

# Analysis of Nitrosamines in Processed Meat Products in Medan City by Liquid Chromatography-Mass Spectrometry

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## Abstract

**BACKGROUND:** Nitrosamine is a carcinogen and the maximum level in processed meat products set by WHO.

**AIM:** The purpose of this study was to determine nitrosamine levels in meat products in Medan City and compared nitrosamine levels with standards set by WHO.

**METHODS:** The samples analysed were obtained from Berastagi Supermarket, sausages, burgers, corned beef and smoked beef. Nitrosamine levels were determined by reverse phase liquid chromatography-mass spectrometry.

**RESULTS:** The results showed that only 5 out of 20 samples of N-nitroso-thiazolidine-4-carboxylic acid (NTCA) nitrosamine ranged from 501.290 to 4227.492 µg/kg. The highest level of NTCA was found in smoked beef (Chiefs), which is 4227.492 µg/kg. N-nitroso-2-methyl-thiazolidine-4-carboxylic acid (NMTCA) was contained in all the samples analysed which ranged from 20.50 to 989.175 µg/kg. The highest NMTCA nitrosamine content was found in smoked beef (Chiefs), which is 989.175 µg/kg.

**CONCLUSION:** From this study reveal that nitrosamines in meat products exceed the maximum standards set by WHO (10 µg/kg).

## Introduction

Nitrite and nitrate are generally found widely in soil, water, and food (especially in vegetables) [1]. Also, nitrite and nitrate are also intentionally added to some foods such as processed meats as preservatives and colouring agents [2]. The use of nitrite in processed meat products may react with alkyl amines to form carcinogen nitrosamines. The maximum level in processed meat set by WHO WHO is 10 µg/kg [3], [4], [5], [6]. There are several factors affecting the formation of nitrosamines, namely the concentration of nitrite, acidity, temperature, storage condition, the alkalinity of amines, and the presence of catalysts or inhibitors. So it is very

necessary to analyse the content of nitrosamines in food, especially in meat products [7], [8], [9], [10], [11]. Nitrosamine analysis in food can be done by high-performance liquid chromatography and gas chromatography with a detector system. The method of high-performance liquid chromatography using mass spectroscopy is very selective and sensitive and can be used as an alternative method compared to other chromatographic methods [12], [13], [14], [15], [16].

The purpose of this study was to determine nitrosamine levels in meat products in Medan City and compared nitrosamine levels in meat products with standards set by WHO.

## Material and Method

### Materials

The materials used in this study were acetonitrile, formic acid (98%), methanol. Nitrosamines standard are *N-Nitrosodiethylamine* (NDEA), *N-nitroso-2-methyl-thiazolidine 4-carboxylic acid* (NMTCA) and *N-nitroso-thiazolidine-4-carboxylic acid* (NTCA).

### Samples Preparation

The samples used in this study were meat products (sausages, corned beef, burgers, and smoked beef) were obtained from Berastagi Supermarket in Medan city. Samples that have been taken and then placed in a *cooling bag* containing ice, then stored in the Laboratory in the freezer (temperature -20°C) before testing.

### Formic Acid Solution 0.1% in Purified Double Distillation Water

Transferred 0.5 mL formic acid (E. Merck) in a 1000 mL beaker glass, then added distilled water up to 500 mL. The mixture was stirred and filtered with 0.2 µm of cellulose nitrate filter membrane. The results obtained was 0.1% formic acid solution in distilled water.

### Formic Acid Solution 0.1% in Acetonitrile

Transferred 0.5 ml of formic acid (E. Merck) in a 1000 ml beaker glass, then added acetonitrile to 500 ml. The mixture was stirred and filtered with a 0.5 µm polymeretrafluoroethylene (PTFE) filter membrane. The results obtained was 0.1% formic acid solution in acetonitrile.

### Preparation of NDEA Standard Solution

Weighed 100 mg NDEA standard, transferred into a 10 ml volumetric flask, dissolved with acetonitrile to the marked line and shaken. Then, it was stirred for 5 minutes until it dissolved to obtain NDEA 10000 µg/ml solutions.

### Preparation NMTCA Standard Solution

Weighed 10 mg of NMTCA standard, transferred into a 10 ml volumetric flask, dissolved with acetonitrile to the mark and shaken. Then, it was stirred for 5 minutes until it dissolved to obtain NMTCA 1000 µg/ml solutions.

### Preparation of NTCA Standard Solution

Weighed 10 mg of NTCA standard, transferred into a 10 ml volumetric flask, dissolved with acetonitrile to the mark and shaken. Then, it was stirred for 5 minutes until it dissolved to obtain NDEA 1000 µg/ml solutions.

### Chromatographic Condition

The nitrogen generator was turned on until it is ready to produce nitrogen gas used for mass spectrometry detectors. Then high performance liquid chromatography (*Agilent 1290 HPLC*) mass spectrometry (ABSciex API 4000 Q TRAP type) was turned on, by: turning on the mass spectrometry detector, setting positive detection, leaving it for a while until the mass spectrometry conditions become vacuum, run the pump on the type mixture of mobile phase, comparison of ratio mobile phase and predetermined mobile phase flow rate, as well as column oven temperature set at predetermined conditions, then the mobile phase was flowed until a stable pressure was obtained, which indicates that the high-performance liquid chromatography system of mass spectrometry has been stable and ready used for analysis [14].

### Mobile Phase

The mobile phase used was a mixture of 0.1% formic acid solution in water and 0.1% formic acid solution in acetonitrile. The ratio of the mobile phase in the mixture were 10:90, 30:70, 50:50, 70:30 and 90: 10. Each of this mobile phase was used to separate the standard solution to determine optimum mobile phase composition [3], [12].

### Nitrosamine Extraction

Five (5) g of mashed meat samples were weighed and transferred into 25 ml volumetric flask. Acetonitrile was added until 25 ml and then homogenised. Then, it was transferred into a tube and then centrifuged for 10 minutes at 8000 rpm. Five (5) ml of supernatant was filtered through a 0.2 µm filter membrane; then the solution was transferred into a vial. Ten (10) µL of solution was injected into a high-performance liquid chromatography system through an automatic injector [3], [12].

### Calibration Curves

From the standard solution (NDEA, NMTCA, and NTCA) with a concentration of 1.25 mg/ml, pipetted 0.4 ml; 1 ml; 2 ml; 4 ml, and 10 ml, respectively. Each solution was transferred into volumetric flask 25 ml, then added acetonitrile to the marked line. The concentration of nitrosamine solutions (NDEA, NMTCA, and NTCA)

is 20; 50; 100; 200; and 500 ng/ml). Each solution was filtered through a membrane filter polytetrafluoroethilen (PTFE) of 0.2 µm. Then, the filtered solutions were transferred into a vial. Ten (10) µL of solution was injected into a high-performance liquid chromatography system through an automatic injector. The peak area of chromatogram was obtained. Then a calibration curve is made by plotting peak area versus concentration. Then, the regression line equation ( $Y = aX + b$ ) determined.

### Analysis of Nitrosamines in Processed Meat Products

Five (5) g of mashed meat samples were weighed and transferred into 25 ml volumetric flask. Acetonitrile was added until 25 ml and then homogenised. Then, it was transferred into a tube and then centrifuged for 10 minutes at 8000 rpm. Five (5) ml of supernatant was filtered through a 0.2 µm filter membrane; then the solution was transferred into a vial. Ten (10) µL of solution was injected into a high-performance liquid chromatography system through an automatic injector. MS/MS detection was done by Multiple Reaction Monitoring (MRM). The mass spectrometer is operationalised using electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) technique. The ion monitored in mass spectrometry is a positive ion that has a mass of 103 for NDEA, and negative ions that have a mass of 175 for NMTCA, and 161 for NTCA. Chromatogram and peak area were obtained, and then the concentration was calculated by substituting the peak area into the regression equation ( $y = ax + b$ ) obtained from the calibration curve on linearity testing [3], [12]. Nitrosamine levels in solution can be calculated by the regression equation  $Y = aX + b$ .

Nitrosamine concentration was calculated using this equation:

$$C = \frac{X \times V \times Fp}{\text{sample weight (5 g)}}$$

Notes: Y = Peak Area

C = Nitrosamine concentration in the sample (µg/kg); X = Nitrosamine levels in sample solution (ng/ml); V = Volume of sample solution before dilution (25 ml); Fp = Dilution Factors (1).

## Results

### Determination of Mobile Phase Composition

Determination of the mobile phase composition was done to get the best separation method. NDEA, NMTCA, and NTCA standard

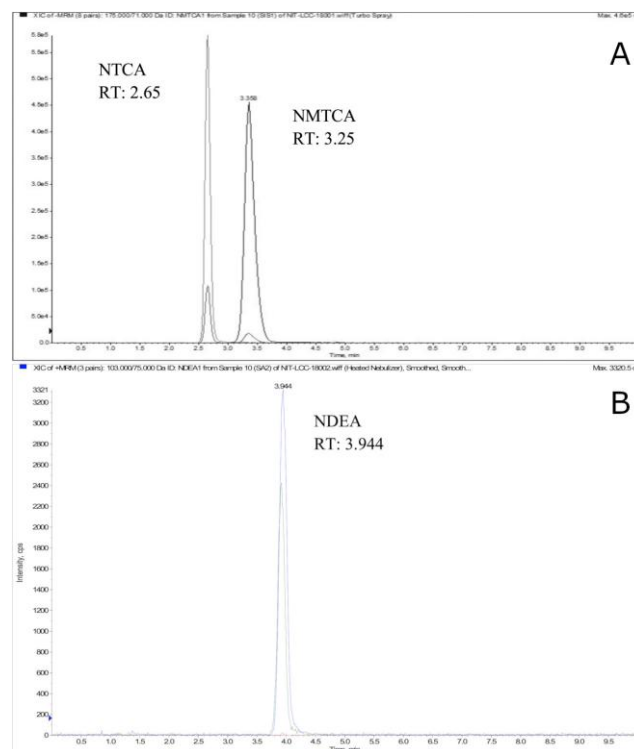
solutions in water and acetonitrile (ratio 10:90, 30:70, 50:50, 70:30) were injected into LCMS- MS/MS system through an automatic injector. The ideal chromatogram peak was shown by a Gaussian form with asymmetric value (tailing factor) which of 1. Tailing factor data with a different ratio of mobile phase for the analysis of NDEA, NMTCA, and NTCA can be seen in Table 1.

**Table 1: Tailing Factor with different ratio of mobile phase for analysis of NDEA, NMTCA, and NTCA**

Motion Phase	Tailing Factor		
	NDEA	NMTCA	NTCA
0.1% Formic acid in water: 0.1% Formic acid acetonitrile (10:90)	NA	NA	1.2
0.1% Formic acid in water: 0.1% Formic acid acetonitrile (30:70)	0.95	NA	0.93
0.1% Formic acid in water: 0.1% Formic acid acetonitrile (50:50)	NA	NA	1.5
0.1% Formic acid in water: 0.1% Formic acid acetonitrile (70:30)	1.08	1	1
Water: Acetonitrile (Format acid 0.1%) 0.1% format 90:10	NA	NA	1.07

(Description: NA = Not applied)

In Table 1 it is shown that the best chromatogram mobile phase ratio is 70:30 (water: acetonitrile) because a symmetrical and sharp peak was produced and meets the requirements for tailing factor of 1.08 for the NDEA, 1 for NMTCA and NTCA. The best mobile phase with a ratio of 70:30 was then used for analysis of mixed solutions of NDEA, NMTCA, and NTCA. Chromatogram of NTCA, NMTCA, and NDEA using optimum mobile phase detected by Multiple Reaction Monitoring (MRM) Mass Spectrometry can be seen in Figure 1.



**Figure 1: Chromatogram of NTCA, NMTCA, and NDEA using optimum mobile phase detected by Multiple Reaction Monitoring (MRM) Mass Spectrometry (A); N-nitroso-thiazolidine-4-carboxylic acid (NTCA) and N-nitroso-2-methyl-thiazolidine 4-carboxylic acid (NMTCA) detected with ESI ionisation and (B); N-Nitrosodiethylamine (NDEA) with APCI ionisation**

As seen Figure 1, it is shown that the optimum mobile phase for the analysis of both volatile and non-volatile nitrosamines is 0.1 % formic acid in water: 0.1% formic acid in acetonitrile (70:30) with flow rate 1 ml/minute. The standard mixture of NDEA, NMTCA, and NTCA eluted the optimum mobile phase shows a symmetrical and sharp peak. NDEA is analysed better using APCI compared to ESI. This is indicated by the high signal response obtained when NDEA standard solutions were analysed using APCI compared to ESI. APCI is usually used for the analysis of volatile nitrosamine compounds. On the other hand, NMTCA and NTCA are analysed better using ESI because higher signal responses were obtained [3], [12].

### Calibration Curve

The concentration used for calibration curve preparation are: 20 ng/ml; 50 ng/ml; 100 ng/ml; 200 ng/ml; and 500 ng/ml for NDEA, NMTCA, and NTCA. Calibration curve of NDEA can be seen in Figure 2.

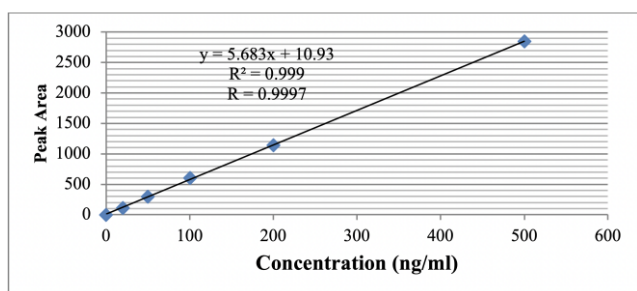


Figure 2: Calibration Curve of NDEA

In Figure 2 it can be seen that the calibration curve obtained has a linear relationship between peak area and concentration. The regression line equation obtained is  $y = 5.683x + 10.93$  with a correlation coefficient ( $r$ ) of 0.9997. This shows a linear correlation between the area of the chromatogram and the concentration of NDEA [5], [6]. The calibration curve of NMTCA can be seen in Figure 3.

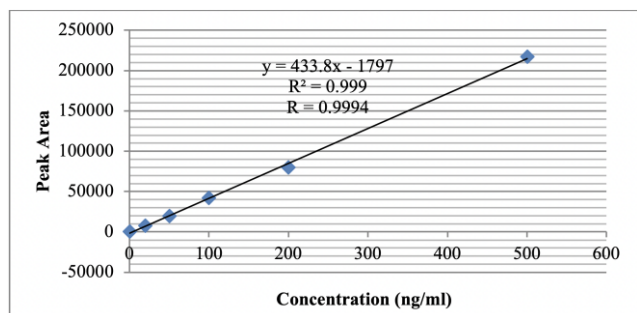


Figure 3: Calibration Curve of NMTCA

In Figure 3 it can be seen that the calibration curve obtained has a linear relationship between peak area and concentration. The regression line equation obtained is  $y = 433.8x - 1797$  with a correlation

coefficient ( $r$ ) of 0.9994. This shows a linear correlation between the area of the chromatogram and the concentration of NMTCA [5], [6]. The calibration curve of NTCA can be seen in Figure 4.

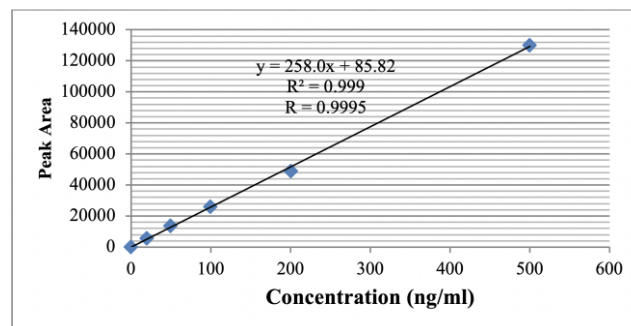


Figure 4: Calibration Curve of NTCA

In Figure 4 it can be seen that the calibration curve obtained has a linear relationship between peak area and concentration. The regression line equation obtained is  $y = 258x + 85.82$  with a correlation coefficient ( $r$ ) of 0.9995. This shows a linear correlation between the area of the chromatogram and the concentration of NTCA [5], [6].

### Nitrosamine Levels in Various Types of Processed Meat Products

Determination of nitrosamines levels was carried out using reverse-phase high-performance liquid chromatography method which is then characterised by a mass spectrometer. Nitrosamine levels in various types of processed meat products can be seen in Table 2.

### Discussion

Based on Table 3, it can be seen that there are differences in nitrosamine levels (NDEA, NMTCA, NTCA) in various types of processed meat products. There are 20 samples consisting of 5 types of sausage brands, 5 types of corned beef brands, 5 types of burger brands, and 5 types of smoked beef brands.

Of all samples analysed, NDEA (a volatile nitrosamine) was not detected. There are 2 possibilities, either NDEA is not present in the samples, or it is present in the sample, but below the limit of detection. On the other hand, non-volatile nitrosamine compounds such as NMTCA and NTCA are found in the samples and vary greatly. There are also some samples where these compounds are not detected. The highest nitrosamine level is NTCA (4227.492  $\mu\text{g}/\text{kg}$ ) which is found in smoked beef (Chiefs).

**Table 2: Nitrosamine Levels (NDEA, NMTCA, and NTCA) in Various Types of Processed Meat Products**

A. SAUSAGE			
No.	Nitrosamine	Brand	Nitrosamine $\pm$ SD ( $\mu\text{g}/\text{kg}$ )
1	NDEA	Fiesta	-
		Delicious	-
		Kenfood	-
		Kimbo	-
		So good	-
2	NMTCA	Fiesta	36.91 $\pm$ 6.2210
		Delicious	39.41 $\pm$ 3.0015
		Kenfood	35.64 $\pm$ 6.3940
		Kimbo	41.99 $\pm$ 6.6020
		So good	108.14 $\pm$ 6.5348
3	NTCA	Fiesta	585.45 $\pm$ 23.8477
		Delicious	-
		Kenfood	-
		Kimbo	-
		So good	2565.44 $\pm$ 60.8385
B. CORNED			
No.	Nitrosamine	Brand	Nitrosamine $\pm$ SD ( $\mu\text{g}/\text{kg}$ )
1	NDEA	Kornetku	-
		Pronas	-
		Mili	-
		Libbys	-
		Bernardi	-
2	NMTCA	Kornetku	20.54 $\pm$ 0.2951
		Pronas	20.50 $\pm$ 0.2604
		Mili	48.05 $\pm$ 2.8778
		Libbys	46.84 $\pm$ 37.3362
		Bernardi	90.52 $\pm$ 84.3906
3	NTCA	Kornetku	-
		Pronas	-
		Mili	-
		Libbys	-
		Bernardi	1508 $\pm$ 2975.7
C. BURGER			
No.	Nitrosamine	Brand	Nitrosamine $\pm$ SD ( $\mu\text{g}/\text{kg}$ )
1	NDEA	Kimbo	-
		Bernardi	-
		Vitalia	-
		Hemato	-
		Abbys	-
2	NMTCA	Kimbo	20.705 $\pm$ 0.0077
		Bernardi	20.703 $\pm$ 0.0043
		Vitalia	35.79 $\pm$ 2.7469
		Hemato	20.705 $\pm$ 0.0062
		Abbys	20.700 $\pm$ 0.0118
3	NTCA	Kimbo	-
		Bernardi	-
		Vitalia	-
		Hemato	-
		Abbys	-
D. SMOKED BEEF			
No.	Nitrosamine	Brand	Nitrosamine $\pm$ SD ( $\mu\text{g}/\text{kg}$ )
1	NDEA	Fiesta	-
		Yona	-
		Chiefs	-
		Bernardi	-
		Farmhouse	-
2	NMTCA	Fiesta	20.703 $\pm$ 0.0113
		Yona	20.702 $\pm$ 0.0107
		Chiefs	989.175 $\pm$ 116.403
		Bernardi	20.708 $\pm$ 0.0078
		Farmhouse	20.697 $\pm$ 0.0224
3	NTCA	Fiesta	-
		Yona	-
		Chiefs	4227.492 $\pm$ 605.5445
		Bernardi	501.290 $\pm$ 108.1681
		Farmhouse	-

Note: (-): Not Detected, the data is an average of three Repeat times

20.697  $\mu\text{g}/\text{kg}$  (Farmhouse).

Besides that, it is also found that NTCA in some samples exceeds the limit allowed by the WHO. Those samples are Fiesta sausage (585.45  $\mu\text{g}/\text{kg}$ ) and So Good (2565.44  $\mu\text{g}/\text{kg}$ ), corned beef Bernardi (1508.00  $\mu\text{g}/\text{kg}$ ), smoked beef Chiefs (4229.492  $\mu\text{g}/\text{kg}$ ) and smoked beef Bernardi (501.29  $\mu\text{g}/\text{kg}$ ).

Based on previous research conducted by Herrmann (2014), nitrosamines levels obtained were also varied. The highest nitrosamine content found is NTCA which is 4030  $\mu\text{g}/\text{kg}$ . The results of this study are the same as previous studies, where NDEA levels are not detected in all analysed samples. But in this study nitrosamine levels are higher than previous studies. The highest nitrosamine level is NTCA which is 4227.492  $\mu\text{g}/\text{kg}$  (smoked beef Chiefs). The highest NMTCA and NTCA levels are found in smoked beef products. This is also corresponding to the previous study conducted by Herrmann (2014) where the levels of nitrosamines reach the maximum level of 2034-4030  $\mu\text{g}/\text{kg}$ . The formation of these two nitrosamines is high in smoked beef samples because the high temperature applied during the smoking process triggers NMTCA and NTCA formation. It is also likely to occur due to several factors such as the concentration of nitrite, temperature, storage condition, the presence of catalysts or inhibitors. The higher the concentration of nitrite and amine compounds, the easier the formation of nitrosamines will be. However, the toxicological properties of both NMTCA and NTCA (nonvolatile nitrosamine) compounds have not been detected. Nonvolatile nitrosamines are weak carcinogens, but not enough data to determine the toxicological properties of these two compounds. However, NMTCA and NTCA nitrosamine levels in processed meat products need to be considered to prevent cancer prevalence [3], [9], [11], [12], [16], [17], [18], [19], [20].

The level of NMTCA in all samples analysed varies greatly, ranging from 20.50 to 989.175  $\mu\text{g}/\text{kg}$ . Whereas NTCA was only found in 5 of the 20 samples tested, ranging from 501.290-4227.492  $\mu\text{g}/\text{kg}$ .

In this study, it is found that the levels of NMTCA in all samples exceed the limit set by the WHO, which is 10  $\mu\text{g}/\text{kg}$  body weight. In sausage samples, NMTCA levels detected are 36.91  $\mu\text{g}/\text{kg}$  (Fiesta), 39.41  $\mu\text{g}/\text{kg}$  (Delicious), 35.64  $\mu\text{g}/\text{kg}$  (Kenfood), 41.99  $\mu\text{g}/\text{kg}$  (Kimbo) and 108.31  $\mu\text{g}/\text{kg}$  (So Good). In corned beef samples, NMTCA levels detected are 20.54  $\mu\text{g}/\text{kg}$  (Kornetku), 20.50  $\mu\text{g}/\text{kg}$  (Pronas), 48.05  $\mu\text{g}/\text{kg}$  (Mili), 46.84  $\mu\text{g}/\text{kg}$  (libbys), and 90.52  $\mu\text{g}/\text{kg}$  (Bernardi). In the burger samples, NMTCA levels detected are 20.705  $\mu\text{g}/\text{kg}$  (Kimbo), 20.703  $\mu\text{g}/\text{kg}$  (Bernardi), 35.79  $\mu\text{g}/\text{kg}$  (Vitalia), 20.705 (Hemato), and 20.70  $\mu\text{g}/\text{kg}$  (Abbys). In smoked beef samples, NMTCA levels detected are 20.703  $\mu\text{g}/\text{kg}$  (Fiesta), 20.702  $\mu\text{g}/\text{kg}$  (Yona), 989.175  $\mu\text{g}/\text{kg}$  (Chiefs), 20.708  $\mu\text{g}/\text{kg}$  (Bernardi), and

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