A DISSERTATION ON

Validation of QuEChERS Method for Extraction of Nitrosamines in Noodles by LC-MS/MS

SUBMITTED TO THE
DEPARTMENT OF BIOENGINEERING
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FOR THE
B.Tech.-M.Tech. Dual Degree Biotechnology
IN
Biotechnology

BY

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B.Tech.-M.Tech. Dual Degree Biotechnology (**X Semester),**
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UNDER THE SUPERVISION OF
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DECLARATION FORM

I, **Naveera Khan**, a student of **B.Tech.-M.Tech. Dual Degree Biotechnology** (5 Year/ X Semester), Integral University have completed my six months dissertation work entitled **"Validation of QuEChERS method for Extraction of Nitrosamines in Noodles by LC-MS/MS"** successfully from **CSIR-Indian Institute of Toxicology Research,Lucknow-226001** under the able guidance of **Dr. Nasreen Ghazi Ansari.**

I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

Naveera Khan

Dr.Ashish
(Course Co-ordinator)

सीएसआईआर-भारतीय विषविज्ञान अनुसंधान संस्थान
CSIR-INDIAN INSTITUTE OF TOXICOLOGY RESEARCH

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यह प्रमाणित किया जाता है कि **सुश्री नवीरा खान** [Enrollment No1700100128] **B.Tech + M.Tech (बायोटेक्नालॉजी)**, इंटीग्रल यूनिवर्सिटी, कुर्सी रोड, लखनऊ, उत्तर प्रदेश 226026 लखनऊ में अध्ययन कर रही है । इन्होने विषय **Validation of QuEChERS Method for Extraction of Nitrosamines from Noodles using LC-MS/MS** पर दिनांक **17/01/2022 से 16/07/2022** तक शोधकार्य का प्रशिक्षण **डॉ नसरीन गाज़ी अंसारी,** वरिष्ठ वैज्ञानिक, सीएसआईआर-आईआईटीआर लखनऊ, उत्तर प्रदेश के पर्यवेक्षण में प्राप्त किया है।

हम सभी **सुश्री नवीरा खान** के उज्जवल भविष्य की कामना करते है।

To Whomsoever it May Concern

This is to certify that **Ms Naveera Khan** [Enrollment No 1700101181] student of **B.Tech + M.Tech (Biotechnology)**, Integral University, Kursi Road, Lucknow 226026 Uttar Pradesh, has undergone dissertation training on **Validation of QuEChERS Method for Extraction of Nitrosamines from Noodles using LC-MS/MS** during **17/01/2022 to 16/07/2022** under **Dr. Nasreen Ghazi Ansari,** Principal Scientist, CSIR - Indian Institute of Toxicology Research, Lucknow. Uttar Pradesh

We wish **Ms Naveera Khan** success in her future endeavours.

Professor V P Sharma

26/07/2022

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Certificate that Ms **Naveera Khan** (Enrollment Number 1700101181) has carried out the research work presented in this thesis entitled **"Validation of QuEChERS method for Extraction of Nitrosamines from Noodles using LC-MS/MS "** for the award of .**B.Tech.-M.Tech. Dual Degree Biotechnology** from Integral University, Lucknow under my supervision. The thesis embodies results of original work and studies carried out by the student himself/herself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/Institution. The dissertation was a compulsory part of **B.Tech.-M.Tech. Dual Degree Biotechnology**

I wish her good luck and bright future.

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Principal Scientist

Analytical Chemistry Department

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I wish her good luck and bright future.

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I wish her good luck and bright future.

Dr. Alvina Farooqui
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Naveera Khan

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LIST OF ABBREVIATIONS

S NO.	Abbreviation	Meaning
1	℃	Degree Centrigade
2	µg	Microgram
3	kg	Kilogram
4	mL	Milliletre
5	g	Gram
6	L	Litre
7	LOD	Limit of Detection
8	LOQ	Limit of Quantification
9	NSA	Nitrosamines
10	NDMA	N-Nitrosodimethylamine
11	NDEA	N-Nitrosodiethylamine
12	NDBA	N-Nitrosodibutylamine
13	NPIP	N-Nitrosopiperidine
14	NPYR	N-Nitrosopyrrolidine
15	NDPA	N-Nitrososipropylamine
16	NPRO	N-Nitrosoproline
17	NHPRO	N-Nitrosohydroxyproline
18	SPE	Solid Phase Extraction
19	SPME	Solid Phase Microextraction
20	LLE	Liquid Liquid Extraction
21	LLME	Liquid Liquid Microextraction
22	ACN	Acetonitrile
23	GC	Gas Chromatography

24	TEA	Thermal Energy Analyzer
25	LC	Liquid Chromatography
26	MS	Mass Spectrometry
27	MS/MS	Tandem Mass Spectrometry
28	DED	Direct Extraction Device
29	SIM	Single Ion Monitoring
30	MRM	Multiple Reaction Monitoring
31	NFDM	Non Fat Dry Milk
32	MAE	Microwave Assisted Extraction
33	SIS	Single Ion Storage
34	HPLC	HighPerformance LiquidChromatography
35	VNSA	Volatile Nitrosamine
36	NVNSA	Non-Volatile Nitrosamine
37	APCI	Atmospheric Pressure Chemical Ionization
38	APPI	Atmospheric Pressure Photo Ionization
39	QQQ	Triple Quadrapole
40	TQMS	Triple Quadrapole Mass Spectrometry
41	QTOF	Quadrapole Time Of Flight
42	UPLC	Ultra-Performance Liquid Chromatography
43	IS	Ionization Source
44	CXP	Cell Exit Potential
45	DP	Declustering Potential
46	CE	Collision Energy
47	CAD	Collision Associated Dissociation
48	EF	Enrichment Factor

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ABSTRACT

For a near Century, Nitrosamines in foods have posed a serious threat to human community because of their carcinogenic properties. However because of certain matrix complexity, extraction and analysis of NSAs has been a real challenge. So a quick, easy, cheap, effective, rugged and safe (QuEChERS) method was developed coupled with liquid chromatography-triple quadrapole mass spectrometry for their determination in mainly two kinds of food consumed widely all over the world. The standard preparations were performed and extraction conditions were optimized and investigated thoroughly. The matrix effect was estimated in the organic solvent and the actual samples by comparing slopes of their caliberation curves. The NSAs can be completely separated in 4 minutes. The recoveries off all the Nitrosamines ranged from 62-144%. The LOD was in the range of 0.12 to 0.56 µg/mL, while the LOQ was in the range of 0.12 to 3.11 µg/mL. The described method is rapid, sensitive, cost effective, highly selective and precise.

1.INTRODUCTION

Nitrosamines have been studied by scientists for more than 100 years. Carcinogenic properties of nitrosamines were detected in 1954 by Barnes and Magee (Nawrocki and Andrzejewski, 2011) in various human diets and other environmental media. Nitro-sating agents found in various system like food, water, air reacts with 1° and 2° amines for the formation of NSAs (Bhangare et al., 2015) . which are known to be causing cancer, mutations like symptoms (Al-Kaseem et al., 2014) and effects organs like nose, skin, kidney, esophagus, brain and nervous system(Herrmann et al., 2014). Nitrosamines have been found in various food products such as meat varieties like pork, bacon, salami, spiced meat, preserved sausages,(Yurchenko and Mölder, 2007, Herrmann et al., 2015) various dairy products like non-fat dried milk, dried butter milk(Libbey et al., 1980) cheese, yogurt (Vanginkel, 1970) other products like beans, turkey, hotdogs, rice, pasta(Griesenbeck et al., 2009) different alcoholic beverages like beer, wine, malt beverages, whiskey (Goff and Fine, 1979, Griesenbeck et al., 2009)].

Various surveys and researches have been conducted for determination of carcinogenic properties of nitrosamines in various countries all over the world like in Korea the range of NSAs were estimated from 0.3 to 1.54 µg/kg in processed meats and from 0.56 to 0.72 µg/kg in dairy products (Park et al., 2015) in London the range was estimated up to 1-9 µg/kg in meat products like bacon, and up to 1-4µg/kg in different varieties of cheese (Crosby et al., 1972) in Italy surveys were conducted in which amount of nitrosamines present came out as 0.35 to 2.5 ppb in canned meat and up to 0.19 to 0.72 ppb in alcoholic beverages were observed (Gavinelli et al., 1988) in Denmark the exposure of nitrosamines by the consumption of processed meat was found to be 0.34 to 1.1 ng/kg (Herrmann et al., 2014) in France different meat samples like smoked bacon, ham, pork were studied in which total nitrosamines concentration estimated was 0.25 to 0.79 µg/kg (Mavelle et al., 1991) in Germany the current daily exposure of nitrosamines in meat products is 0.2 to 2.5 µg/kg(Tricker et al., 1991) in Canada, when milk and milk products were tested, nitrosamines were found at a level of average of 0.4 µg/kg(Weston, 1983).

Nitrosamines can be classified in basically two categories that are volatile and non-volatile nitrosamines (Ramezani et al., 2015)volatile and non- volatile nitrosamines differ on the basis of their polarity and molecular weights. Volatile N-nitrosamines include N-

nitrosodimethylamie (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosodibutylamine (NDBA), N-nitrosopiperidine (NPIP), N-nitrosopyrrolidine (NPYR), N-nitrosomorpholine (NMOR), and N-nitrosodiphenylamine (NDPhA) and non-volatile N-nitrosamines includes N-nitrosoproline (NPRO) and N-nitrososarcosine(Seo et al., 2016).

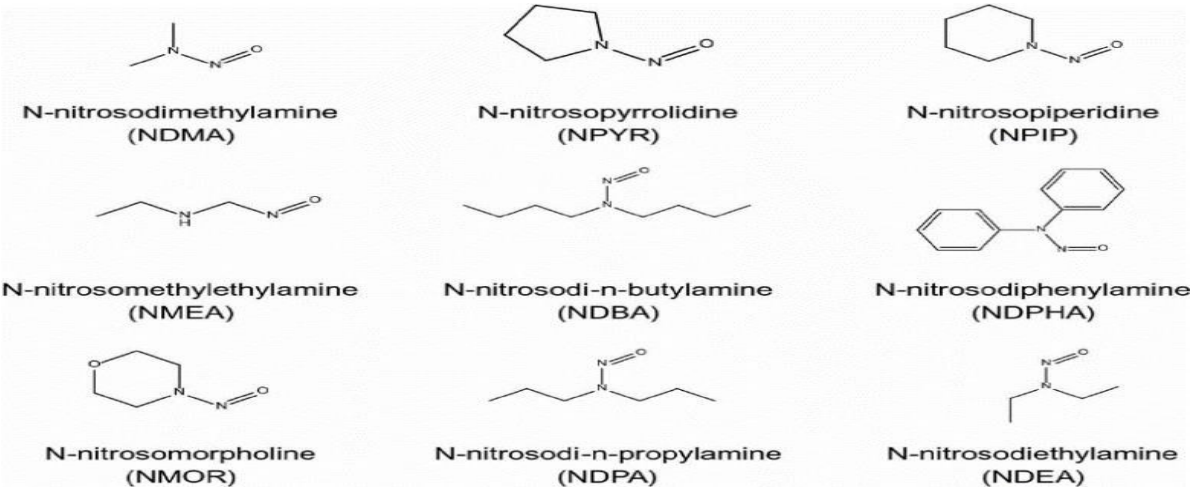


Fig-1 structures of different volatile n-Nitrosamines

When the different experiments were performed, the NSA with highest carcinogenic property came out to be N- nitrosodiethylamine (NDEA), followed by NDMA, with less potency, NPYR and NPIP lesser than NDMA and some NSAs were proven to be harmless like (NPRO) and NHPRO(Weston, 1983).

For the analysis and detection of nitrosamines various steps are involved in which the extraction method to refine and condense the nitrosamines is said to be rate limiting step in analysis.(Li et al., 2021) various extraction procedures like solid phase extraction (SPE), liquid-liquid microextraction(LLME), solid phase micro-extraction(SPME) have been bought to light by various researchers (Boyd et al., 2011). SPE has been used as an effective method for purification of NSAs in water analysis(Ozel et al., 2010) and in certain food products like sausages and milk in which amount of sample and solvent required for analysis has been exceptionally decreased and the detection limit came out to be 0.3 ppb[20]. Solid phase microextraction method used for analysis of water for detection of volatile nitrosamines have been proven to be a reliable and easy technique with detection limit upto 30 ng/lt(Grebel et al., 2006) and it is advantageous over solvent extraction as it provides more accurate results with high sensitivity as in presence of NDEA and NDMA in cured meat and sausages with detection limit upto 10µg/kg(Andrade et al., 2005).according to the various reviews and researches DLLME has been proved to be a

novel method compared to all the other procedures for extraction of nitrosamines with more recovery, less time consuming, inexpensive and high selectivity and sensitivity as in estimation of nitrosamines in sausages and salami with detection limit up to 0.1 to 9.0 ng/g (Scheeren et al., 2015).

As its name suggests, QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) is conducted quickly, easily, and safely through the use of non-halogenated solvents and a straightforward approach, giving it various advantages over other extractions. The QuEChERS method constitute to important steps: a LLE step and a cleaning step using a dispersive SPE.. In the first step, an aqueous-based sample is extracted using an organic solvent such acetonitrile (ACN), and the miscible aqueous and acetonitrile phases are separated using salts. A d-SPE sorbent is added in the subsequent phase to bind unwelcome substances and produce a cleaner sample for analysis.

2.REVIEW LITERATURE

In this study, headspace sampling by SPE and GC with TEA detection were used for determining volatile NSAs in sausages. Two fused silica fibres, one coated with poliacrylate (PA) and the other with polydimethylsiloxane-divinylbenzene (PDMS-DVB), were compared. The effects of equilibrium time, ionic strength, extraction time, and temperature on the extraction of four NSAs in sausages were assessed using a factorial fractional design. For the purpose of GCTEA N-nitrosamine analysis in cured meat, SPME showed to be a beneficial extraction procedure. The technique is straightforward, enables quick analysis, is solvent-free, and has a good level of selectivity(Grebel et al., 2006).

For agricultural food , the quantitative analytical procedures for seven N-nitrosamines were developed. For fatless matrices, a combination of solid-supported LLE with Extrelut NT and SPE with Florisil was used. LLE was established for a matrix containing fat, and only SLLE without SPE was used for an alcohol matrix. for ion source ammonia gas was used and the extract was examined using (GC-PCI-MS/MS). The range of LOD and LOQ for all N-nitrosamines under investigation was 0.10 to 0.18 g/kg for rice , 0.10 to 0.19 g/kg for juice, 0.10 g/kg for maize oil, and 0.10 to 0.25 g/kg for 20% alcohol. As a result, the established technique(Seo et al., 2016).

In this study in different meat products extraction and analysis of NSAs was performed in which values for LOD, LOQ, linearity, repeatability were studied using gas chromatography and nitrogen chemiluminescence detector. different types of processed meat like salami, kebab and sausage were analyzed which detected combined concentrations of NSAs in them. The range of the six NAs' combined concentrations in the examined beef samples was 0.45 to 16.63 g/kg(Ozel et al., 2010).

A class of carcinogens known as N-nitrosamines (NAs) have been found in a number of meat products. Between 2001 and 2005, 386 different meat samples were tested for the presence of five NSAs NDMA, NDEA, NDBA, NPIP, and NPYRThe investigation comprised beef products that were marinated, roasted, broiled, barbecued, and preserved. Extrelut and Florisil sorbents were utilised in a two-step SPE for sample cleaning. Using ammonia as the reagent gas, Column chromatography was used to separate the NAs, and positive-ion chemical ionisation was used to identify them.. In 386 examined meat samples,

the total amounts of NAs ranged from undetectable to 30 lg/kg. Fried food samples contained the highest concentrations of NAs(Ozel et al., 2010).

This study was done on irradiated papperoni and salami sausages in which gas chromatography along with thermal energy analyzer(TAE) was used for detection of NDMA and NPYR. Sausages were kept at 4 degree Celcius and irradiated at 0, 5. 10 and 20 kGy for four weeks. Irradiating the fermented sausage considerably lowered the levels of NDMA and NPYR.it was observed that in irradiated sausages NSAs concentration were found less as compared to non irradiated one, According to the study, storage of fermented sausage included lower amounts of the cancer-causing N-nitrosodimethylamine and N-nitrosopyrrolidine after irradiation. GC-TEA was able to accurately measure these volatile Nnitrosamines even at these low trace levels. s. According to this study, food radiation can improve food quality and can also lower the toxicity in foods(Ozel et al., 2010).

In this study, 150 samples of canned foods were taken from food processing plants in various areas of Poland between 2000 and 2001 to measure their N-nitrosamine level. These items included canned beef, seafood, and offal. The investigations were carried out using a thermal energy analyzer and gas chromatography. About 61 percent of the individual samples contained detectable quantities of N-nitrosamines. NDMA was present in almost 58 percent of samples, with an estimated value of 3.01 g/kg. NPIP was detected in roughly 6% of specimen. There were no other volatile Nnitrosamines found. It should be noted that the discovered levels of nitrosamines are reasonably low and comparable to the values found in other countries when taking into account the results of volatile Nnitrosamine estimate in samples of canned meat and canned offals(Ozel et al., 2010).

The main objective of this work was to determine whether NA could be detected from a sample matrix utilising an exposure time as brief as feasible in order to assess the potential application of SPME-DED-GC-MS as a screening analysis method in practical scenarios.. SPME along with DED was used as an extraction method for nine volatile nitrosamines in food. Linearity and effectiveness of extraction of NSA was checked at 4 degree and 25 degree temperatures. For analysis Gas chromatography-mass spectrometry (GC-MS) was used in SIM mode. The findings imply that for all investigated NA, greater chromatographic areas were achieved with longer extraction times. So, even at refrigerated temperatures and with quick extraction durations, SPME-DED-GC-MS appears to be an

appropriate technique to perform an initial and subjective study of volatiles NA frequently observed in foods(Ozel et al., 2010).

This study looked for nitrosamines in nonfat dry milk that had been heated indirectly using steam and fired directly. Electron capture gas chromatography was used to identify the nitrosamines in nanograms per gram of non fat dry milk. With the help of this technique, nitrosamines added to NFDM at a rate of 10 ng/g could be isolated and found (10 ppb). An analysis of NFDM samples revealed that there are no steam distillable nitrosamines in NFDM at 10 ppb and that it makes no difference whether the heat source is direct gas fired or indirect steam. No volatile nitrosamines were detected in freshly kept dry milk or kept for 30 days with or without heating(Ozel et al., 2010).

Dimethylnitrosamine (DMN) and the other six volatile NSAs were extracted from meat items in this investigation using a sensitive method called microwave-assisted extraction (MAE) combined dispersive micro SPE. Systematically examining the factors that affect MAE and D-SPE efficiency. 30 ml of 0.025M NaOH solution was used to wash the 5 gm meat sample which was extracted at 100 C for 10 min. The NAms were shaken vigorously for 30 minutes, and after that, 200 l of dichloromethane was used to desorbate them.(GC-CI-MS) in the selected-ion-storage (SIS) mode was used to analyse a 10 lL sample. The procedure offers high linear range, low ng/g level detection limits, and outstanding precision and sensitivity(Ozel et al., 2010).

This paper describes a quick extraction method for analysing volatile NSAs in alcoholic drinks. GC along with thermal energy analyzer is used for the analysis with extraction procedure LLE were dichloromethane is used to extract eluate. NDMA was found in the range of 0.2g/L and NPYR with a range of 0.5 g/L in a 15 ml sample. The presence of NDMA ranged from 0.4 to 7.0 g/liter in all the beers and from 0.3 to 2.0 g/liter in six of the seven Scottish whiskies. VNSAs were not found in any examines alcoholic liqueurs like vodkas and rums. Consequently, it may be said that in vodkas, liqueurs, gins and brandies NSAs are not the Cancer causing agents(Goff and Fine, 1979).

In addition to initiatives to eliminate nitrosamines from water, this review highlights the presence of nitrosamines in water, their abundance, and their constituents in drinking and wastewaters. The article also discusses the ways by which nitrosamine forms in water technologies. when dichloromethane reacts with dimethylamine it forms unsymmetrical

dimethylhydrazine which oxidizes to form NDMA and this process can be assumed to form chlorination in drinking water and creation of NDMA in it.. The current approaches for nitrosamine measurement are based on nitrosamine enrichment via SPE, extraction of the analytes with chloromethane, amount of the extract to be less than 1 ml, and GC or HPLC analysis with Mass Spectrometry detection. Both LLE and SPE procedures frequently result in low NSAs recoveries. Significant amounts of NMDA may also be produced by ozonation (and other oxidizing agents), especially if certain components are present in processed waters. Fortunately, the majority of the NSA is removed by biological filtering, which frequently comes after ozonation in potable water technologies. There are several approaches being researched to destroy NSAs. It appears that photolytic techniques have the most promise for use. (Nawrocki and Andrzejewski, 2011).

This study primarily focuses on numerous Korean foods in which nitrosamines are found in varied amounts utilizing various analytical techniques, such as In place of distillation for the cleanup processes, solid supported LLE and SPE are used, and MS/MS detection is used which is highly sensitive and selective. NH3 gas was used as an ion source in a gas chromatography-positive chemical ionisation tandem mass spectrometer (GC-PCI-MS/MS), the extract was examined. Fresh vegetables and mushrooms had maximum NDMA concentrations of 6.1 and 4.9 g/kg, respectively, and mushrooms had the highest NDBA concentration of 6.11 g/kg. Cheese's NDMA concentration was 0.72 g/kg, but that of other foods like bakery products was less than 0.56 g/kg.. Furthermore, beef and meat products had the highest rate of NDMA detection. NDMA in meats was found in the range of 0.31 to 1.54 g/kg which was lot less than in vegetables(Crosby et al., 1972).

In this study, nitrosamines were found in 19 marine food products that were procured from the fishing areas along the western coasts of Maharashtra, India. 20 gram of the material were thoroughly mixed with n-Hexane, methanol, water, 1.5 M sulfuric acid, and 1 percent sulfamic acid (50:40:8:2). Prior to centrifugation, With 25 ml of 1 M Potassium Hydroxide, the aqueous layer was alkalized, and methylene chloride was used to extract it. An HPLC method was optimized. for the measurement of five VNSAs. For several NAs, it was discovered that the LOD and LOQ were in the range of 0.2 to 0.4 ng. With an average value of 0.82 g/kg, the levels of total NSAs ranged from Non-Detected to 2.29 g/kg. Additionally, it was found that storage time and temperature had a big impact on how N-nitrosamines

formed. As a result, the HPLC approach for nitrosamine determination is straightforward, quick, and applicable to routine analysis(Bhangare et al., 2015).

According to a study on processed meat products conducted in Denmark, 20 gram and 16 gram of different meat products are consumed daily by adults in Denmark (15–75 years old) and children in Denmark (4-6 years old) (95th percentile). Ham, salami, boiled and seasoned pork flank, sausages, and salami are the main food items that make up the consumption. The majority of the total consumption comes from sausages (25–30%), with salami making up the second-highest consumption for both categories of kids (13–20%). The third most popular commodity is either ham (6-14 and 15-75 years) or pork flank (4-5 years).. Thus, the Danish population's top consumers, as measured by the 95th percentile, consume around 1.5 times as much (20 g) as is said to have an impact on mortality. Due to this usage, adults and children, respectively, are exposed to 33 and 90 ng/kgof NVNSA per day, The volatile NSAs were in the span of 0.34 to 1.1 ng/kg in adults and children(Libbey et al., 1980).

3.OBJECTIVES

The dissertation entitled **"Validation of QuEChERS method for Extraction of Nitrosamines from Noodles Using LC/MS-MS"** was designed with the main aim of obtaining Nitrosamines from food matrix. My objectives for this study were:

- Development of sensitive method for trace level nitrosamine estimation in packaged food.
- Qualitative and Quantitative analysis of samples on Liquid Chromatography/Tandem Mass Spectrometry.

4.MATERIALS AND METHODS:

4.1.INSTRUMENTATION

4.1.1.LC-MS/MS (LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY)

The analytical chemistry method known as LC-MS/MS combines mass spectrometry's mass analysis skills with liquid chromatography's physical separation capabilities (MS). Mixtures with many components are separated by liquid chromatography whereas for identifying structural idea of an analyte with high sensitivity and selectivity , mass spectrometry is performed. Using this tandem method, different inorganic and organic substances frequently present in composite samples with biological and sustainable origins can be analysed. As chromatographic column is not comapatible to MS source, there is requirement of interfaces. The interfaces in the LC-MS system efficiently transfer the divided parts from the chromatographic column into the MS source. The mechanically straightforward LC-MS interface preserves the chemical identity of the chromatography products while transferring as much analyte as is physically possible, eliminating a substantial portion of the mobile phase utilised in LC (chemically inert).

atmospheric pressure chemical ionisation (APCI), electrospray ionisation (ESI) and atmospheric pressure photo-ionization (APPI). Are certain ionization technique in LC/MS-MS. By connecting two mass analyzers that are run in series, it is possible to further enhance sample identification and precise quantification.

triple quadrupole mass spectrometers (QQQ or TQMS and quadrupole time-of-flight (QTOF) are most used mass spectrometers. the TQMS consists of three main parts that is Q1 q2 and Q3 in which Q1 and Q3 qre mass analuzers while q2 is a collion cell. The precursor iions in Q1 are fragmented by collision cell using high energy collisions with gases like helium argon or nitrogen. An accurate m/z value can be monitored through scanning within a mass range using the two analyzers

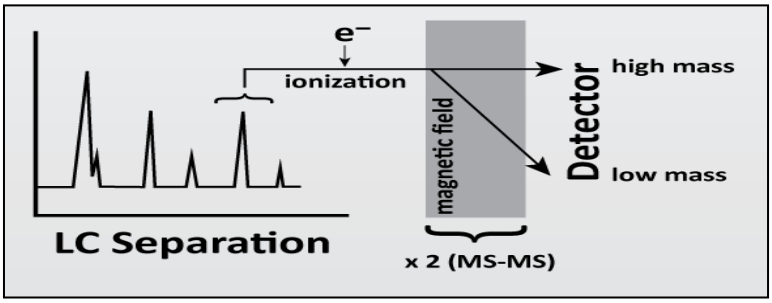


Fig-2 LC/MS-MS spectrometry

4.1.2.WEIGHING BALANCE

A beam balance, sometimes known as a beam scale, is a tool used to quantify mass or weight.

The conventional scale is made up of two plates or bowls suspended from a fulcrum at equal intervals. The plates level off until static equilibrium is reached, which occurs when the masses on the two plates are equal. One plate is used to support an object with an unknown mass (or weight), while the other plates are filled with known masses. The ideal scale rests at zero. A known-stiffness spring will be used in a spring scale to calculate mass (or weight). The spring will lengthen by a specific amount when a certain mass is suspended, depending on the spring constant. According to Hooke's law, the spring stretches more the heavier the object is. There are more scales with various physical principles at work. Instead of reading in units of mass like kilogrammes, some scales can be calibrated to read in units of forces (weight), such as newtons. Since many products are sold and packed in bulk, scales and balances are frequently employed in commerce.

A type of balance called an analytical balance is made to measure minuscule masses down to milligrammes. A transparent cage with doors surrounds the measurement pan of an analytical balance (0.1mg or better), keeping dust out and preventing the room's air currents from interfering with the balance's performance. This barrier is frequently referred to as a draught shield. A mechanically ventilated balance safety container and specifically created Acrylic airfoils are used to create a smooth, turbulence-free airflow, which eliminates balance fluctuation and allows the measurement of mass up to 1 g without changes.

Fig-3 Weighing Balance

4.1.3.CENTRIFUGE

A centrifuge is a machine that employs centrifugal force to separate different fluid constituents. this is accomplished by rapidly spinning the fluid inside of a container to separate fluids with various densities or liquids from solids. Denser materials and particles travel outward in a radial direction as a result of its action. Less dense things travel to the centre and are dispersed at the same time. Denser particles sink to the bottom of sample tubes used in laboratory centrifuges while low-density materials ascend to the top due to the radial accelerate. They differ greatly in terms of speed, capacity, temperature regulation, and other features. Many different fixed-angle and swinging bucket rotors that can hold various numbers of centrifuge tubes and are rated for particular maximum speeds can be used in laboratory centrifuges.There are several different rotor options for ultracentrifuges that are suited for a wide range of experiments. The majority of rotors are made to accommodate tubes that house the samples. As the rotor begins to accelerate, swinging bucket rotors enable the tubes to hang on hinges so they can realign to the horizontal.The tubes are held in cavities that have been punched at a predetermined angle by fixed angle rotors, which are formed of a single piece of material. Instead of tubes, zonal rotors are made to hold a sizable amount of sample in a single core hollow. Some zonal rotors have the ability to load and unload samples dynamically while the rotor is spinning rapidly.

Fig-4 Centrifuge

4.1.4.ROTOSPIN

The rotospin rotary mixers are pieces of digital electronics that are used to mix different liquids and liquid mixes. They are specially made tubes, and in some circumstances, bottles, that may be loaded with samples onto the machine. Different speeds and durations of time can be used to create the movement, and both of these parameters are programmable. In more sophisticated versions. It is possible to create movements and motions other than rotating

motions. Rotating bar tubes range in diameter from 10 to 30 mm, and the time limit is 99 hours, 59 minutes. It has spring clips with a plastic coating.

Fig-5 Rotospin

4.1.5.VORTEX MIXER

A vortex mixer, sometimes known as a vortexer, is a straightforward tool used frequently in laboratories to mix tiny vials of liquid. It comprises of an electric motor positioned somewhat off-center, with the drive shaft vertically coupled to a cupped rubber portion. The rubber component rapidly oscillates in a circular motion as the motor turns. When a test tube is shoved into the vortex, a motion is provided to the content in test tube inside the rubber cup. Different vortex mixers have different speed range ranging from 100 to 3200 rpm and when a downward pressure is applied it can run continuously with configuration**.**

Fig-6 Vortex

4.1.6.PH METER

A pH meter is a scientific instrument that measure the hydrogen ion activity in water-based solution indicating its acidity or basicity expressed as pH.the pH meter measures the difference I electrical potential between a pH electrode and a referece electrode, and so the pH meter is sometimes referred to as a "potentiometric pH meter". The difference in electrical potential relates to the acidity or pH of the solution. The Ph meter is used in many applications ranging from laboratory experimentation to quality control**.**

Fig-7 PH Meter

4.1.7.OTHER INSTRUMENTS

1) Micropipette
2) Eppendrof
3) Glasswares-: beakers, conical flasks, volumetric flasks
4) Tarsons

4.2.Reagents and Solutions:

Acetonitrile, sodium acetate, magnesium sulfate, florisil were purchased from Honeywell (Germany); all solvents were of LC-MS grade except sodium acetate, magnesium sulfate , florisil that were of analytical grade. Water was purified with an Elga Purelab Option R7 (Labtec, Villmergen, Switzerland). Nitrosamines analytical standards were obtained from Sigma-Aldrich (Darmstadt, Germany) Mixed solutions of 1 ppm was prepared in which 1 ml of seven nitrosamines standards were mixed that is N-nitrosodimethylamie(NDMA), N-nitrosodiethylamine (NDEA), N-nitrosodibutylamine (NDBA), N-nitrosopiperidine (NPIP), N-nitrosopyrrolidine (NPYR), N-Nitrosonornicotine (NNN), Nitrosopiperidene (NPIP) in acetonitrile. These solutions were stored at -18°C in the dark to minimize analyte degradation.

4.2.1.STANDARD PREPARATION

Standards were prepared gravimetrically

- The stock standards of 10,000 µg/L were prepared by dissolving 10mg of individual nitrosamine in different 10ml calibrated volumetric flask with methanol.
- Respective working and intermediate standards of nitrosamine 500, 100,10 and 1 µg/L by diluting 0.100ml from 10,000 µg/L in 10ml, 2ml from 500 µg/L in 10ml, 0.200ml from 500 µg/L in 10ml and 1ml of 10 µg/L in 10ml.
- Mix of 7 Nitrosamine standards of 1 µg/L concentration were prepared by mixing 1ml of each of 7 standards, of 10 µg/L and make up to 10ml.
- The standards were weighed along with the lid, soon after mixing and shaking. The weight was noted down after its stable.
- All standard were stored in 4^{o}C and in dark conditions. Each volumetric flask was properly labelled with name of the compound, concentration and date of preparation. Gravimetric records were maintained after each withdrawal.

4 . 2 . 2 . SAMPLE COLLECTION

For the determination of NSAs in this study a total number of 100 noodle samples were collected which were categorised as branded and non-branded ones consumed on daily basis by majority of population.

Samples were purchased from approximately 10 -15 areas in Lucknow which were selected based on population density. The noodles of 40 branded were collected to be used for the estimation of nitrosamines. The 30 non-branded noodles were collected from various stalls and restaurants from local areas of the city.

4.2.3.Chromatographic conditions:

UPLC system (Acquity - Waters, Milford, USA) was used for chromatographic separation.. NSAs were separated by using the column Agilent ZORBAX RRHD Extend C18 (2.1mm x 150mm, 1.8µm). In order to acquire the best chromatographic resolution, UPLC conditions were tuned. We examined various mobile phase pH and compositions. The final chromatographic settings were 5 µL injection volume, 0.4 mL min-1 column flow, 35 °C column temperature, and 4 °C autosampler temperature. The mobile phase included 95 percent B and 5 percent A (0.1 percent formic acid in Milli-Q) (0.1 percent Formic acid in ACN). NSAs were eluted using an isocratic procedure that ran for a total of 4 minutes.

4.2.4.LC/MS-MS Analysis:

Mass spectrometry detection was performed using API 4000 triple quadrupole mass spectrometer from ABSCIEX in Framingham, Massachusetts, USA. Multiple reaction monitoring (MRM) modes were used for the acquisitions. Transitions to product ions were seen with positive polarity for MRM detection.

Electrospray voltages (IS), different temperatures (TEM), and gas flows were tried in order to optimise the source parameters. collision associated dissociation (CAD) gas was 12 psi, turbo gas (GS2), nebulizer gas (GS1), curtain gas was 10 psi, Dessolvation gas temperature was 400°C, and ionspray voltage was 5500 V in positive. These were the optimum values. The following are some of the Nitrosoamine's compound parameters, including Declustering Potentials (DP), Collision Energies (CE), and Cell Exit Potential (CXP):

Table 1: Optimized compound parameters of the Nitrosamines on LC-MS/MS

Compound	m/z (Q1/Q3)	Dwell time (msec)	DP (volts)	EP (volts)	CE (volts)	CXP (volts)
N-Nitrosodimethylamine	74.9/43.3	50	55	6	19	6
	74.9/46.9	50	60	6	9	6
N-Nitrosodiethylamine	103.1/74.9	50	45	10	12	16
	103.1/29.4	50	45	10	30	10
N-Nitrosodibutylamine	158.7/57.3	50	42	4	21	11
	158.7/41.5	50	27	4	35	7
N-Nitrosopyrrolidine	100.7/55.2	50	24	12	20	10
	100.7/59.3	50	21	7	20	6
N-Nitrosopiperidine	115.2/69.3	50	29	5	19	13
	115.2/41.4	50	35	8	33	8
N-Nitrosodipropylamine	130.9/88.9	50	40	5	13	8
	130.9/73.0	50	20	5	12	6

4.2.5.Calibration and Analytical Parameters:

The seven NSA were dissolved in v/v ACN at a concentration of 200 µL each to create the calibration standard. Curves for calibration were created with one point driven through zero. Before using the calibration standard, the system is calibrated using a number of injections of a sample.

4.2.6.Extraction Procedure:

Quechers (Quick, Easy, Cheap, Effective, Rugged, and Safe) approach with modifications was used to extract NSAs from the samples.. An amount of 1 gm of homogenised noodles were weighted in 15 ml centrifuge tubes. 2 ml acetonitrile were added with 100 mg $MgSO_4$ and 50 mg sodium acetate. The solution was mixed well on rotaspin for 10 minutes at 50 rpm. The solution was then vortexed for 2 min to solubilize all the nitrosamines. The test tube was shaken well and centrifuged at 5000 rpm for 3 minutes. The upper layer was then diluted with 50 gm florisil and then centrifuged at 8000 rpm for 2 minutes. The upper layer was then placed at -20°C. LC/MS-MS was used for the analysis with the conditions.

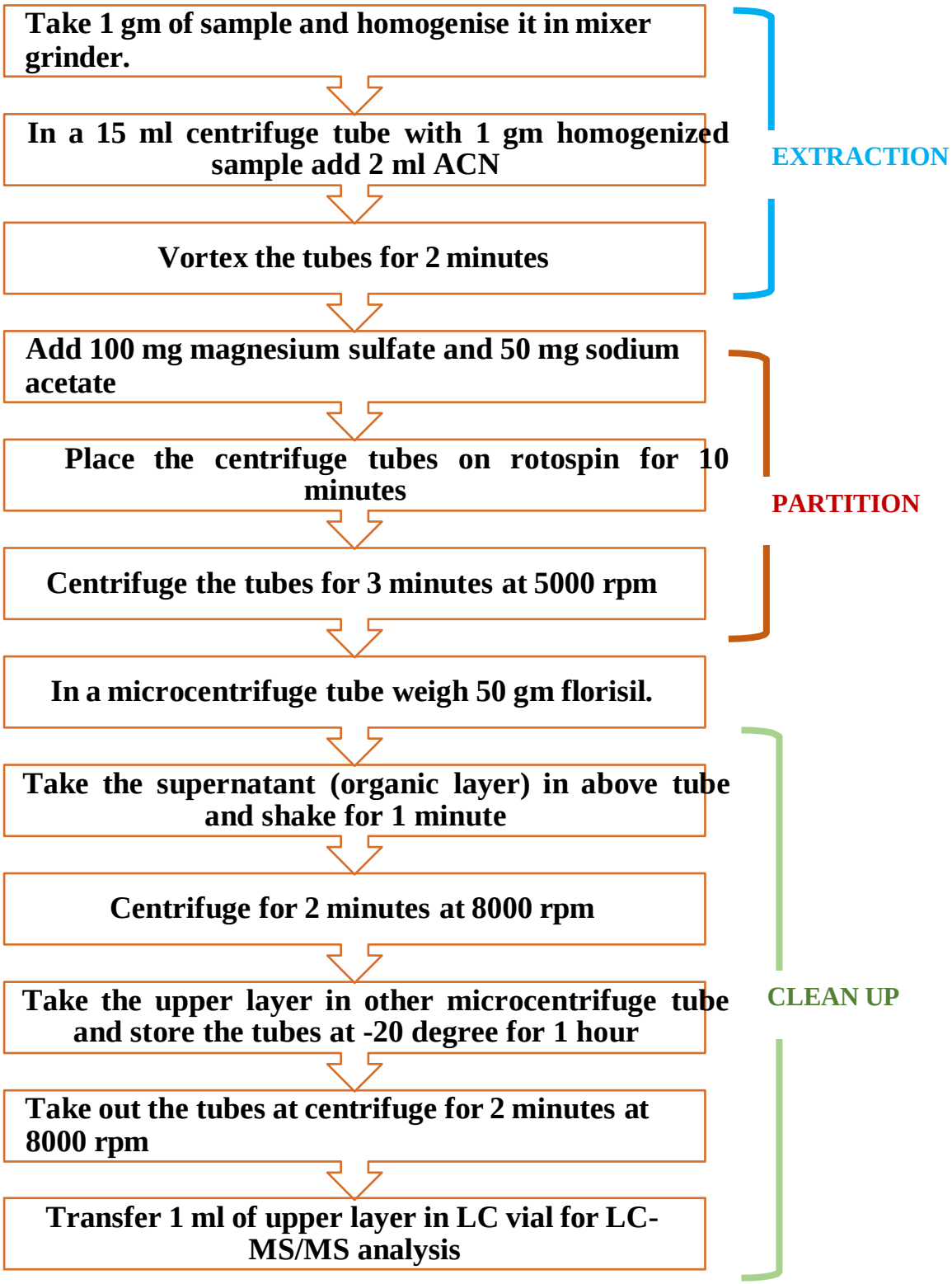


Fig: QuEChERS EXTRACTION METHODOLOGY

5.RESULT & DISCUSSION

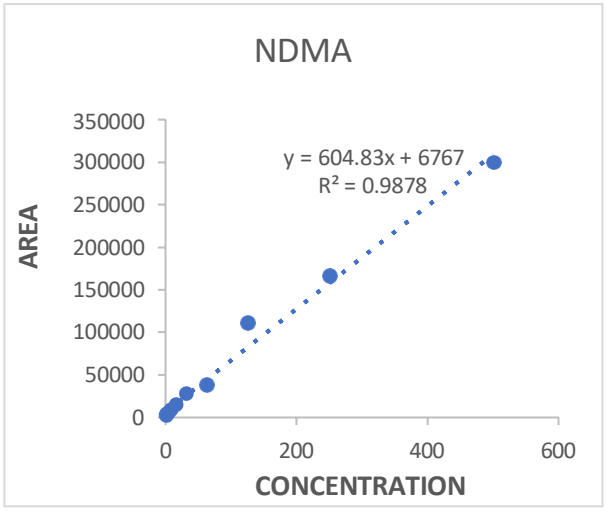
5.1.Linearity of Nitrosamines:

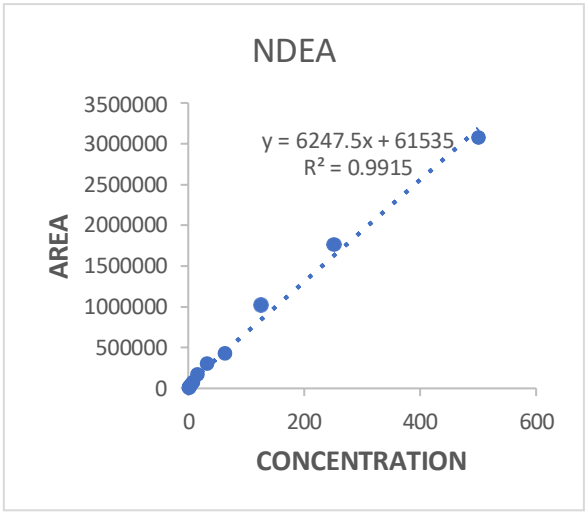
The standard followed the linear equation with regression coefficient near to unity. The linearity of six NSAs NDMA, NDEA, NDBA, NPYR, NPIP, NDPA are in the range of 0.48 to 500 ppb.

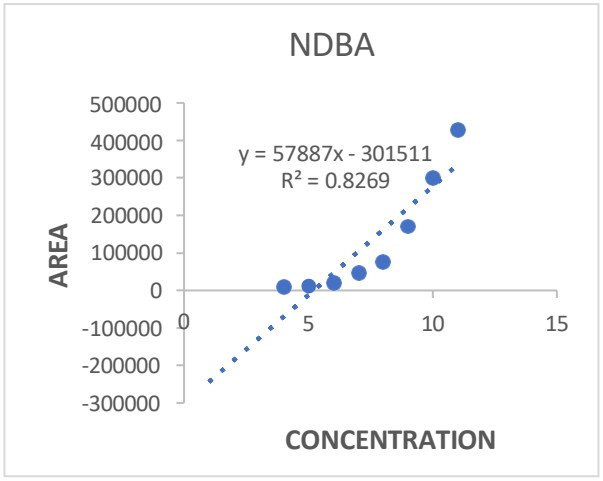
Table 2: Optimized analytical performances for NSA measurement using the QuEChERS method in combination with mass detection and liquid chromatography (LC-MS/MS); Limits of Quantification (LOQ) and LOD (limits of detection).

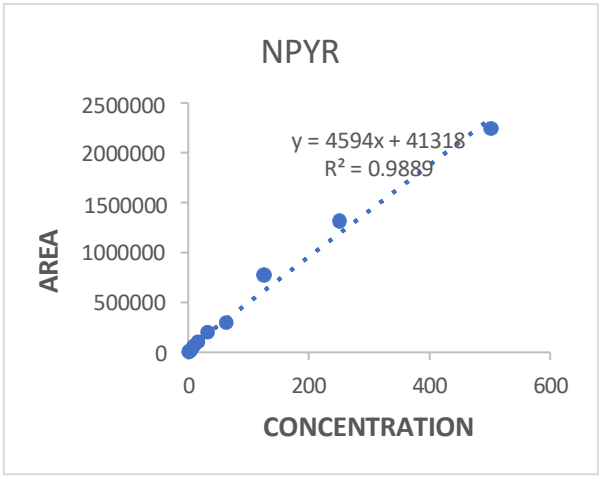
ANALYTE	LINEAR EQUATION	R^2	LOD	LOQ
NDMA	y=604.86x+6767	0.9878	0.402728	1.329002
NDEA	y=6247.5x+61535	0.9918	0.562103	1.855073
NDBA	y=57887x+301511	0.8269	0.129956	0.43318
NPYR	y=4594x+41318	0.9889	0.9332	3.110666
NPIP	y=12062x+90279	0.9931	0.374277	1.247589
NDPA	y=20132x+54857	0.9987	0.038307	0.127691

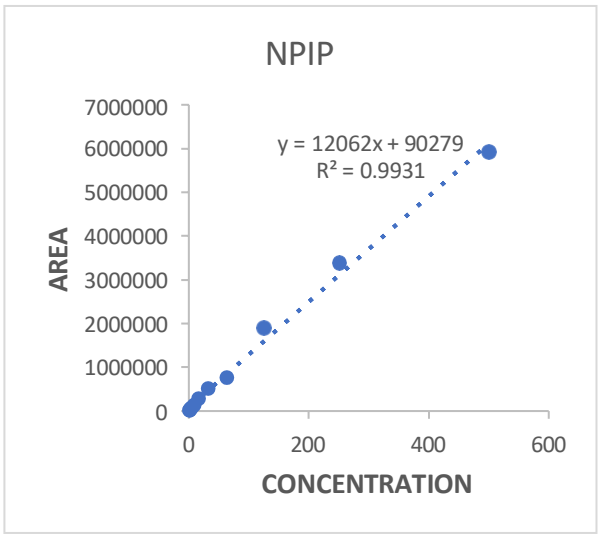
The Linearity of the six nitrosamines are depicted by the graphs below:

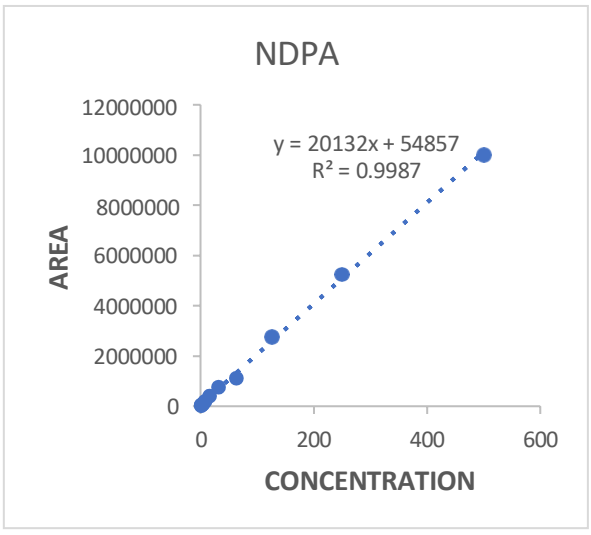












5.2.Optimization of QuEChERS Extraction Method:

Single-factor tests were used to examine the effects of extraction factors, such as the extraction solvent (acetonitrile), extraction solvent amount (2 ml), purifying agent (florisil), and salt type (sodium acetate and magnesium sulphate), on the recoveries. Each experiment was carried out three times(triplicate) the recovery was determined using the equation below.

$$R = \frac{(\text{analyte})_{\text{spiked sample}} - (\text{analyte})_{\text{non-spiked sample}}}{(\text{analyte})_{\text{added}}}$$

Table 3: Recovery, intraday, interday precision (relative standard deviation) at different five spiked levels (ppb) in analysis of Nitrosamines by the QuEChERS method:

Analyte	**{RR±Intraday)(%RSD)]N=6**				
	100	**200**	**300**	**400**	**500**
NDMA	115±9	66±23	64±9	64±9	65±7
NDEA	144±3	82±10	75±5	70±9	70±4
NDBA	117±6	66±2	62±12	64±9	62±4
NPYR	144±16	88±12	81±11	74±8	74±4
NPIP	131±6	81±12	73±7	70±7	68±2
NDPA	122±4	70±12	67±10	64±7	63±5

Analyte	[RR±Interday)(%RSD)]N=6				
	100	**200**	**300**	**400**	**500**
NDMA	611±125	-165-227	-236±.7	-175±.9	-141-.2
NDEA	45.8±.4	6.7±112	5.5±26	4.7±45	.2±352
NDBA	56.9±.5	6.7±84	1.9±70	0.3±135	.09±170
NPYR	37±.3	3±84	3.3±54	2.9±44	-.5-83
NPIP	40±.4	4.7±90	5±23	3.5±41	.24±245
NDPA	37±.7	2.5±131	2±23	0.4±377	-0.8-27

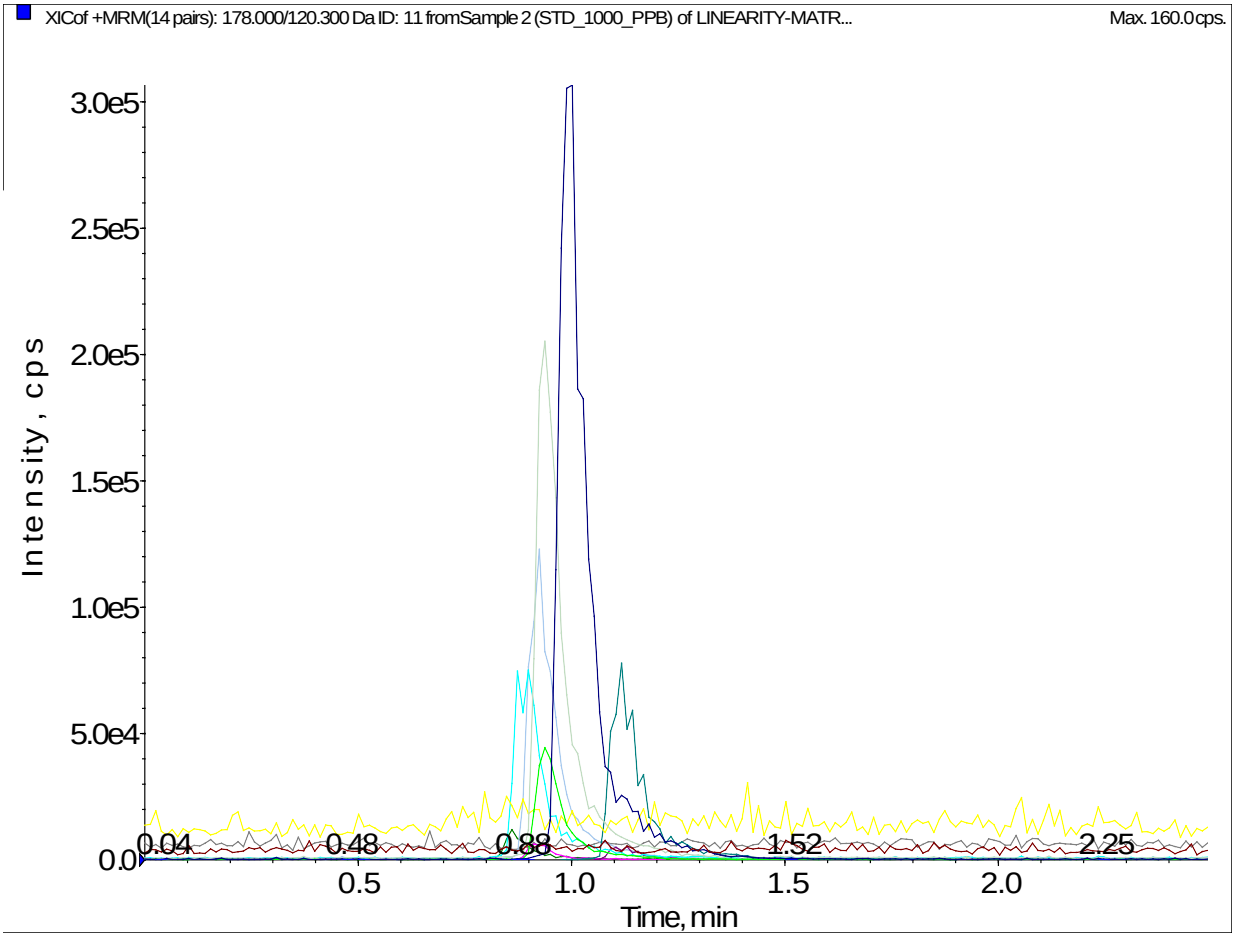


Figure8; Typical chromatogram of six Nitrosamines N-nitrosodimethylamine(NDMA),N-nitrosodiethylamine(NDEA),N-nitrosodibutylamine(NDBA), N-nitrosopyrrolidine(NPYR), nitrosopiperidine(NPIP), N-nitrosodipropylamine(NDPA) under optimum conditions.

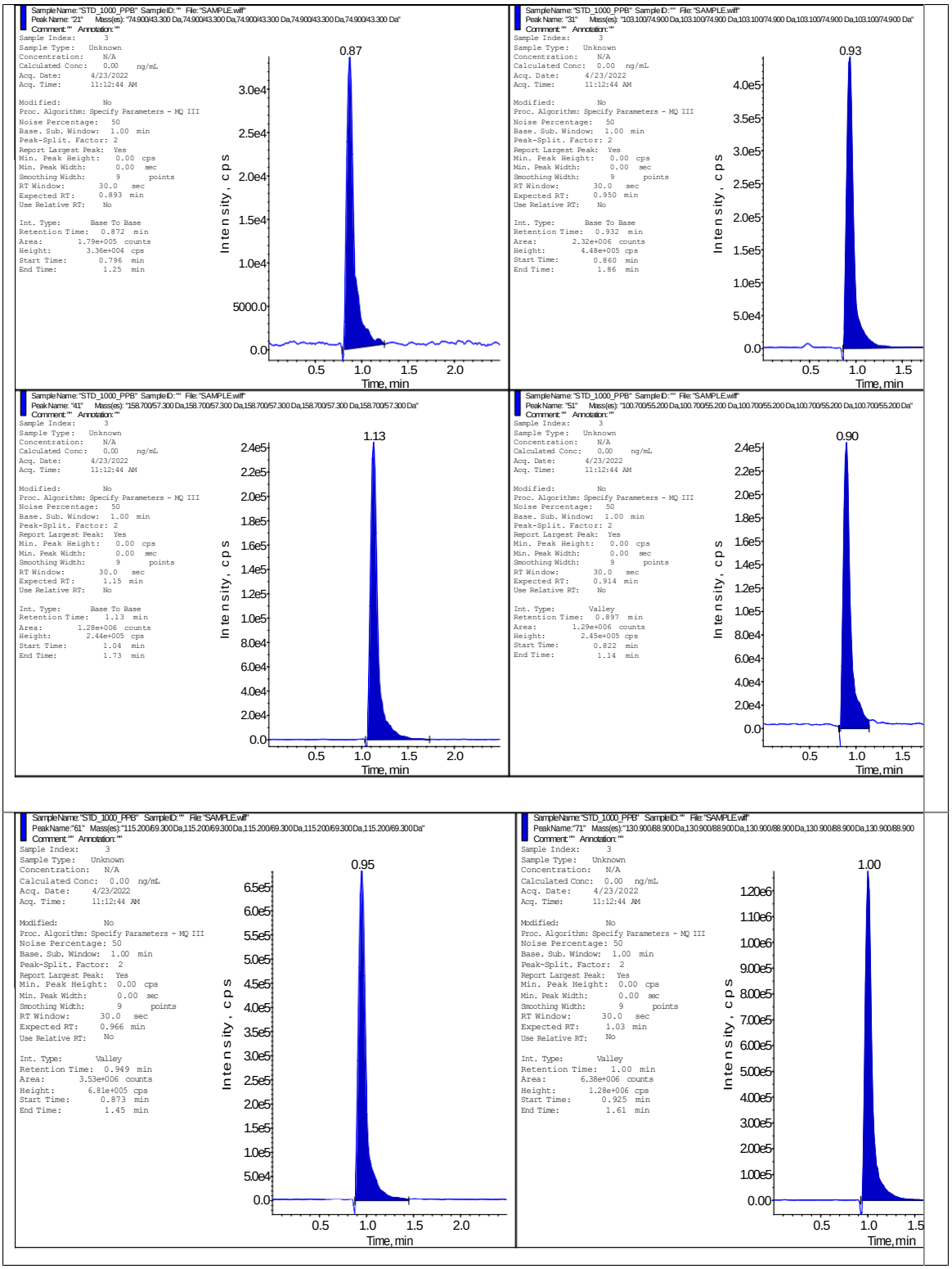


Figure9: The individual chromatograms of six Nitrosamines Nitrosamines N-nitrosodimethylamine(NDMA),N-nitrosodiethylamine(NDEA),N-nitrosodibutylamine(NDBA), N-nitrosopyrrolidine(NPYR), nitrosopiperidine(NPIP), N-nitrosodipropylamine(NDPA) with Retention time 0.87, 0.93, 1.13, 0.90, 0.95, 1.00 respectively.

5.3. Matrix Effect of Nitrosamines:

The accurate identification of target molecules in a complex sample is significantly impacted by the matrix effect. For the purpose of removing the matrix effect, standard solutions and sample solutions are made using a blank matrix devoid of NSAs. The following equation was used to gauge the matrix effect's influence:

$$\text{Matrix effect (\%)} = \frac{X1 - X2}{X2} \times 100\%$$

In this scenario, X1 represents the slope of the graph created in the matrix, while X2 represents the slope of the graph created in the solvent.

Table 4: Matrix effect of six Nitrosamines under optimum conditions using QuEChERS method combined with liquid chromatography and mass spectrometry

Analyte	Matrix effect (%)
NDMA	-76.85
NDEA	-76.66
NDBA	-98.12
NPYR	-73.92
NPIP	-78.42
NDPA	-80.94

5.4.Enrichment Factor:

The percentage of the final sediment phase concentration (Cfin) to the original target element concentration in the sample is known as the enrichment factor (EF). The equation is shown in the following format:

$$EF = C_{fin} - C_{in}$$

Table 5: Enrichment factor of six Nitrosamines (ppb) under optimum conditions using QuEChERS method combined with liquid chromatography and mass spectrometry

Analyte	**Enrichment factor(EF)**				
	100	**200**	**300**	**400**	**500**
NDMA	1.15	0.33	0.21	0.16	0.13
NDEA	1.44	0.44	0.25	0.17	0.14
NDBA	1.17	0.32	0.20	0.16	0.12
NPYR	1.44	0.44	0.27	0.18	0.15
NPIP	1.31	0.40	0.26	0.17	0.13
NDPA	1.22	0.35	0.24	0.16	0.12

5.5.Method Validation:

By assessing LOD, LOQ, linearity, matrix effect, accuracy and precision, the analytical method was proven to be accurate. By contrasting the calibration curves created in the matrix and the solvent, the matrix effect was investigated. Curves constructed with NSA concentrations of 0.48, 0.97, 1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 125, 250, and 500 g/mL in a blank matrix were used to access linearity. The QuEChERS method was used to extract and purify the NSA-free solution that made up the blank matrix. After examining three duplicates at each concentration, the accuracy in terms of recovery was assessed using blank samples spiked at 100, 200, 300, 400, and 500 µg/mL. By assessing three spiking samples twice a day, and three separate days, intra-day and inter-day precisions were assessed.

5.6.Discussion:

NSA in samples of noodles and snacks was determined using the QuEChERS technique in conjunction with LC/MS-MS. 2 mL of ACN was utilised as the extracting solvent under optimal extraction conditions. For the purification of the extracts, florisil was used. In 4 minutes, the nitrosamine was separated using chromatography.

All of the NSAs had recoveries between 62 and 144 percent.The signal to noise ratios of 3 (S/N = 3) and 10 (S/N = 10) were used to calculate LOD and LOQ respectively. Six NSAs had LOD values between 0.12 and 0.56 µg/mL and LOQ values between 0.12 and 3.11 µg/ML, as shown in the table.

By analysing samples twice a day and three times a week, respectively, the intra-day and inter-day precisions were determined. There were for both the inter-day and intra-day precisions (n=6). By examining the samples spiked with five different concentrations of the six NSAs (100, 200, 300, 400, and 500 g/mL), accuracy was assessed. This method's enrichment factor ranges from 0.12 to 0.14.

6.Conclusion:

A brand-new, modified QuEChERS-based LC/MS-MS technique for NSA in food analysis. The technique allowed for the quick extraction of NSA from samples of noodles and snacks. The suggested LC/MS-MS technique demonstrated excellent linearity, great sensitivity, decent recoveries, and precision. This approach has the benefits of being quick, simple, and ecologically friendly, and it uses very little reagent.

The QuEChERS technology can manage a complicated matrix and is very easy to use and convenient to operate because the composition of noodles and snacks contains complex extracts, fats, oils, and other components. This straightforward, inexpensive, and highly suspectible analytical technique may be useful for analysing NSA residues in food matrices.

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