300°C . The oven program was as follows: 40°C , held for 3 min; ramp to 100°C at $10^{\circ}\text{C}/\text{min}$, held isothermally for 1 min; ramp to 250°C at $15^{\circ}\text{C}/\text{min}$, 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.

2.6. Conditions for Autoclave Treatment

Conditions for autoclave treatment were adopted as our previous study (Kaseem, Assaf and Karabeet, 2013) [32], half milliliter of the N-nitrosamine Mix standard containing 50 $\mu\text{g/L}$ of each nitrosamine was diluted with 10 mL of 1 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 10 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 original 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 \times 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.

2.7. Extraction of N-Nitrosamine from Meat

The method of preparation of meat samples was described in our previous work [32]. Approximately one gram of meat sample was placed in the Pyrex tube into which 10 mL of sodium hydroxide 1N was poured. The tube was capped tightly and autoclaved at 121°C for 10 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 as in the case of the standard solution (Section 2.5).

2.8. Method Validations

The matrix effect was studied by comparing the slope of the aqueous standards and standard additions calibration graphs obtained for the sex different fast food samples, namely, raw meat, cured meat, smoked meat, grilled meat, fried meat and canned meat, no statistically differences were observed, and so quantification was carried out by external calibration. Calibration curves were obtained by least squares linear regression analysis for the peak area versus the analyte concentration using six concentration levels in duplicate. The method was validated to demonstrate that it is suitable for its intended purpose by the standard procedure to evaluate adequate validation characteristics [33]. Retention times of selected N-nitrosamines were determined by using standard solutions of each nitrosamine. **Figure 1** shows the chromatogram obtained by GC-FID of 50 ppb (part per billion) standard solution of each N-nitrosamine. **Figure 2** shows the chromatogram obtained by unspiked meat sample.

2.8.1. Linear Range, Limit of Quantification (LOQ) and Limit of Detection (LOD)

A series of working standards at the concentrations of 4, 10, 50, 100, 500 and 1000 $\mu\text{g/L}$ were prepared and analyzed under the selected chromatographic conditions. The linear equations and the correlation coefficients for the seven N-nitrosamines are shown in **Table 2**. The limits of quantification with a signal-to-noise ratio of 10 for the seven nitrosamines were from 0.26 to 0.6 ppb ($\mu\text{g}/\text{kg}$) with limits of detection with a signal-to-noise ratio of 3 for the seven nitrosamines were from 0.077 to 0.18 ppb.

2.8.2. Recovery and Precision

The recovery and precision were conducted by spiking different blank meat samples including, raw meat, cured meat, smoked meat, grilled meat, fried meat, and canned meat. At three spiking levels of 1, 5, 10 $\mu\text{g/L}$ to the

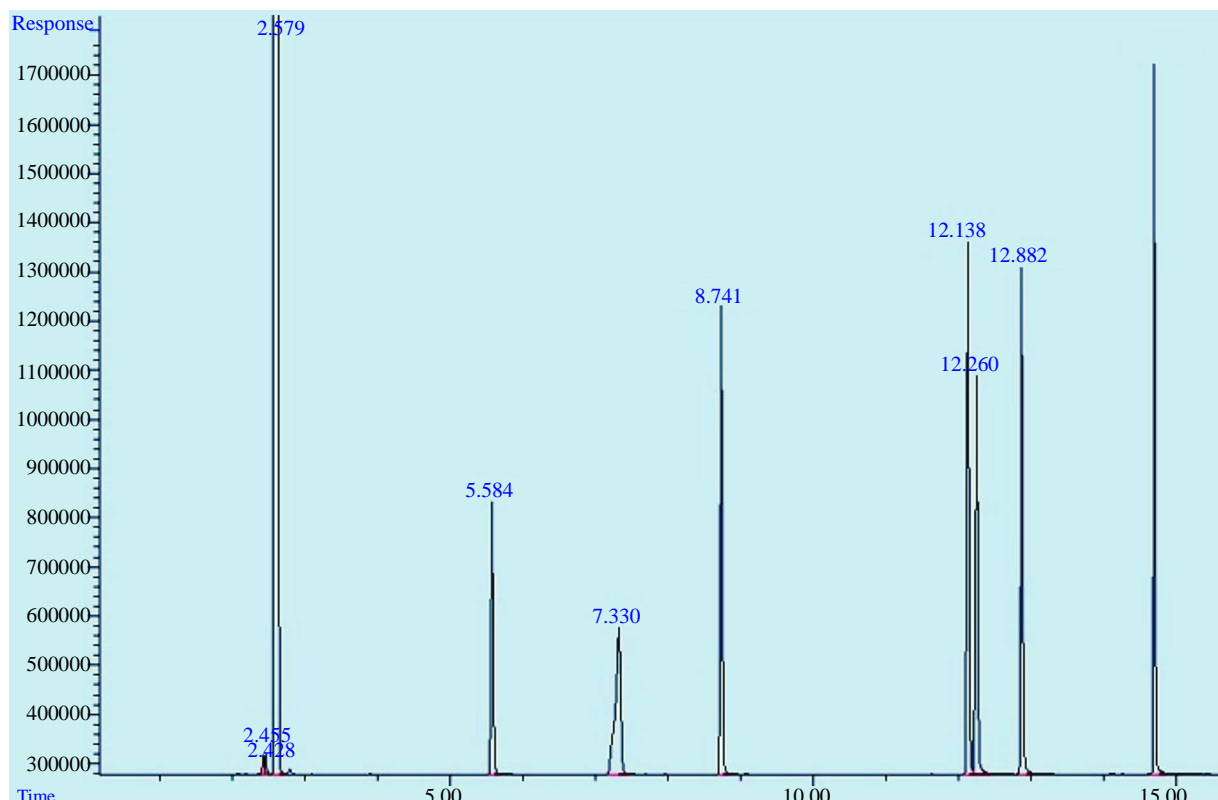


Figure 1. Chromatogram of 50 µg/L of N-nitrosamine Mix (GC-FID).

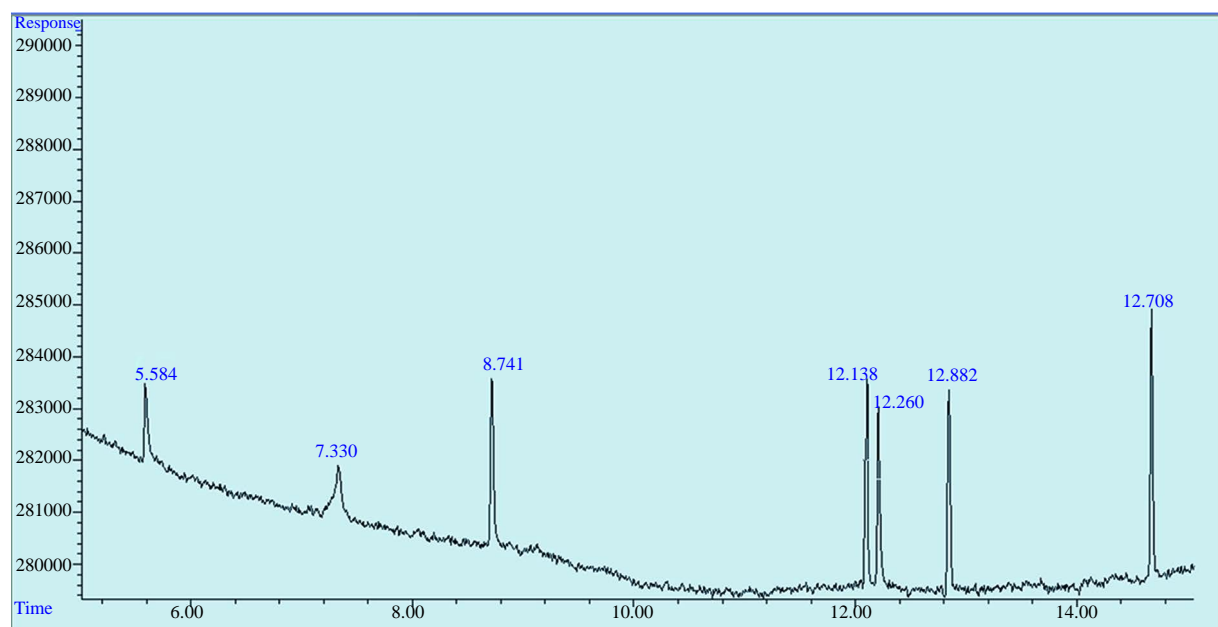


Figure 2. Chromatogram of spiked fried meat sample.

prepared samples, six replicates at each level, the mean recoveries were from 82% to 105.5% with the relative standard deviations (RSDs) from 2.8% to 4.4%. The results are shown in Table 3 and spiked sample chromatogram is shown in Figure 2.

2.8.3. Selectivity

The selectivity of the method was judged from the absence from the interfering peaks (false peaks) at the analyte elution times for blank chromatograms of different unspiked samples after irradiating them to UV light

Table 2. Linear equations, linear ranges and correlation coefficients of seven volatile nitrosamines.

N-nitrosamine	RT* GC-FID	Linear equation	Linear range µg/L	Correlation Coefficients	LOQ µg/kg	LOD µg/kg
NDMA	5.617	$y = 4161.7x - 9274.8$	0.6 - 500	0.9997	0.6	0.18
NMEA	7.344	$y = 5816.1x - 14015$	0.43 - 500	0.9994	0.43	0.13
NDEA	8.733	$y = 7095.3x - 13214$	0.33 - 500	0.9996	0.33	0.1
NPYR	12.104	$y = 8699.3x - 8839.9$	0.26 - 500	0.9995	0.26	0.08
NDPA	12.224	$y = 6674.6x - 3555.2$	0.25 - 500	0.9981	0.25	0.077
NPIP	12.853	$y = 7992.3x - 141000$	0.34 - 500	0.9995	0.34	0.105
NDBA	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 3. Results of spiked recoveries for seven volatile nitrosamines in meat samples (n = 6).

NA	Spiked Level (µg/L)	Raw meat		Cured meat		Grilled meat		Fried meat		Canned meat		Smoked meat	
		Recovery	RSD	Recovery	RSD	Recovery	RSD	Recovery	RSD	Recovery	RSD	Recovery	RSD
NDMA	1	98.00	2.82	82.20	2.81	84.45	2.92	85.00	2.89	82.09	3.00	83.90	3.09
	5	91.00	2.86	84.50	3.09	87.56	3.01	87.34	2.98	83.67	3.04	82.01	4.06
	10	89.10	3.23	88.08	3.12	90.98	3.11	90.88	3.18	89.00	3.30	89.90	3.98
NMEA	1	102.00	3.87	100.89	3.13	101.78	3.12	89.07	3.17	100.09	3.34	100.00	4.40
	5	89.90	3.98	88.09	3.76	85.21	3.80	85.51	3.79	89.07	3.90	89.00	4.23
	10	89.00	2.99	90.05	4.10	90.45	3.00	91.00	2.93	88.99	3.09	89.50	3.09
NDEA	1	105.50	4.1	91.89	4.39	102.10	4.22	102.09	4.29	104.9	4.34	91.89	4.39
	5	89.20	4.23	98.06	2.98	93.08	3.01	94.01	3.04	90.05	2.99	90.09	4.11
	10	82.01	4.06	89.89	2.89	96.06	2.90	95.90	2.91	83.04	3.03	83.03	4.34
NPYR	1	100.00	4.40	83.03	4.34	100.90	4.40	101.01	4.35	100.90	4.40	101.01	4.27
	5	89.00	4.23	90.89	4.09	90.08	4.08	90.10	4.01	90.00	3.88	90.07	4.09
	10	89.70	3.88	88.78	4.39	90.78	4.30	91.02	4.29	90.09	4.11	89.98	4.07
NDPA	1	101.00	3.98	95.01	4.09	101.05	4.19	100.99	4.20	101.01	4.27	99.98	4.40
	5	94.00	4.38	99.08	4.39	94.09	4.28	94.80	4.32	95.00	4.40	94.77	4.38
	10	92.00	4.31	99.67	4.33	92.78	4.38	93.03	4.12	92.93	4.20	93.01	4.19
NPIP	1	101.00	3.89	100.78	2.89	102.01	2.99	101.99	3.09	99.98	3.10	99.77	3.09
	5	90.10	3.06	91.89	2.95	90.09	2.98	91.01	3.06	90.87	3.10	91.00	3.07
	10	89.50	3.09	102.45	3.01	90.05	3.00	89.90	2.91	90.07	3.02	90.15	3.13
NDBA	1	89.30	3.87	89.98	4.09	90.01	4.08	89.97	3.99	90.81	4.04	89.95	4.23
	5	92.60	3.56	85.34	4.31	93.00	4.34	94.00	4.40	91.05	4.38	88.99	4.39
	10	94.00	3.58	88.23	4.08	94.06	4.10	93.77	4.09	94.00	4.08	95.01	4.40

NA; nitrosamine, Recovery and RSD are expressed as (%).

(wavelength 366 nanometer) for three hours to destroy nitrosamines if they are present [34]. The original signals were wrongly positive if the signals from the samples are

not remarkably diminished after the irradiation. No matrix compounds existed that might give a false positive signal to the blank samples as shown in [Figure 3](#).

3. Results and Discussion

In this work, the level of seven nitrosamines, namely, NDMA, DNEA, NDPA, DDBA, NMEA, DPYR and NPIP, in 180 samples of most popular meat products in fast food resultants in Syria was determined. Data on nitrosamines level in various samples are given in **Table 4**. The total concentrations of the seven nitrosamines in the studied meat samples ranged from 2.60 to 13.28 $\mu\text{g}/\text{kg}$. The highest levels of nitrosamines were found in fried and smoked meat. Relatively high level was found in grilled meat. In the opposite the low level of nitrosamine was detected in cured meat and canned meat. Nitrosamines were not detected in raw meat (beef and poultry) and this result might be related to the absence of preservatives (Sodium nitrite) and effect of cooking, which may promote nitrosamine formation. The level of

total nitrosamines in canned meat (beef and luncheon) was lower than the level in all other samples including the cured meat (cured beef). The level of total nitrosamines in fried meat (poultry) was very high and exceeds 13 $\mu\text{g}/\text{kg}$, the concentration of NDMA exceeds 1 $\mu\text{g}/\text{kg}$ in fried and smoked meat, while NPYR and NPIP were detected in all positive samples with high concentrations ranged between 1.12 and 8.40 $\mu\text{g}/\text{kg}$ for NPYR, and from 0.25 and 1.65 $\mu\text{g}/\text{kg}$ for NPIP. The variations in the levels of total nitrosamines in the tested samples may related to many factors including cooking methods, time and temperature of cooking [12], food additives especially sodium nitrite and to the other ingredients of the meal (paprika pepper) [35]. This can explain the negative results which obtained for 20 samples of raw meat because of the absence of all these factors, while the high level of nitrosamines in fried meat may be related to the

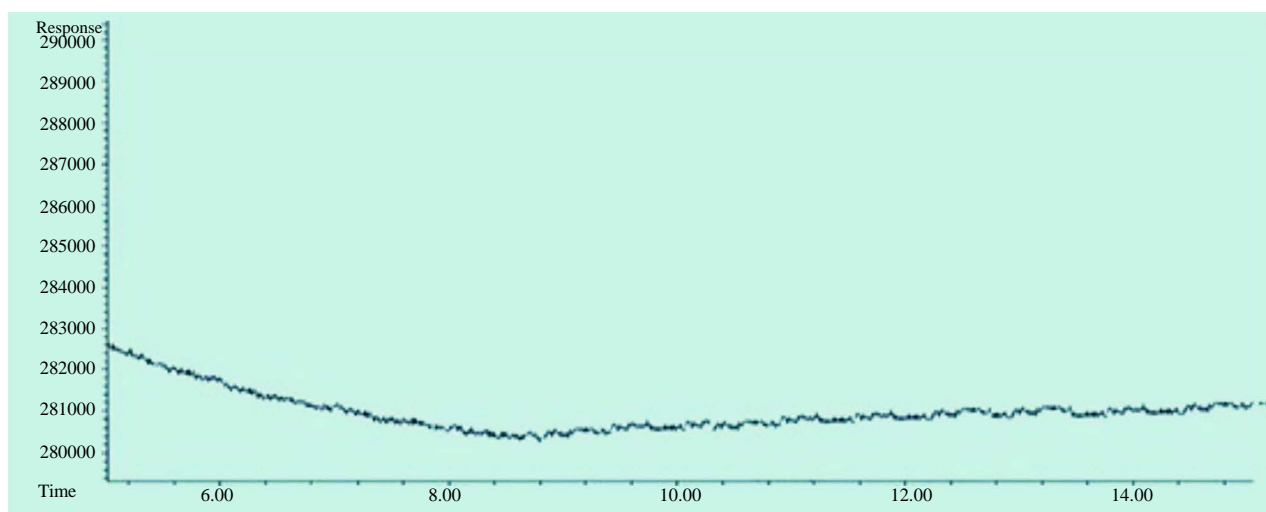


Figure 3. Chromatogram of unspiked fried meat sample after irritation (366 nanometer, 3 hours).

Table 4. Results on nitrosamine results in meat samples.

Products	No. of Samples	Mean concentration (n = 3) of N-nitrosamine $\mu\text{g}/\text{kg}$						Sum of seven N-nitrosamine $\mu\text{g}/\text{kg}$	
		NDMA	NDEA	NDPA	NDBA	NPYR	NPIP		
Raw meat	Beef	10	nd*	nd*	nd*	nd*	nd*	nd*	nd*
	Poultry	10	nd*	nd*	nd*	nd*	nd*	nd*	nd*
Cured meat	Beef	10	nd*	nd*	nd*	nd*	3.20	nd*	3.20
	Sausage	30	1.12	0.66	nd*	nd*	4.20	1.13	7.11
Smoked meat	Luncheon	30	1.05	0.54	0.28	0.31	5.12	1.65	8.95
	Sausage	20	0.89	0.34	nd*	0.32	2.50	0.98	5.03
Grilled meat	Poultry	20	0.78	0.38	nd*	0.42	1.12	0.75	3.45
	Poultry	20	1.16	1.23	0.81	0.45	8.40	1.23	13.28
Canned meat	Beef	20	0.20	nd*	nd*	nd*	2.10	0.23	2.92
	Luncheon	10	nd*	nd*	nd*	nd*	2.60	nd*	2.60

nd* not detected.

effect of cooking temperature, which usually exceed 170°C [12], and the conventional use of pepper in Syria as spice for fried and grilled meat which may contain precursors for NPYR [36] and this may explain the high levels of NPYR in smoked, grilled and fried samples which contain paprika pepper.

The canned meat usually preserved using sodium nitrite, which is normally added to canned meat such as canned meat and luncheon to prevent the formation of toxins produced by *Clostridium botulinum*, nitrite is also responsible for the development iron reaction with the some meat pigments to produce the desirable red color, and flavor characteristics for these products [37]. However, Nitrite is also converted to nitrosating agents that may react with amines and amino acids in meat to produce nitrosamines. The high levels of nitrosamines in sausage could be explain that, sausage is usually made from ground meat, ground fat, salt and spices (sometimes with other ingredients such as herbs) and preserved by sodium nitrite or smoking which serves as nitrosating agents [17]. It was found that NDBA and NDPA were the least observed nitrosamine in meat samples in this study and their concentrations were quite low compared to those of other nitrosamines. These results support earlier studies showing that NDMA and NDEA are commonly found in sausages (Byun *et al.*, 2004; Yurchenko and Molder, 2007) [1,12]. These two nitrosamines were found in the highest concentrations in sausage samples even though these samples were seen to have the lowest sum of Nitrosamines overall. The other meat products containing nitrite and nitrate interestingly had lower NDMA and NDEA values. This might be explained by the differences in their processing conditions and compositions. 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 negative samples (raw meat), 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 and grilling and frying) in the formation of these carcinogenic compounds in smoked meat.

4. Conclusion

In this study, seven nitrosamines were separated and analyzed successfully by using GC-FID after extraction under autoclave conditions. The total concentrations of the seven nitrosamines in the studied meat samples ranged from 2.60 to 13.28 µg/kg. The highest levels of nitrosamines were found in fried and smoked meat. Relatively high level was found in grilled meat. In the opposite, the low level of nitrosamine was detected in cured

meat and canned meat. The level of total nitrosamines in fried meat (poultry) was very high and exceeded 13 µg/kg, the concentration of NDMA exceeded 1 µg/kg in fried and smoked meat, while NPYR and NPIP were detected in all positive samples with high concentrations ranging between 1.12 and 8.40 µg/kg for NPYR, and from 0.25 to 1.65 µg/kg for NPIP. This study has been important in revealing the high content of nitrosamines in meat-fast food.

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