Journal of
Food and Nutrition

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Fast, Simple, Novel and Economical Method for Ultra Trace Determination of Nine Nitrosamines in Sausage by Gas Chromatography-Flameionization Detector and Ascorbic Acid and Sodium Nitrite Effect on Nitrosamine Formation

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Received Date: December 06, 2019 **Accepted Date: January** 08, 2020 **Published Date: January** 10, 2020

Citation: Mehdi Nabi (2020) Fast, Simple, Novel and Economical Method for Ultra Trace Determination of Nine Nitrosamines in Sausage by Gas Chromatography-Flameionization Detector and Ascorbic Acid and Sodium Nitrite Effect on Nitrosamine Formation. J Food Nutr 6: 1-12.

Abstract

A simple, fast and low-cost method have been applied for the determination of nine nitrosamines in sausage. This method is based on a liquid-liquid extraction, which examinations were performed by gas chromatography-flame ionization detector (GC-FID) and high-performance liquid chromatography diode array detection (HPLC-DAD) by post-column photolysis and derivatization was used for confirmation and validation of the results. Initially, partitioning of samples was carried out in a water-hexane mixture and then the organic phase was separated and evaporated to dryness, substituted by ethanol and was injected. The detection limit was 0.4 μg Kg^{-1} and the average relative standard deviation (RSD %) variations for repeated experiments was 6.3%. The extraction recovery variation for the spiked samples was 63-97%. The method was stable and consistent and successfully applied for nitrosamines determination in sausage.

Keywords: Nitrosamines; Postcolumn photolysis-Derivatization High-Performance Liquid Chromatography; Gas Chromatography; Stability; Consistency; Sausage

Introduction

The presence of a large amount of free water in meat has been caused on one hand it has high nutritional value; on the other hand, meat is prone to microbial growth and decay. Diverse meat products, such as Sausage, with different flavors, and more lifetime are produced from meat treatment with suitable ingredients. Since antique times, the use of salt for keeping safe of meat revealed that the nitrate existence in salt, as a contaminant, had a meaningful function in the restraint of microorganism growth. The nitrate is converted to nitrite and improves the curing of meat. Nowadays, to prevent the formation of Clostridium botulinum bacterium, nitrite and nitrate salts are commonly added in curing mixtures in order to cause to slow down lipid oxidation and give to treated meats their distinctive taste, color, and odor. There is, however, a lot of controversy over the safety of meat products [1].

Nitrite, in the process of meat manufacturing, is one of the most important and common additives that it is caused desirable texture and color, delay in lipid oxidation, and particularly preventing from the formation of clostridium botulinum [2,3].

In foodstuffs, nitrosamines are produced by the reaction of nitrogen oxide and amines. In acidic medium, hydronitrogen oxide ($H2NO2+$) is formed from nitrite in food. The source of nitrite is the reduction of nitrate fertilizer or added as a preservative [4]. The reaction of hydro nitrogen oxide with another molecule of nitrite form nitrogen anhydride after dehydration. Nitroso group from nitrogen anhydride reacts with amines in food to form nitrosamines.

Stable nitrosamine is formed by secondary amines, while nitrosamines produced from primary amine is destroyed quickly; it is also known that nitrosamine formation from tertiary amine is difficult [4].

But nitrite reaction with amines and amino acids and as a result, N-nitrosamines formation is an important concern. Cured meat products, tobacco, rubber products, cosmetics, other consumer products, and environmental matrices are a source of nitrosamines [5]. Nitrosamines are carcinogenic, mutagenic and teratogenic. Due to this cause, meat improving by the addition of these nitrosating agents is completely regulated and monitored in some countries. Moreover, some compounds such as ascorbic acid are added to several foods during the processing because these have an inhibitory role in the nitrosation reaction. The substantial reason is the reduction of nitrite by ascorbic acid and following that changing the reactions of nitrite. The several groups have been investigated mechanisms of the reactions of ascorbic acid with nitrite [6]. The N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) have been classified as most carcinogenic and N-nitrosodibutylamine (NDBA), N-nitrosopiperidine (NPIP) and N nitrosopyrrolidine (NPYR) as possibly carcinogenic to humans by International Agency for Research on Cancer (IARC) [7].

The chemical structure of nitrosamines is as $R1N(–R2)–N=O$, that is, a nitroso group bonded to an amine as following.

Distribution of seven nitrosamines has been studied in different foodstuffs such as agricultural products, seafood, milk, and milk products, meat and meat products, processed meats, oils, butter and margarine, soybean paste, soy sauce, seasoning, sauce, and alcoholic beverages [4].

Nitrosamines have been determined in various matrices by different analytical methods, such as gas chromatography-tandem Mass Spectrometry, [8] capillary electro-chromatography, [9] micellar electro-kinetic capillary chromatography,[10] gas chromatography-nitrogen/phosphorus detector and nitrogen chemiluminescence detector, [11] gas chromatography-flame ionization detector, [12] gas chromatography with nitrogen chemiluminescence detection, [13] gas chromatography-mass spectrometry detector, [14] liquid chromatography-atmospheric pressure ionization tandem mass spectrometry, [15] high-performance liquid chromatography with post-column photo-hydrolysis and colorimetric detector, [16] high-performance liquid chromatography with fluorescence detector[17]. The nitrosamines have been usually determined by gas chromatography-thermal energy analysis (TEA) [18].

The various methods for nitrosamine extraction and extract clean-up in the complicated food matrices have been applied, including supercritical fluid extraction,18 solvent extraction, [19] solid-phase extraction, [20] and solid-phase micro-extraction (SPME) [21].

Recently, nitrosamines have been determined by Online coupling of tandem liquid-phase extraction with HPLC-UV, [22] high-performance liquid chromatography, [23] vortex-assisted reversed-phase dispersive liquid-liquid microextraction and liquid chromatography with mass spectrometry, [24] liquid chromatography-tandem mass spectrometry, [25] stir bar-supported micro-solid-phase extraction coupled with gas chromatography-mass spectrometry, [26] automated headspace solid-phase microextraction coupled with gas chromatography-mass spec-

trometry (automated HS-SPME/GC–MS), [27] and liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry (LC-APCI-MS/MS) [28]. Hyoung S. Lee has also compiled level and measurement methods of eight nitrosamines in processed meat and poultry products, based on 25 references published for the period of 1985 to 2018 from 14 countries [29].

The purpose of the present work was development and validation of an accurate and easy method for the determination of nine volatile N-nitrosamines in sausage (fast food) using gas chromatography-flame ionization detector and ascorbic acid measurement by high-performance liquid chromatography-diode array detector. In addition, in this article, sodium nitrite and ascorbic acid effect on nitrosamine formation have been investigated.

To the best of our knowledge, this article describes the first determination of nine nitrosamines by GC-FID and then method validation by HPLC-DAD with post-column photolysis and derivatization with a low detection limit for the stable and consistent determination of nitrosamines in foodstuffs.

Experimental

Chemicals and materials

Nitrosamines mix, certified reference material, was purchased from Sigma Aldrich (2000 mgL^{-1} each component in methanol, PubChem CID: 5959, 502138 Supelco). Organic solvents such as methanol (PubChem CID: 887), ethanol (PubChem CID: 720), acetone (PubChem CID: 180), pentane (PubChem CID: 8003), hexane (PubChem CID: 1140), benzene (PubChem CID: 241)and dichloromethane (PubChem CID: 6344) were purchased from Merck (Germany). All the above chemicals were of the analytical grade for GC and HPLC. Ammonium acetate (PubChem CID: 517165), sodium sulfate (PubChem CID: 24436), orthophosphoric acid (PubChem CID: 1004), 85 %, N-(1-Naphthyl) ethylenediamine dihydrochloride (PubChem CID: 15106), sulfanilamide (PubChem CID: 5333), potassium ferrocyanide trihydrate (PubChem CID: 161067), zinc acetate dehydrate (PubChem CID: 2724192), borax (PubChem CID: 10219853), sodium nitrite (PubChem CID: 23668193), potassium dihydrogen phosphate (PubChem CID: 516951), hydrochloric acid (PubChem CID:313) were purchased from Merck (Germany) of analytical grade. Ultrapure water (electrical resistivity 18.3 MΩ) was used for the preparation of required aqueous solutions.

2.1.1 Griess reagent: 0.25 g N-(1-naphthyl) ethylenediamine dihydrochloride was dissolved in water and in a volumetric flask was diluted to 250 ml. 4.0 g of sulfanilamide was dissolved in 250 ml of a 5% aqueous solution of 85 % orthophosphoric acid. These reagents were mixed in an amber glass bottle and the mixture was kept in dark.

No.	Name	Polarity	Boiling point (°C)	CAS
1	N-Nitrosodimethylamine (NDMA)	log K_{ow}^{*}= -0.57	154.0	62-75-9
2	N-Nitrosomethylethylamine (NMEA)	log K_{ow} = 0.04	170.0	10595-95-6
3	N-Nitrosodiethylamine (NDEA)	log K_{ow} = 0.48	176.9	55-18-5
4	N-Nitrosodi-n-propylamine (NDPA)	log K_{ow}= 1.360	206.0	621-64-7
5	N-Nitrosodibutylamine (NDBA)	log K_{ow} = 2.63	237.0	924-16-3
6	N-Nitrosodiphenylamine (NDPhA)	Log K_{ow}= 3.13	268.0	86-30-6
7	1-Nitrosopiperidine (NPIP)	log K_{ow} = 0.36	219.0	100-75-4
8	1-Nitrosopyrrolidine (NPYR)	log K_{ow} = -0.19	214.0	930-55-2
9	Nitrosomorpholine (NMOR)	log K_{ow} = -0.44	225.0	59-89-2
*K_{ow}'s have redistributed from,[30]				

Table 1. Nitrosamines specifications

Working standards

Ascorbic acid: A 1000 mg L^{-1} stock standard was prepared by dissolving 10mg ascorbic acid in 10 mL ultrapure water and used for preparing working standards.

Nitrosamines: A secondary storage solution with a concentration of 1.0 mg $L^{-1}$was prepared by diluting nitrosamines mix the standard solution. Then, by sequential dilution of the secondary storage solution in methanol, working standards with concentrations of 1, 2, 5, 10 and 20 µgL-1 were obtained.

Instrumentation

High-performance liquid chromatography

Ascorbic acid was measured by HPLC, Agilent 1100, C18 reverse-phase analytical column (250 mm × 4.6 mm), photodiode array detector (DAD), injection volume 100µl, total flow 1 mlmin-1, column oven temperature 25°C and detection wavelength 265 nm. The solvents used as the mobile phases were previously filtered by a 0.2 µm filter.

Nitrosamines were measured by HPLC, Agilent 1100, with the following configuration:

C18 reverse-phase analytical column (250 mm × 4.6 mm), photodiode array detector (DAD), total flow 0.5 mL min-1, column oven temperature 35 °C and detection wavelength 540 nm.

An injection system provided with a 100 µL loop connected to column oven (at the right hand of the HPLC) and then to the column. Column outlet connected to a photochemical reactor equipped with a transparent PTFE tube (10 m length, 0.3 mm internal diameter) coiled around UV lamp emitting at wavelength 254 nm. Then its outlet linked to a T-shaped connection. The derivatization reagent (Griess reagent) transferred by another HPLC pump at the flow of 0.5 mLmin-1to the other branch of the T-shaped connection. Mixing of solutions performed in the T-shaped connection. The T-shaped outlet connected to the HPLC column oven (at the left hand of the HPLC) set at 50 oC. The outlet of the T-shaped connection connected to the detector. All tubes and connections were as short as possible in order to prevent band broadening. Configuration and operation details had been presented in ISO.31

Gas chromatography

Samples, for nitrosamines, were determined using GC, Agilent 7890, FFAP analytical column (30 m × 0.25 mm, 0.25 µm), and flame ionization detector (FID). The operating conditions under which the chromatograms were obtained, have been summarized in table 2.

Operating parameters	Values
Injection volume (µL)	2
Inlet temperature (°C)	280
Flow (ml min^{-1})	1.0
Temperature programming	5 min isothermal at 60 °C, programmed to rise at 5 °C min^{-1} to 160 °C, rise at 20 °C min^{-1} to 240 °C,15 min isothermal at 240 °C
Detector temperature (°C)	300
Makeup-gas flow (N_2) (mL min^{-1})	20
Carrier gas	Nitrogen
Mode	Pulsed splitless - Constant flow

Table 2. Typical operating conditions for GC-FID

Optimization of the nitrosamines extraction parameters

The extraction efficiency depends on various parameters such as type and volume of extraction solvent, nitrosamines solvent and salt addition. The peak area was utilized for the evaluation of the above-mentioned parameters. All measurements were carried out three times and their mean was considered.

Selection of type and volume of extraction solvent

Extraction efficiency depends on the selection of extraction solvent significantly. Extraction ability, strength and quality of desire analyte, and finally weak solubility in water are very important and effective. In this work, three solvents were surveyed. These solvents were different in water solubility and its density which includes n-hexane, benzene, and dichloromethane. Solvent selectivity for extraction was examined on nitrosamines aqueous solution with a concentration of 10.0μg L-1. In this regard, extraction was carried out in section 2.4. After extraction, solvents injected and the peak area as the analytical index was estimated and monitored. On the base of the achieved results, dichloromethane had compared to the other solvents. However, for the next examinations, hexane was chosen as an extraction solvent because of environmental problems.

The peak area relevant to analytes was surveyed as a function of hexane volume. When the volume of hexane increases, the signal reduces. Dilution of the extract is the reason of the reduction.

Nitrosamines solvent

Dissolving and diluting of nitrosamines standard solutions were performed in different solvents such as methanol, ethanol, acetone, pentane, hexane, and dichloromethane. The highest signal and the best peaks obtained by using ethanol as solvent.

Salt addition effect

Generally, the increase of the ionic strength can cause a decrease in the solubility of the analytes in the sample solution and enhance extraction efficiency. To evaluate the possibility of the salting-out effect, the extraction efficiency was studied with adding sodium sulfate ranging from 0.5% to 5%. Due to the salting-out effect, the peak area increased as the amount of Na_2SO_4 increased from 0.5% to 2% owing to very high solution ionic strength and from 2.5% to 5% had negligible changes. By increasing the ionic strength, increasing the extraction recoveries was observed. Based on the experimental results, salt was added in the next experiments.

Method validation and performance characteristics

Performance characteristics of nitrosamines determination

The optimum parameters were selected as discussed in previous sections. The system suitability of the suggested method was evaluated by investigating figures of merit using the best conditions. Working solutions from nitrosamines mix standard were prepared by ultrapure ethanol in the range of 0.01-500.0 mg L^{-1} and instrumental parameters and performance characteristics were determined after extraction/clean-up and quantitation. The analytical sample (2 μL) was injected and the retention time of the analytes came into view between 11.5 and 31.1 min. Finally, the analytes areas against the concentration of the analyte were drawn, and the least-squares method was applied for acquiring the calibration curve equations. On the base of achieved results, nitrosamines were linear in the range of 0.05 -500.0 mg L^{-1}. The typical determination coefficient (R2) for the examined analytes was higher than 0.99. The detection limit was calculated by using the standard definition, DL = 3s/N, thus the detection limits were lower than 0.4 μg kg^{-1}. The calibration data are listed in Table 3.

Accuracy

Precision

Results of three replicates of the nitrosamines solutions at low, medium and high levels (1.0, 5.0 and 10.0 μg L−1) during three days were used for evaluation of the method precision indices such as repeatability (intra-day) and intermediate (inter-day) and reproducibility (Intra/inter-day) [32,33]. The average of repeatability relative standard deviation (RSD), intermediate RSD and reproducibility RSD were 6.3%, 4.2%, and 7.5%, respectively.

Bias

Samples were spiked by nitrosamines at various levels including 1, 5 and 10 μg Kg^{-1}. Three duplicates for 3 sequential days were performed for each level of nitrosamines. Recoveries were calculated for evaluating the method bias. The results have been presented in table 4. Recoveries had been distributed in the range of 63-97%for nitrosamines.

Performance Characteristics						
Nitrosamine	RT	Linear dynamic range, LDR (mg L^{-1})	The determination coefficient (R^2)	Limit of detection, LOD μg kg^{-1}	Limit of quantification, LOQ μg kg^{-1}	Relative standard deviation, RSD (%)
NDMA	11.5	1.0-10	0.997	0.4	1.0	7.2
NMEA	12.6	1.0-10	0.989	0.4	1.0	8.6
NDEA	13.3	1.0-10	0.988	0.4	1.0	8.2
NDPA	16.7	1.0-10	0.984	0.4	1.0	10.5
NDBA	21.0	2.0-10	0.982	0.7	2.0	11.7
NDPhA	21.4	1.0-10	0.990	0.4	1.0	5.0
NPIP	22.2	2.0-10	0.995	0.7	2.0	6.7
NPYR	23.4	1.0-10	0.992	0.4	1.0	4.2
NMOR	31.1	1.0-10	0.993	0.4	1.0	4.2

Table 3: Analytical performance data for nitrosamines determination

N-nitrosamine	Spiked Level(μg/L)	Raw sausage		Fried sausage	
		Recovery	%RSD	Recovery	%RSD
NDMA	1	70	5	72	5
	5	85	4	83	4
	10	95	3	97	3
NMEA	1	68	5	70	6
	5	87	5	85	4
	10	92	3	95	5
NDEA	1	71	4	68	6
	5	85	5	85	3
	10	95	3	95	4
NDPA	1	70	6	67	4
	5	85	4	83	4
	10	95	3	96	3
NDBA	5	85	5	85	4
	10	92	3	95	3
NDPhA	1	77	3	75	5
	5	80	5	84	6
	10	93	7	90	3
NPIP	5	88	6	85	7
	10	94	3	97	5
NPYR	1	63	5	66	4
	5	75	6	78	8
	10	89	3	93	4
NMOR	1	71	6	74	7
	5	86	4	88	5
	10	92	3	96	2

Table 4: Results of recoveries for nine spiked nitrosamines in sausage samples (n = 3).

Quality Assurance

Stability

The variation in bias over time is stability. A stable measurement process is in statistical control with respect to time. Stability is the total change in the measurements obtained with a measurement system on the same or similar quality control sample when a single parameter is examined over a long time period. Based on the obtained results, drift was %10.

Consistency

The degree of repeatability variation over time is consistency. A consistent measurement process is in statistical control with respect to time. Based on the obtained results, consistency was %7.3.

Stability and consistency results have achieved during 20 consecutive weeks. Based on the results, the method is stable and consistent. It means results are in statistical control in over a long time. Therefore, the method is robust. These parameters are not often checked in research works and validation results can hold in a short time.

Real sample

A homogeneous sausage sample was prepared by Elka company contained 120 mg kg^{-1} sodium nitrite and 240 mg kg^{-1} ascorbic acid. Determinations were carried out on this batch and ascorbic acid and sodium nitrite effect on nitrosamine formation were examined by the use of this sample.

Ascorbic acid, sodium nitrite, and nitrosamines were determined in raw, freeze and fried sausage in various time intervals, each seven days once, during 2 months.

Ascorbic acid determination

About 5 g sample was transferred to a beaker. Extraction was carried out by a 20 ml phosphoric acid (1.7%). The mixture was transferred to a 50 ml flask and diluted to volume by extraction solvent. Then the mixture was centrifuged and filtered by filter syringe and injected to HPLC. Potassium dihydrogen phosphate, pH = 2.9, was used as a mobile phase.

Nitrite determination

A test portion with hot water was extracted, the proteins were precipitated and filtered. By the addition of sulphanilamide and N-1-naphthyl ethylenediamine dihydrochloride to the filtrate, in the presence of nitrite, red color was developed and photometric measurement was carried out at a wavelength of 538 nm.

Nitrosamines

Extraction and preconcentration procedure: Sample was mixed by grinding in a mixer and homogenized. Then 50g ± 0.01g sample was transferred to a round-bottom flask and 100 ml water was added. The mixture was mixed and shaked for 30 min by shaker and then filtered in a 100 ml flask. 2 g sodium sulfate was added and was mixed until was dissolved. After that, 10 ml hexane was transferred and shaked completely. Afterward top phase (organic layer) was transferred to a rotary evaporator flask and the solvent was evaporated entirely. Flask was washed with 1 ml ethanol and was transferred to a vial for GC injection. 8 batches of sausage samples were tested for nitrite, ascorbic acid, and nitrosamines using the method developed in this work. Some Nitrosamines were found in sausage samples at concentrations 2.6 to 17.3 μg Kg^{-1}.

Therefore, the suitability of the developed method in this study was demonstrated for the determination of nine nitrosamines in real samples.

Confirmatory test for nitrosamines verification

The extraction of nitrosamine analytes from samples is carried out with ultrapure water. Dichloromethane was used for clean-up. The cleaned solutions were determined by HPLC with post-column photolysis and derivatization configuration. Nitrosamines separation from the matrix is performed using reversed-phase liquid chromatography. The N-nitroso bond splitting is accomplished by UV photolysis and the nitrite ion was formed, online. Griess reagent reacts with the nitrite functional group at the presence of sulfanilamide in an acid medium. Then N-(1-naphthyl) ethylenediamine dihydrochloride (NED) is linked to it and is produced a purple color that is measured at wavelength 540 nm, quantitatively.

The verification and confirmation of nitrosamines presence was performed by analyzing the analytical sample with the photolysis stage (turning UV lamp on) and without photolysis stage (turning UV lamp off, thus N-nitroso bond splitting and nitrite ion formation has not occurred). When the UV lamp is off, if the nitrosamines peak does not appear at the desired retention time, peak monitored in the photolysis stage does not match to nitrosamines.

The mobile phase was 0.02 mol L-1 ammonium acetate solution.

Comparing the new method with other methods

Some analytical performance characteristics have been presented in table 5 such as linear dynamic range, LOD, and recovery values obtained in this work and they have been compared with those determined in various literary works including analysis of nitrosamines in foodstuffs. For the analytes measurement in trace levels, mainly, a sample treatment and preconcentration method have been combined with a separation technique such as GC, GC-MS, HPLC or LC-MS. But, because these methods have deficiencies such as the need for expensive and toxic solvents and equipment, expensive equipment, a long time for sample treatment and analysis, tiresome step for derivatization, they may not be appropriate for routine sample analysis. Considering some figures of merit reported in this work with those presented antecedently for nitrosamines determination in comparable samples show the relative superiority of this method, in spite of the fact that comparison of complicate matrices and different methods are difficult.

The proposed method, relatively, simple, sensitive, fast and low-cost with short analysis time, good repeatability, reproducibility, and recovery for the nitrosamines determination in sausage relative to the previously suggested methods. Therefore this method could readily be used for routine nitrosamines analysis.

Conclusions

In this work, GC-FID was used for the development of a new accurate, novel and inexpensive method for the determination of nitrosamines in sausage. The efficiency was improved by changing the type and volume of extraction solvent, nitrosamine solvent, and salt addition effect. In this regard, nitrosamines detection at 0.4μg Kg^{-1} levels becomes possible. Therefore, this method could be utilized for quantifying ultra-trace residues of nitrosamines in sausage with good accuracy.

The quantity of N-nitrosamines in raw and fried sausage considered and the trend of nitrosamines concentration at the presence of sodium nitrite and ascorbic acid monitored. The

Method	Quantitation limit (μg Kg^{-1})	Detection Limit (μg Kg^{-1})	Recovery (%)	Real sample	Linear range (μg Kg^{-1})	Ref.
GC-NCD	0.26 to 0.6	0.077-0.18	82-105.5	Fast food, meat	0.25-500	[3]
GC-EID	0.003	0.001	103	Potato	-	[35]
SFE and MEKC	400	-	20-81.6	Sausages	-	[18]
GC-MS-PCI	0.13	400-1600	82-111	Water	10000-200000	[36]
SPE-GC-CI-MS	0.03-0.36	0.01-0.12	60-105	Meat products	0.25-500	[6]
GC-GC-NCD	6.96-16.71	1.66–3.86	76-85	Meat products	2-300	[3]
GC-PCI-MS/MS	-	0.10-0.30	80-120	Foodstuff	-	[7]
GC-MCI-MS	0.5-1.24	0.15-0.37	70-114	Processed meats	1-100	[1]
HPLC-APCI-MS/MS	0.08-2.76	0.03-1.26	80-110	Food	5-50	[28]
HPLC-UV	0.26-1.82	0.08-0.55	74-117	Food products	0.3-100	[37]
GC-CI-MS	0.50-1.24	0.15-0.37	70-114	Processed meats	1-100	[38]
HS-SPME-GC-MS		0.00078-0.0112	97.44-107.16	Water	0.1-100	[27]
GC-FID	0.5-1.0	0.4	63-97	Sausage	50-500000	This work

Table 5: Method analytical figures of merit for nitrosamines determination in sausage at a comparison to other methods

nitrosamines (NDMA, NDEA, and NDBA)amount in the fried sausage was higher than raw sausage and there was a significant difference between them ($P < 0.05$) and during the time, nitrosamines quantity increased both in raw and fried sausage. The other nitrosamines(NMEA, NDPA, NDPhA, NPIP, NPYR, NMOR) were negligible or were not found in sausage samples. Nitrite and ascorbic acid levels in raw and fried sausage samples decreased during the time and the rate of reduction was more significant in fried ones. Reduction in ascorbic acid amount was more significant than a reduction in nitrite amount, that this fact shows some of the ascorbic acids is used for preventing nitrite participation in nitrosamine formation.

Therefore, amines react with nitrites that exist in sausage and nitrosamines formation occurs by thermal processes. All trends have been presented in figure 1.

In this research, the amount of nitrite, ascorbic acid, and nine nitrosamines was measured in sausage samples during the time. The concentration range of the nine nitrosamines in the monitored samples was from 2.6 to 17.3 μg kg^{-1}.

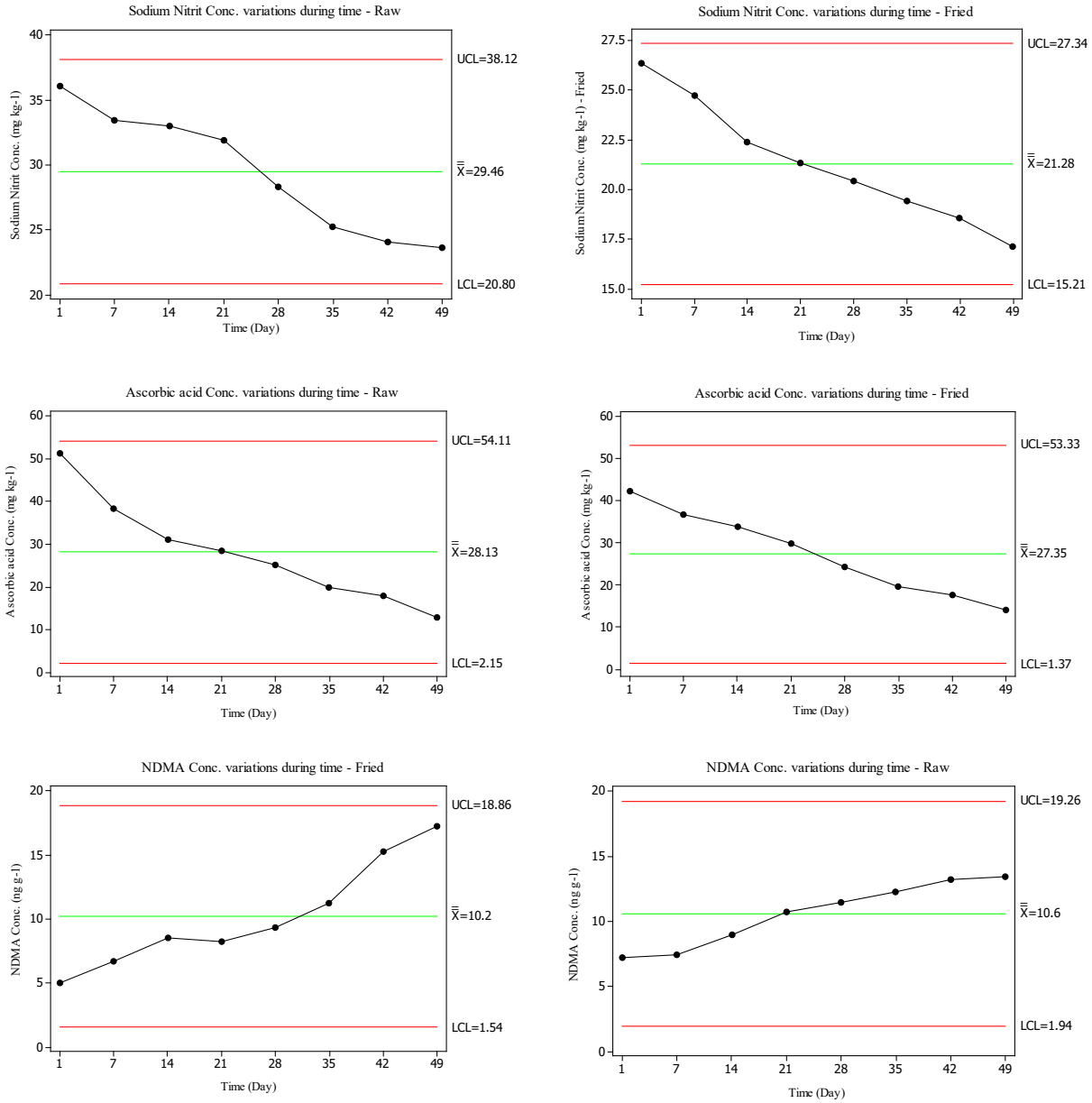

Figure 1: Ascorbic acid and sodium nitrite effect on nitrosamine formation in during time

Acknowledgments

The authors are grateful for financial support from the Meyar Danesh Pars Company and also the University of Kashan with Grant No. of 52798.

Compliance with Ethical Standards

Ethical Approval This article does not contain any studies with human or animal subjects.

Informed consent Publication has been approved by all individual participants.

Conflict of Interest Mehdi Nabi declares that he has no conflict of interest. Mohsen Behpour declares that he has no conflict of interest. Sayed Mehdi Ghoreishi declares that he has no conflict of interest.

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