A COUPLING OF SUPERHEATED WATER AND SOLID-PHASE EXTRACTION OF *N*-NITROSAMINE FROM FRANKFURTERS

INTRODUCTION

Nitrosamines are a class of chemical compounds that were firstly described in chemical literature over 100 years ago. In 1956 two British scientists, John Barnes and Peter Magee, reported that dimethylnitrosamine produced liver tumors in rats. The discovery caused scientists around the world to pay more attention to the carcinogenic properties of other nitrosamines and *N*-nitroso compounds. Intensive studies revealed that they have been found to be carcinogenic in a wide variety of experimental animals even at low concentrations, by inducing tumors in various organs, i.e. liver, lung, kidney, bladder, pancreas, esophagus and tongue depending on the species. In addition, they are potentially mutagenic and teratogenic for animals. Since nitrosamines are metabolized similarly in both human and animal tissues, humans are highly susceptible to their toxicities.

The *N*-nitroso compounds are amines containing two organic group (R) and one NO group bonded to a central nitrogen (Figure 1). They are generally stable, only slowly decomposed by light or acid.

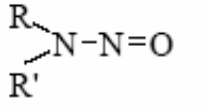


Figure 1 General formula of nitrosamines.

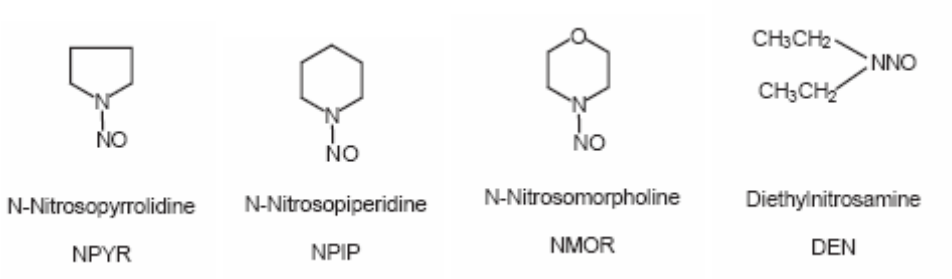


Figure 2 Example of nitrosamine compounds.

Unfortunately, these substances can be formed directly in foods, such as bacon, fish, cheese, beer, water, ham, cured meat and milk products, by the reaction between primary, secondary or tertiary amines that naturally generated from amino acid breakdown and nitrosating agents (nitrite, nitrate, nitrogen oxide or nitrous acid). Nitrite and nitrate are additives for meat to prevent outgrowth and toxin formation by *Clostridium botulinum*. Nitrite is also used as flavorings which reacts with pigments in meat to impart a desirable pink color. Nitrosamines can be also formed endogenously in human stomach or small intestine by an interaction of nitrosating agents and ingested amine.

$$HNO_2 + H^+ \leftrightarrow H_2O + NO^+$$
$$NO^+ + (CH_3)_2NH \rightarrow (CH_3)_2N{-}N = O + H^+$$

Figure 3 Nitrosation: Nitrosodimethylamine (NDMA) formation.

As carcinogenic and mutagenic substances, they are oxidized and generated carbonium ions, which promote alkylation of DNA as the example shown in Figure 4. Different species of *N*-nitroso compound respond to different organ(s) depending on chemical structure of the carcinogen. In addition, the size and frequency of dose and the administration route can change the affected organ, which is difficult to predict.

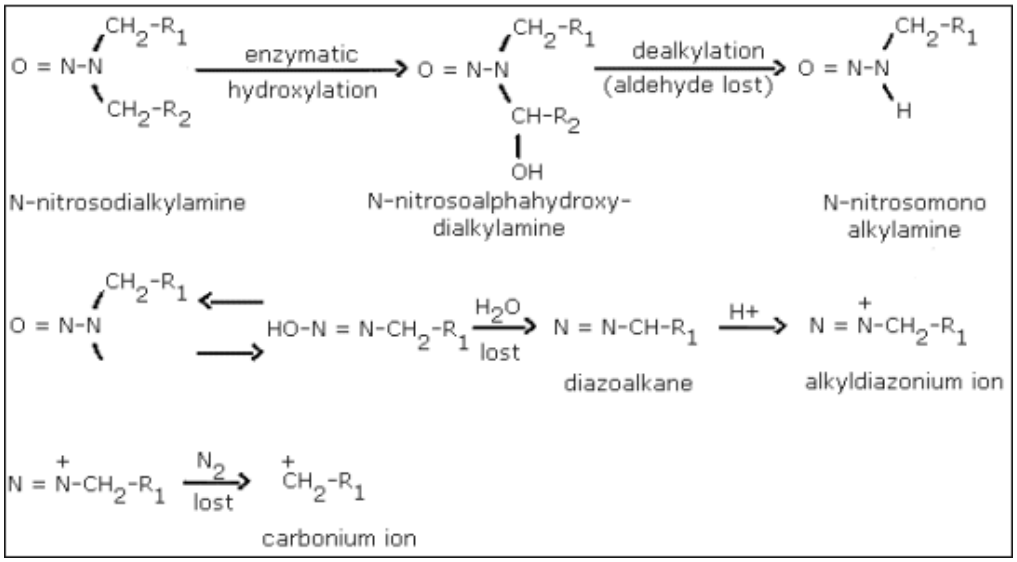


Figure 4 Metabolic activation on *N*-nitrosodialkylamines.

For analytical purposes the nitrosamines are divided into non-volatile compounds, i.e. nitrosoproline, nitrosohydroxyproline, nitrosothioproline, nitrosopipecolic acid, nitrososaroosine and volatile compounds, such as nitrosodimethylamine nitrosodiethylamine and nitrosopyrrolidine. The analysis method for non-volatile compounds usually uses high performance liquid chromatography-mass spectrometry and voltammetry. The volatile nitrosamines are a group of relatively nonpolar, low-molecular weight compounds, which can be removed from food matrix by distillation. So volatile *N*-nitrosamines are easily extracted and analyzed by distillation and gas chromatography, respectively. Isolation and identification of volatile nitrosamines is relatively easy. Most attentions have been given the volatile group because the non-volatile one appears to be non-carcinogenic.

In food the concentration of volatile *N*- nitrosamines is very low, consequently their human exposure is quite small, but people do not ignore because of two reasons. Firstly, their carcinogenic potency in experimental animals is highly significant. Secondly, nitrosamines may be more sensitive to humans than experimental animals. Regulation of volatile nitrosamine permission level in food has been set in several

countries, for example in the US less than 10.0 µg/kg of *N*-nitrosopyrrolidine (NPYR) is allowable in bacon, 2.0-4.0 µg/kg of sum of *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) allowance in meat products in Russia, and 0.002 and 0.004 mg/kg of *N*-nitrosamines permission level in fresh and smoked foodstuffs, respectively, restricted by the World Health Organization.

Great concerns towards these compounds challenge researchers to develop satisfactory methods for extraction and determination of nitrosamines in foods. The extraction of nitrosamines in foods shows several difficulties. Firstly, they have to be detected at very low levels and in a large variety of foods with different compositional characteristics. Secondly, isolation and detection of nitrosamines are problematic, such as possibility of interferences with similar chemical compounds. Therefore, it is necessary to develop sensitive, efficient and specific methods for nitrosamines analysis. In food, methods of analysis of volatile nitrosamines involve many preliminary steps, such as sampling, extraction, clean-up and pre-concentration. Several methods available for extraction and clean-up of volatile nitrosamines are solvent extraction on a dry celite column, supercritical CO_2 extraction, steam distillation and vacuum distillation in a mixture of mineral oil and base. Most methods require large amount of toxic solvent such as, dichloromethane. Using supercritical CO_2 extraction, the higher lipid co-extraction with the analytes was occurred. The distillation method is greatly time consuming, high energy and sample throughput so it is not suitable for routine analysis. Determination of volatile N-nitrosamines in samples has been typically carried out by gas chromatography-thermal energy analyzer (GC-TEA). The detector is a modified chemiluminescence detector, based on a photon decay released from a reaction of ozone with nitrogen oxide radical generated from a thermal cleavage of the N-N bond of nitrosamine. Although GC-TEA is sensitive and specific for *N*-nitroso compounds, it is very expensive, not usually available in most laboratories, besides, its operation is complicate. From all above reasons including US Environmental Protection Agency regulations designation to reduce usage of organic solvents, particularly those containing halogens, that are potentially harmful to the environment and to reduce costs of solvent disposal, better methods of extraction are needed. So in our approach

subcritical water extraction or supercritical water extraction (SWE), a new technique for extraction, was developed for the determination of nitrosamines. Because of GC-TEA is not available in most laboratories. Therefore, GC-FID and GC-MS with ion monitoring have been used for the identification and quantitation of nitrosamines.

Subcritical water extraction, is also called hot water extraction, pressurised (hot) water extraction, superheated water extractions or hot liquid water extraction, and high-temperature water extraction. It is a sample preparation and extraction technique that combines elevated temperature and pressure with liquid solvents to achieve fast and efficient extraction of the analytes from the solid matrix. This technique based on the use of water as extraction solvent at temperatures between 100 and 374 ^{o}C (critical point of water, 374 ^{o}C and 22 MPa) and at a pressure which is high enough to keep it in the liquid state. Under such conditions the dielectric constant of water is lowered. This means that at elevated temperatures and moderate pressures the polarity of water is reduced and the water can act as ethanol or methanol. So the water can replace another organic solvent to extract medium-polarity or low-polarity compounds. Increasing temperature at moderate pressure also reduces the surface tension and viscosity of water, thereby increasing the solvent's ability to wet the matrix and solubilize the target analytes. Temperature also assists in breaking down analyte–matrix interaction. Pressure has been reported to play no role other than to keep the extraction solvent liquid at the high-temperatures. However, in applications involving highly adsorptive matrices, a high pressure can help to enhance efficiency by forcing the solvent into the matrix pores.

Superheated water extraction can be performed in static mode, dynamic mode, or a combination of both. In the static mode, sample and solvent are maintained for specific time at constant pressure and temperature, whereas in the dynamic mode the solvent flows through the sample in a continuous manner. Several studies have shown that a combination of both extraction modes can result in improved extraction. In this study the combination of two modes was used. SWE has many advantages. For example water is non-toxic and inexpensive so the method is environmentally friendly. The equipment is relatively simple. The method requires short extraction time and

low solvent consumption. In addition, it offers a wide range of polarities extraction by changing the temperature.

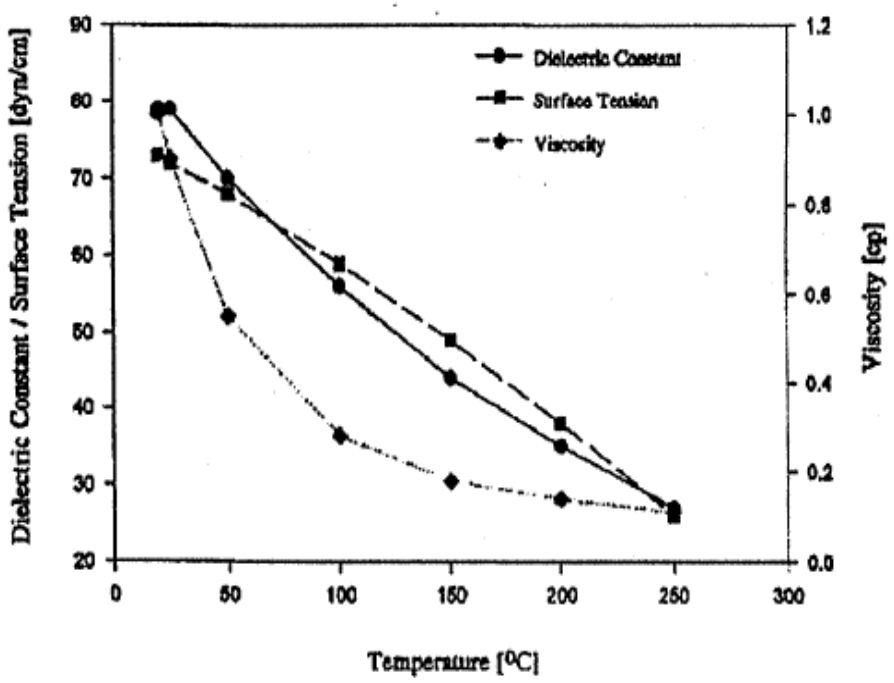


Figure 5 Temperature influence on physical properties of water.
Source: API soil & ground water research bulletin (1998)

In this research, a new efficient, rapid and inexpensive method was developed for extraction and clean-up of selected volatile nitrosamines compounds, namely *N*-nitrosodiethylamine (NDEA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosopiperidine (NPIP) and *N*-nitrosomorpholine (NMOR) from frankfurters by using superheated water extraction (SWE) coupled with solid-phase extraction (SPE). In the study, some parameters affecting SPE, such as sorbent type, eluting solvent system and volume, were optimized to obtain the highest yield, as well as to remove lipid from the extract sample. The coextraction of lipid can cause some serious problems during the extraction and a subsequent analysis. So the use of fat-selective florisil adsorbent is necessary. The superheated water extract was pre-concentrated and cleaned up by solid-phase extraction with florisil as adsorbent. In addition, various factors affecting SWE, such as flow rate, extraction temperature dynamic time and static time were

also investigated. GC-MS with ion monitoring mode was used for chromatographic separation and detection step, because of its sensitivity and its specificity. A full factorial design with three replicate was used to study the influence of several parameters on SWE in terms of recovery. The use of the design is necessary to study new systems where several factors interact and more information is obtained with few runs by varying several factors at once.

The purposes of this research are:

1. To propose a new application for superheated water extraction.
2. To develop a rapid, efficient and inexpensive method for extraction and clean-up of volatile nitrosamines from frankfurters.
3. To improve extraction method of nitrosamines quantitative determination.
4. To investigate various factors affecting superheated water extraction, i.e. temperature, static time and flow rate etc., by using full factorial design to optimize conditions.
5. To study some parameters affecting solid-phase extraction, e.g. sorbent type, eluting solvent system and volume.
6. To determine the extraction efficiency of the method operated under the optimized conditions.
7. To compare the results obtained from superheated water extraction with those from standard and conventional method.

LITERATURE REVIEW

To date a number of extraction and clean-up procedures for determining N-nitrosamines in food have been described, these included solvent extraction on a dry celite column, low temperature vacuum distillation and steam distillation, mineral oil distillation, supercritical fluid extraction and solid-phase extraction. In addition, a derivatization of nitrosamines prior to detection was feasible, since it could occur via N-N bond cleavage to form secondary amines, thus the sensitivity of detection could be improved. Several methods were utilized for quantifying nitrosamines after extraction from foods. The most common methods used are gas chromatography (GC) and high-performance liquid chromatography (HPLC) combined with a detection by using thermal energy analyzer (TEA) or mass spectrometer (MS). A number of those analytical methods of nitrosamines in food were summarized chronologically as follows.

Newell and Sisken (1972) extracted nitrosodimethylamine (NDMA) from apples and milk. The NDMA and other volatile components were removed from the non-volatile fraction of the sample by a vacuum distillation. The analytes in the distillate were removed from the water by percolation through a column of polymer beads, which was heated to evaporate the absorbed analyte passing to a gas chromatography column. The NDMA was catalytically reduced to ammonia, which was microcoulometrically titrated. To find the efficiency of recovery, samples of cooked apples and milk were fortified with 10 ppb of NDMA prior to vacuum distillation, resulting the recovery of 70% or more with a very low sensitivity of 3 ppb.

Cox (1973) applied HPLC to couple with two established gas chromatographic methods for the determination of three N-nitrosamines namely *N*-nitrosodimethylamine (NDMA), *N*-nitrosopyrrolidine (NPYR) and *N*-nitrosodiethylamine (NDEA) in foods. Two GC methods for nitrosamine determination were developed. One method utilized an initial steam distillation of sample. After this step, relied upon two distillations, one from alkali, the other from acid, followed by electrochemical reduction of the N-nitrosamines to corresponding

amines. The other method of analysis used only one steam distillation, after that the distillate was extracted with dichloromethane. The two methods showed low detection limits between 1 and 10 μg/kg. It was found that the methods required long extraction time, high volume of organic solvent and the yield of corresponding amines obtained from electrochemical reduction were low.

Maxwell *et al.* (1993) applied supercritical fluid extraction (SFE) technology coupling with an offline trapping technique for extraction of nitrosamines from frankfurters. The optimum SFE conditions were as follows: extraction temperature at 40^{o}C, valve temperature at 110 ^{o}C, static time for 2 minutes, dynamic time for 20 minutes, CO_2 flow rate of 3.0 L/min and pressure of 680 bar. In this work, solid-phase extraction with silica gel was used for clean-up. *N*-nitrosamines were eluted from the column by using 30%ethyl ether in dichloromethane, and then determined by GC-TEA. The results showed SFE recoveries of nitrosamines from fortified frankfurters in a range of 84.3 to 104.8%.

Bellec *et al.* (1996) extracted *N*-nitrosamines from gastric juice and alcoholic beverages. Gastric juice samples were extracted triplicately with dichloromethane for 30 min in an agitator. Those in beer samples were adsorbed by using a Chem-Elut column with dichloromethane eluting solvent. *N*-Nitrosamines were separated by reversed-phase HPLC and then were quantitatively photohydrolysed in a UV photoreactor in aqueous solution to give the nitrite ion. The ion was determined via its acid-catalyst diazotation of sulfanilamide, followed by coupling with N-l-naphthylethylenediamine di-HC1 to give an azodye, which has a maximum absorbance at about 546 nm. The yield of photohydrolysis depended upon pH and time of exposure under UV light. The response was shown to be linear in the 0-200 ng range with a limit of detection of 8 and 20 pmoles injected for N-dialkyl nitrosamines and N-nitrosamines bearing two phenyl groups, respectively. Although *N*-nitrosamines could be detected at 230 nm without post-column reaction, such the reaction enhanced specificity of the detection in biological matrices, such as gastric juice or alcoholic beverages.

Kataoka *et al.* (1996) developed a selective and sensitive method for the determination of volatile *N*-nitrosamines by gas chromatography (GC) with flame photometric detection (FPD). The method was based on denitrosation of *N*-nitrosamines with hydrobromic acid to produce corresponding secondary amines that subsequently converted to their N-diethylthiophosphoryl derivatives and then measured by GC using a DB-1701 capillary column with FPD. The reaction conditions for denitrosation and subsequent N-diethylthiophosphorylation were investigated to establish an optimum derivatization method for *N*-nitrosamines. The denitrosation of these *N*-nitrosamines proceeded rapidly in a minimum excess of ca. 10^{3} mol of hydrobromic acid per mole of *N*-nitrosamine, while N-diethylthiophosphorylation of secondary amines with diethyl chlorothiophosphate (DECTP) occurred rapidly in aqueous alkaline media. The reaction was completed in 5 min at 60 °C. Separation of N-nitrosamines and secondary amines were achieved by an extraction with 25% 2-propanol in diethyl ether. Overall recoveries of N-nitrosamines added to tested cigarette smoke samples were 83-110%.

Raoul *et al.* (1997) developed a rapid solid phase extraction (SPE) method to analyze volatile *N*-nitrosamines in foods. The extraction procedure was based on two extraction/concentration steps using Extrelut and Florisil SPE. The nitrosamines were eluted from the Extrelut column with 40% dichloromethane in hexane. Quantitative elution of the polar nitrosamines from the Florisil cartridge was achieved with 95:5 (v/v) dichloromethane/methanol. The amount of food sample and solvent required in this method were reduced compared to the conventional vacuum distillation method without affecting sensitivity which was at 0.3 ppb detection limit. An application of the SPE method to a survey of volatile nitrosamines content in sausages and dried milk powder revealed no contamination (<0.3 ppb) in either of these food samples.

Mitacek *et al.* (1999) extracted volatile nitrosamines namely NDMA, NPYR and *N*-nitrosopiperidine (NPIP) from fresh and preserved food that widely consumed in Thailand. Food sample was mixed with phosphate-citric acid buffer pH 4.5, ascorbic acid and N-nitrosopropylamine (NDPA) as an internal standard. The aqueous filtrate was further extracted with dichloromethane and pre-concentrated and cleaned

up with basic alumina with dichloromethane. Afterwards the eluent was analysed by gas chromatography combined with a thermal energy analyzer (GC-TEA). The recovery of volatile nitrosamines was between 60 and 90%, depending on the compound and types of food sample. NDMA was detected at levels ranging from trace amounts to 66.5 µg/kg in several salted and dried fish. NDMA and NPYR were frequently detected in several vegetables at levels ranging between 1- 95.1 µg/kg and 0-146 µg/kg, respectively.

Haorah *et al.* (2001) determined total N-nitroso compounds (NOC) and NOC precursors (NOCP) in extracts of food products, following Walters' method (Walters *et. al.*, 1978). NOC were decomposed to NO by refluxing in HBr/HCl/HOAc/EtOAc and NO was detected by TEA-chemiluminescence in which the NO reacts with ozone to give excited NO_2, which emits infrared light. NOC was determined after sulfamic acid treatment to destroy nitrite, and NOCP was determined after treatment with 110 mM nitrite and then sulfamic acid. Analysis without HBr gave results ≤ 20% of those with HBr. Mean NOC and NOCP results were 5.5 and 2700 µmol/kg of frankfurters, respectively. For fresh meat, mean NOC and NOCP contents were 0.5 and 660 µmol/kg of product And those for dried fish were 5.8 and 5800 µmol/kg, respectively.

EncarnaciÓn *et al.* (2001) applied a spectrophotometric method using a flow-injection system for the determination of NDMA in cured-meats. The method was based on the photochemical cleavage of N–NO bond. In the proposed method, nitrosamines were firstly extracted from food by using hot water at 90–95°C, then photodegraded to yield corresponding amine and nitrite ion. The violet complex was formed by a reaction of the nitrite with the Griess reagent, and it was detected at 542 nm. Factors affecting the hydrolysis, namely concentration of the Griess reagent, pH, flow rate of the sample/Griess reagent and irradiation time were studied. The optimum conditions were as follows: a concentration of 1% Griess reagent in sulfanilamide, pH 6.2, flow rate of the sample/Griess reagent of 2.3 mL/min and 5 min irradiation time. The recoveries achieved for spiked samples analysis were between 91.6 and 105.8% with 2.0 - 3.9% relative standard deviation (RSD).

Komarova *et al.* (2001) developed a method for determination of volatile N-nitrosamines in food by high-performance liquid chromatography coupled with fluorescence detection. N-nitrosamines were quantitatively determined in a form of fluorescent densyl derivatives. Procedure for sample preparation included steam distillation of *N*-nitrosamines from model solutions and real samples, an extraction from the distillate with dichloromethane, and denitrosation reaction. Secondary amines were obtained by denitrosation of nitrosamines with 5-dimethylamino-1-naphthalenesulfonyl chloride (dansyl chloride). A mixture of concentrated hydrobromic acid and glacial acetic acid was proposed as the reducing agent for the denitrosation reaction. Factors affecting the denitrosation procedure, such as, concentration of dansyl chloride and time of the reaction were studied. N-nitrosamines were detected as fluorescence dansyl derivatives that gave excitation and emission maxima at 350 and 530 nm, respectively. The results showed that optimal concentration of dansyl chloride was 1 mg mL^{-1} with optimal reaction time of 40 min. Recoveries of nitrosamines were quite low, ranging between 72 and 78%.

Cárdenes *et al.* (2002) developed a fast microwave-assisted dansylation procedure for derivatization of *N*-nitrosamines prior to high-performance liquid chromatography determination. *N*-Nitrosomorpholine, *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosopyrrolidine and *N*-nitrosopiperidine were initially denitrosated by hydrobromic acid–acetic acid to produce secondary amines that were subsequently dansylated with densyl chloride. Several variables that potentially affect the dansylation efficiency such as, radiation power supplied, maximum pressure inside reactor and reaction time were studied. Optimum conditions for dansylation method were as follows: radiation power of 378 W, reaction time for 5 min and pressure inside the reactor of 1.4 bar. The reaction mixture was separated on a C_{18} column with acetonitrile–water (55:45, v/v) as mobile phase with fluorimetric detection at 531 nm (excitation at 339 nm). The detection limits ranged from 8 to 75 pg for *N*-nitrosomorpholine and *N*-nitrosodiethylamine, respectively. The method was applied to study the recoveries of *N*-nitrosamines in beer, resulting 82.7-98.4%,recoveries with 2.0-12.4% RSD.

Filho *et al.* (2003) developed a new method for extraction, pre-concentration and analysis of nitrosamines in meat derived products. The extraction was carried out by vacuum steam distillation in a rotary vacuum evaporator for 80 min. Nitrosamines were pre-concentrated by solid-phase extraction with active carbon. Variables affecting the SPE, such as adsorbent mass (0.5-1.0 g), adsorption time (15-45 min) and aqueous sample volume (25-200 ml), were studied. A 200 mL of aqueous distillate of solid sample was magnetically stirred with 1.0 g active carbon for 45 min. The compounds were eluted from the adsorbent by an addition of acetone and dichloromethane. Afterwards micellar electrokinetic chromatography was used for separation and determination of different nitrosamines contained in real sample. The effect of buffer composition and instrumental variables were also investigated. Gas chromatography with mass spectrometry detection was used as a confirmation technique. The proposed method allowed the determination of nitrosamines at trace levels with 4.0 to 22%RSD. The recovery of the SPE procedure using solid spiked samples was very low, approximately 40%. The recoveries were obtained in a range of 68.8- 105.0% when studied with synthetic aqueous samples.

Lee *et al*. (2003) analysed *N*-nitrosamines in seven dried (un)cooked seafood products. The cooking methods used were briquet fire, gas range, electric oven, microwave oven, steam cooker and electric coil cooker. In this work samples were extracted by a steam distillation. The sample, sulphamate in sulphuric acid, distilled water and internal standard NDPA solution were distilled by using a steam generator equipped with an electric heating mantle. The distillate was extracted with dichloromethane and analysed by GC–TEA. The results showed that the detection limit of the method was 0.1 μg/kg. Only NDMA was detected, giving 1.0 to 46.9 μg/kg in uncooked products. When these samples were cooked, the content tended to increase, ranging from 1.1 to 630 μg/kg. In general, indirect heating, such as steam cooker and microwave ovening, compared with the direct one, such as gas range and briquet fire, reduced the increase in NDMA content during cooking.

Byun *et al.* (2004) determined volatile NDMA and NPYR in irradiated pepperoni and salami sausages by using GC–TEA. These fermented sausages were

irradiated at 0, 5, 10, and 20 kGy, and then stored for 4 weeks at 4 ◦C. The extraction was performed using steam-distillation on a steam generator and the distillate was extracted three times with dichloromethane. NDPA was added as an internal standard for extraction efficiency. Mean recovery of the standard was 90.2± 3.28%. It was found that the contents of NDMA and NPYR in the irradiated sausage were lower than those of non-irradiated control. Results indicated that high dose of irradiation (>10 kGy) was needed to reduce *N*-nitrosamines in the sausage during storage and GC–TEA analysis was effective in determining *N*-nitrosamines in irradiated meats even at low trace level.

Hyun-Joo Ahn *et al*. (2004) determined volatile nitrosamines in cooked pork sausage. Sausage with aerobic or vacuum packaging was irradiated in a cobalt-60 irradiator at 0, 5, 10 or 20 kGy and immediately stored at 4 ◦C for 4 weeks. For the extraction, the sample was homogenized with distilled water and filtered. The filtrate was then extracted by the method of Raoul (1997) with some modifications using Extrelut NT3 pre-packed glass column added with Extrelut packing materials. NDPA was used as an internal standard for extraction efficiency. Volatile N-nitrosamine was determined quantitatively by GC-TEA. Mean recovery for internal standards obtained from all samples was 94.1± 3.22%. The study was demonstrated that irradiation was an alternative method to reduce residual nitrite and carcinogenic N-nitrosamines during storage. NDMA in vacuum packaging and NPYR under air packaging were reduced by irradiation at 10 and 5 kGy, respectively.

Katarzyna *et al.* (2005) determined *N*-nitrosamine content in 150 samples of tinned foods collected from meat factories in Poland during 2000-2001. Volatile nitrosamines were extracted by low temperature vacuum distillation. *N*-nitrosodiisopropylamine (NDiPA) was added as internal standard to the samples before extraction. The distilled extracts were quantified by using GC-TEA and the analytes were confirmed by gas chromatography coupled with mass spectrometry (GC-MS). It was found that about 58% of samples contained NDMA at a mean concentration of 3.01 μg/kg. The most contaminated products were tinned fish which had total *N*-nitrosamines content at the concentration of 8.20 μg/kg. Tinned meat and

tinned offals contained much lower concentrations of 0.55 μg/kg and 0.39 μg/kg, respectively. This method enabled identification and simultaneous quantification of seven nitrosamines, on the other hand, the extraction time was long. The recoveries were 76-98%.

Andrade *et al.* (2005) developed a method for determination of volatile nitrosamines, namely NDMA, NDEA, NPIP and NPYR, in sausages by using headspace sampling with solid-phase microextraction and gas chromatography-thermal energy analyzer detection (HS-SPME-GC-TEA). Two fused silica fibers, polydimethylsiloxane-divinylbenzene (PDMS-DVB)-coated fiber and poliacrylate (PA)-coated fiber were compared for extraction of nitrosamines in vapor phase. A factorial fractional design was employed in order to evaluate the influence of equilibrium time, ionic strength, extraction time and extraction temperature. The results showed that NDMA and NDEA were commonly detected in sausages, and the PDMS-DVB coated fiber gave higher recoveries for these compounds so it was employed for the study of sausages samples. The optimum HS-SPME extraction conditions were as follows: 45^{o}C temperature, 10 min equilibrium time, 25 min extraction time and no ionic strength adjustment. This method was rapid with adequate linearity, sensitivity and recovery.

Ventanas *et al.* (2006) evaluated the feasibility of solid-phase microextraction (SPME) coupled to a direct extraction device (DED) for an analysis of nine volatile nitrosamines from solid food at different temperatures and extraction times. Efficacy of extraction, linearity of response and sensitivity of the method were determined at refrigeration (4 ^{o}C) and room (25 ^{o}C) temperature. Several extraction times (15, 30, 60,120 and 180 min) were tested. Quantitative analyses were performed using GC–MS in selected ion monitoring (SIM) mode. At 4 ^{o}C all nitrosamines were detected at all studied concentrations ranging from 1 to 50 ng ml^{-1}, except for N-nitrosopyrrolidine (NPYR), N-nitrosomorpholine (NMOR) and N-nitrosodiphenilamine (NDPheA). Better results in terms of extraction efficacy, linearity and sensitivity were obtained at 25 ^{o}C. Extraction time of 15 min was enough to extract all nitrosamines in gelatines at 25 ^{o}C. SPME–DED was a rapid and suitable

technique for extracting nitrosamines from food at both refrigeration and room temperatures.

Yurchenko *et al.* (2006) determined the level of five nitrosamines (*N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosodibutylamine, *N*-nitrosopiperidine and *N*-nitrosopyrrolidine) in 294 various fish samples and in 77 oil samples during 2001–2005. For a clean-up method, two-step solid-phase extraction with Extrelut and Florisil sorbents was used. The sample was initially mixed with NaOH, put into the Extrelut column and eluted with hexane/ dichloromethane solution. The eluent was then applied to the Florisil sorbent and nitrosamine was eluted with dichloromethane/methanol 95:5 (v:v) solution and the eluent was subjected to GC-MS with positive-ion chemical ionization (PCI) using ammonia as reagent gas in the selected ion monitoring mode (SIM) with pulsed splitless injection. In this work, limit of detection and of quantitation of nitrosamines were approximately 0.10 and 0.35 µg/kg, respectively. Sum of the average of five nitrosamines content in cold-smoked fish, hot-smoked fish, fried fish, pickled fish, salted fish and salted/dried fish were found to be 1.92, 4.36, 8.29, 5.37, 3.16 and 3.81 µg/kg, respectively. Their recoveries in smoked fishery products varied from 79 to 88%.

Nitrosamines are widespread in our environment, since their appearance has been confirmed in drinking water and groundwater. In 2002, Mitch *et al.* (2002, 2003) reported a formation of NDMA during a disinfection of water and sewage containing dimethylamine and ammonia ions with chlorine. The original analytical methods for nitrosamines determination in water was developed by the Canadian Ministry of the Environmental (MOE, 1990). It was based on EPA method 607, which described a liquid- liquid extraction in a separatory funnel at neutral pH, using NDMA-d6 as a surrogate/internal standard, followed by isotope dilution SIM GC/MS.

In the US, a standard method for nitrosamine analysis in water was established by using US EPA Method 521 (EPA/600/R-05/054) and referees for seven nitrosamines, namely *N*-nitrosodimethylamine (NDMA), *N*-nitrosomethylethylamine (NMEA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosopyrrolidine (NPYR),

N-nitrosopiperidine (NPIP), *N*-nitroso-n-dipropylamine (NDPA) and N-nitroso-n-dibutylamine (NDBA). The method was based on applying SPE technique for sample preconcentration, where the concentrated sample was analyzed by GC-MS/MS with methanol or acetonitrile chemical ionization at large volume injection. Prior to the pre-concentration procedure, 500 mL water samples were dechlorinated and added with NDMA-d6 as surrogate standard (SS). The water sample then was passed through an SPE column filled with coconut charcoal and eluted with dichloromethane. The dichloromethane extracts were concentrated, enriched with NDPA-d14 internal standard (IS) and adjusted to final volume with dichloromethane. Samples containing nitrosamines were separated using GC-tandem MS operated in CI mode with methanol or acetonitrile as reagent gas. $[M+1]^+$ ions were selected mainly as precursor ions. Method detection limits obtained for the seven nitrosamines were very low and ranged from 0.26 ng L^{-1} for NDEA to 0.66 ng L^{-1} for NPIP. For NDMA and NMEA, method detection limits obtained were 0.28 ng L^{-1}.

Filho *et al.* (2003) developed a new method for separation and quantification of nitrosamines by micellar electrokinetic capillary chromatography (MEKC). Effects of composition of the buffer, concentration of sodium dodecyl sulfate (SDS), γ-cyclodextrin (CD) and pH on separation and migration times of the nitrosamines were investigated. A buffer pH 6.6 was selected in order to achieve satisfactory resolution in a short analysis time. The optimized concentration of SDS was 80 mM and γ-cyclodextrin was 10 mM. Different instrumental variables affecting sensitivity and resolution were also optimized, resultingt electrokinetic injection with 10 kV for 10 s at 236 nm absorption wavelength. Application of this method to aqueous synthetic samples allowed the separation and determination of nitrosamine mixtures at mg L^{-1} level. The recoveries were obtained between 80 and 105% with relative standard deviation (RSD) ranging from 4.5 to 8.5%. The results were generally acceptable, so the proposed method was suitable for determination of nitrosamines in aqueous samples.

Mitch *et al.* (2003) extracted nitrosodimethylamine (NDMA) from natural water. Primary extraction was based on US EPA SW-846 Method 3510C in which

samples were triplicately extracted with dichloromethane in a separatory funnel, but an emulsion was formed during shaking. Therefore, the wastewater samples were extracted by continuous dichloromethane extraction using Corning Accelerated One-Step™ Extractor/Concentrators for six hours. Extracts were analyzed for NDMA using a GC- ion trap MS/MS. When the separatory funnels were used for liquid–liquid extraction, recoveries were 21% ± 10% (12 samples) for NDMA-spiked deionized water samples. Recoveries from continuous liquid–liquid extraction averaged 56% ± 11% (74 samples). These recoveries were relatively low and required long extraction time.

Charrois *et al.* (2004) developed a selective, sensitive and affordable benchtop analytical method for detecting several *N*-nitrosamines at relevant drinking water concentrations (low ng/L range). A solid-phase extraction method using Ambersorb 572 and LiChrolut EN was developed in conjunction with GC/MS with ammonia positive chemical ionization (PCI). The LiChrolut EN, Ambersorb 572, and glass wool were packed at the bottom, middle and top of the SPE column and the analytes were eluted with dichloromethane. From the results, ammonia PCI showed excellent sensitivity and selectivity for *N*-nitrosamines, which were quantified using both isotope dilution/surrogate standard and internal standard procedures. Method detection limits for all investigated *N*-nitrosamines ranged from 0.4 to 1.6 ng L^{-1}. When applying the extraction method to drinking water samples with dissolved organic carbon concentrations of 9 mg/L, *N*-nitrosodimethylamine concentrations ranging from 2 to 180 ng/L could be detected. Furthermore, 2-4 ng L^{-1} *N*-nitrosopyrrolidine and 1 ng L^{-1} *N*-nitrosomorpholine were also detected in selected samples with high recoveries of standard and analytes. The method offered a new approach for investigating several *N*-nitroso compounds at ultratrace levels in drinking water.

Okafor *et al.* (2005) analysed *N*-nitrosamines from fruit juices and sachet water commonly marketed and consumed in Nigeria. Colored samples were cleared with animal charcoal. Nitrosamine was determined after decomposition to nitrite by UV irradiation and nitrite was reacted with sulphanilic acid and N (1-naphltyl)

ethylenediamine hydrochloride to form purple color that developed after 20 min. Spectrophotometic measurement was done at 520 nm. The concentrations of nitrosamines in four out of eight brands of juice samples ranged from 2.75±0.47 to 45.70±3.07 μg L^{-1}.

Ruiz *et al.* (2005) developed a simple, automatic and sensitive HPLC method for determination of nitrosamines (NDMA, NDEA, NPIP, NMOR and NPYR) in water samples. A post-column detection system used two photoreactors. In one reactor, $Ru(bpy)_3^{3+}$ was generated by on-line photo-oxidation of $Ru(bpy)_3^{2+}$ with peroxydisulfate. In the other reactor, the nitrosamines eluted were photodegradated, leading to cleavage of N-NO bond, and generated corresponding amines, which further reacted with $Ru(bpy)_3^{3+}$ to give strong chemiluminescence (CL). Factors affecting the photochemical and chemiluminescent reactions, such as pH, peroxydisulfate ($K_2S_2O_8$) and $Ru(bpy)_3^{2+}$concentration, flow rate, length of photoreactor, were optimized to minimize their contributions to band-broadening. The greatest CL signal was obtained using the following conditions: 2×10^{-3} M $Ru(bpy)_3^{2+}$, 1.5×10^{-3} M $K_2S_2O_8$ buffered at pH 5.7, 100 cm long photoreactor and 1.2 mL min^{-1} flow rate. A solid-phase extraction (SPE) was used in conjunction with HPLC to determine nitrosamines in natural waters. Strata X, or surface modified styrene–divinylbenzene polymer, was used as a sorbent. The retained nitrosamines were eluted with acetone followed by air. In the proposed system, sample preparation, sample concentration, separation and detection were all automated. The results showed the recoveries of nitrosamines extraction in a range of 40-90% and detection limits between 0.03 and 0.76 μg L^{-1}.

Grebel *et al.* (2006) developed a determination method of N-nitrosamine in water by solid-phase microextraction (SPME). Eight parameters, namely SPME fiber coating, extraction mode, NaCl concentration, pH, sample volume and headspace optimization, extraction temperature and time were optimized. Four SPME fibers were examined for NDMA extraction efficiency: polyacrylate (PA), Carboxen/polydi-methylsiloxane (CAR/PDMS), Carbowax/divinylbenzene(CW/DVB), and polydi-methylsiloxane/divinylbenzene (PDMS/DVB). For all fibers, two modes of extraction

were compared, direct aqueous extraction and headspace method. The optimum conditions for SPME were CAR/PDMS with headspace extraction mode, the optimum conditions for NDMA extraction were as follows: 100% saturated NaCl solution, neutral pH, headspace to total volume ratio of 0.6, extraction temperature at 65^{o}C and extraction time for 45 min. The detection limits of this method for NDMA ranged from 30 to 890 ng L^{-1}. The recoveries of nitrosamines extraction were in the range of 73-259%.

Special research attraction has been given to an improvement of analytical methods for determination of several analytes by superheated water extraction. A number of approaches in the extraction methods have been reported as follows.

In recent years, superheated water or subcritical water (SW) has been developed as an analytical extraction solvent for a wide range of organic analytes, such as polar, moderate-polar and non-polar compounds. Most interest has been given, reporting its application, e.g. extraction of PAHs, pesticides from environmental samples, essential oils from plant material, flavours and fragrances from plant material and food. .

The recent analytical interest in superheated water as an extraction solvent began in the work of Hawthorne and co-workers, who were interested in environmentally friendly extraction methods for soils and environmental solids. In 1994, they reported an extraction of polar and non-polar analytes from soil samples by using liquid water in a range of temperatures up to 400 ^{o}C.

Fernandez and Luque de Castro (2003) developed a method based on superheated water extraction for a removal of cholesterol from low- and high cholesterol-content foods. The research involved optimisation of parameters affecting the extraction process by a central composite experimental design as well as an optimisation of preconcentration step. The optimum working conditions for SWE were 135 °C, 3.0mL min^{-1} and 5 min of static extraction time. The time required for total removal of the target compound was 60 min. The best preconcentration factor

was obtained when a C18-bonded cartridge was used and the retained cholesterol was eluted with 2 mL 1:20 methanol–trichloromethane. The method was validated using a certified reference material (NIST–dried whole egg powder CRM 1845) and was used to analyse food samples within a wide range of cholesterol concentrations. The efficiency for the CRM was 105%. The precision (in term of relative standard deviation) of the method was less than 6.5% in all substances.

Lawrence *et al.* (2000) extracted fumonisins B_1 and B_2 from corn products and rice sample by SW. The samples were ground and mixed with an adsorbent material. Fumonisins B_1 and B_2 were extracted in a sequential 5 min static time over a temperature range of 23 to 150°C. The extracts were analyzed by reversed phase liquid chromatography with fluorescence detection after a derivertization with o-phthaldialdehyde-mercaptoethanol. Fumonisins were also extracted by using another different extracting solvent compositions. The results showed that pure water was successfully utilized for extracting fumonisins from most samples. Amount of the sorbent material for effective extraction increased as the percentage of water in extracting solvent increased.

García-Marino *et al.* (2006) studied recoveries of catechins and proanthocyanidins from grape seeds obtained as by-products from wineries using superheated water. Effect of temperature in the extraction process was studied by performing five different assays. In three assays, the samples were individually extracted with water at 50, 100 and 150 °C, and the pressures was around 1500 psi to keep the water in the liquid state. In the fourth assays, the samples were treated twice using sequentially 50 and 100 °C. In the fifth assay, a three-stage sequential extraction was used at 50, 100 and 150 °C. The composition of the extracts was determined by using HPLC–DAD–MS. The results were compared with those obtained using conventional analytical extraction with methanol-water (75:25) at atmospheric pressure. The results showed that superheated water was a good solvent for extracting flavanols, in some cases better than methanol-water (75:25). In general, major recoveries were found when the material was submitted to three sequential extractions at 50, 100 and 150 °C, but selective extractions of compounds with different degrees

of polymerisation could be achieved using one-step extraction at different temperatures. Better recoveries for flavanol dimers and trimers, showing higher antioxidant activity, were obtained using a single extraction at 150 °C. Furthermore, gallic acid, with antioxidant characteristics similar to the catechin and epicatechin monomers, was obtained in greater quantities by a single extraction at 150 °C. The higher temperature the better extraction of gallic acid, which reached approximately 70% of total extracted polyphenols.

MATERIALS AND METHODS

Materials

1. Reagents

Double deionized water that having resistance 18 MΩ was used throughout the studies. It was produced by Elga Maxima HPLC double deionized water apparatus (Elga, Bucks, England). *N*-nitroso-di-n-propylamine or NDPA was purchased from Supelco (Bellafonte, PA, USA) and used as an internal standard by preparing a stock standard solution in analytical reagent grade methanol (Merck, Darmstadt, Germany). Standard nitrosamines, namely *N*-nitrosodiethylamine or NDEA (99.0%) from Fluka (Buchs, Switzerland), *N*-nitrosopiperidine or NPIP (99.0%) from Sigma (Steinheim, Germany), *N*-nitroso- pyrrolidine or NPYR (99.0%) from Aldrich (Milwaukee, WI, USA.) and *N*-nitro- somorpholine or NMOR (5000 μg/mL in methanol) from Supelco (Bellafonte, PA, USA.) were used in this study. They were used to prepare each stock standard solution in AR grade methanol.

For an aqueous extraction step, NaCl (99.5%) employing as a de-emulsifier was obtained from BDH (BDH, Poole, England) and anhydrous Na_2SO_4 used as a drying agent was supplied by Ajak Finechem (Seven Hills, Australia). Organic trapping solvent employed were dichloromethane (99.5%) and ethyl acetate (99.98%) purchased from BDH (Poole, England).

For solid-phase extraction, several kinds of organic solvent applied in this study were as follows: dichloromethane (99.5%), diethyl ether (99.5%), glacial acetic acid and ethyl acetate (99.98%) purchased from BDH (Poole, England), acetone (99.5%) supplied by Ajak Finechem (Seven Hills, Australia), and triethylamine (99.5%) obtained from Aldrich (Milwaukee, WI, USA.). For SPE sorbent, 60-100 mesh Florisil wth a particle size of 149-250 μm and a surface area of 289 m^2/g was obtained from Sigma (Steinheim, Germany). Silica gel 60 with a particle size of 230-

400 mesh or 0.040-0.063 mm was purchased from Merck. All standard nitrosamine properties and chemicals were also shown in Appendix B.

2. Apparatus

The SWE extraction system was an equipment built in our laboratory. It consisted of double deionized water contained in a glass reservoir, a HPLC Constra metric 3200 pump (LDC analytical, USA), a Rheodyne Model 7010 injection valve (Rheodyne, Cotani, CA, USA), a 146 mm. x 4.6 mm. i.d. stainless steel 316 column (Waters, Massachusetts, USA) used as extraction cell, a GC oven (F11, Perkin Elmer, USA) and a collecting vial. Each part was connected with a 0.002 mm id. x 1/16 inch od. tubing (Waters, Massachusetts, USA) except between the extraction cell and the collecting vial a 0.004 mm. id. x 1/16 inch od. tubing was used. The schematic diagram of superheated water extraction was shown in Figure 6.

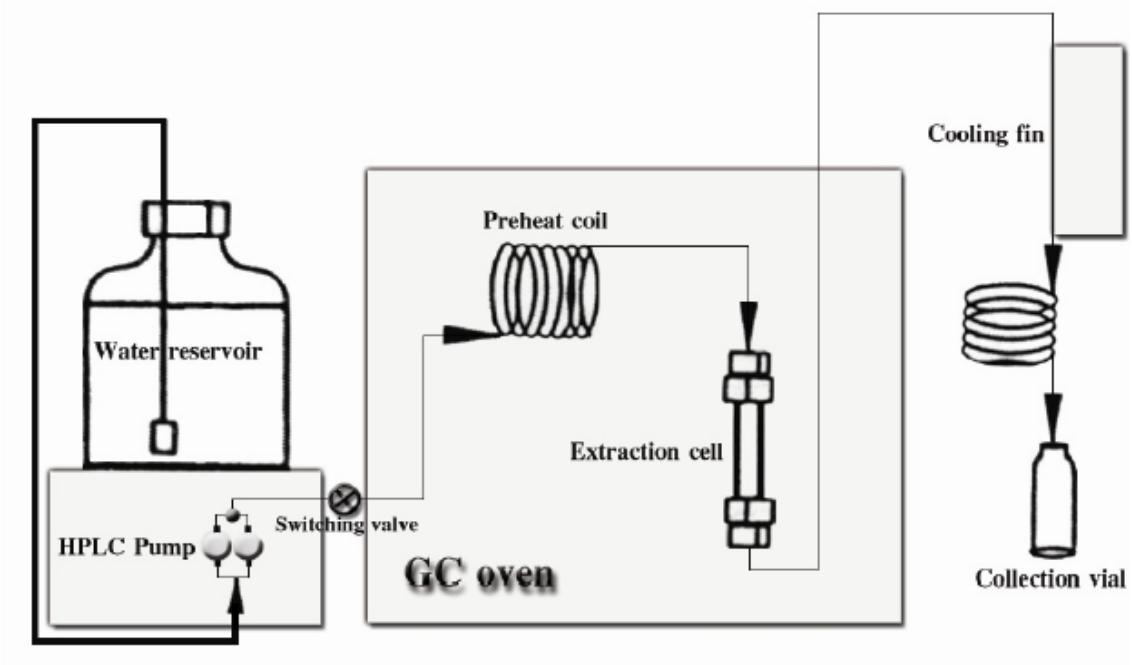


Figure 6 Schematic of superheated water extraction.

Chromatographic separation and detection was carried out using Hewlette Packard 5890 series II Gas chromatograph with flame ionization detector (Hewlette Packard, Polo Alto, CA, USA). GC/MS was performed using a HP5890 gas chromatographic system (Hewlette Packard, Polo Alto, CA, USA) and a HP5989 mass spectrometer (Hewlette Packard, Polo Alto, CA, USA). A 0.32 mm. i.d. x 25 m x 0.52 μm film thickness HP-5 capillary column (Hewlette Packard, USA) was used for separation for both GC-FID and GC-MS instruments.

Methods

1. Standard solution preparation

Nitrosamine standard stock solutions were prepared by dissolving 0.0300 g of each standard analyte with 30.00 mL of methanol, except that NMOR was prepared by pipetting 1.00 mL of 5000 μg/mL NMOR standard into a 5.00 mL volumetric flask and adjusted to the volume by using methanol. The internal standard was prepared from dissolving 0.0200 g NDPA in 20.00 mL of methanol and the final concentration of all nitrosamines were 1000 ppm.

2. Preparation of frankfurter samples

Refrigerated frankfurter samples were purchased from local supermarkets located in Bangkok. After cooling down to room temperature, the sample was chopped into small pieces in and homogenized by hand mixing. Afterthat the chopped frankfurter was weighted into 1.0 g each portion. It was spiked with 100 μg each of nitrosamine stock solution and left for solvent evaporation at room temperature for 10 min.

3. Extraction method

3.1 Superheated water extraction (SWE)

Double deionized water contained in a glass reservoir was sonicated for 1 h to remove oxygen by using a Transsonic model 460/H ultrasonic bath (Elma, Germany). The water was delivered through a Constra Metric 3200 HPLC pump and a Rheodyne 7010 valve at a constant flow rate. It was then passed to a 14.6-cm x 4.6 mm. i.d. stainless steel LC column as an extraction cell containing 1.0 g of fortified frankfurter spiked. The cell was inserted glass wool at both ends to prevent the frit being plugged and was then closed with 2-μm stainless steel frits and screw caps at the either end. Afterwards both preheating coil and extraction cell were placed in an oven that was maintained at a desired constant temperature. The water was flowed through the sample cell at a flow rate of 1.0 mL min^{-1}. Pressure of the extraction system was higher than 300 psi indicated by the HPLC pump and it was controlled by crimping the end of tubing to maintain the extractant in a liquid state. The water was passed out of the cell in the oven to a cooling system consisting of a stainless steel cooling coil wrapped with aluminium cooling fins, which was used to reduce the high temperature of the extractant in order to avoid loss of some volatiles caused by the heat. The aqueous extract was collected in a vial and closely sealed and then was further cleaned up by using SPE.

3.2 SWE parameters optimization

3.2.1 Effect of extraction temperature

Effect of extraction temperature on nitrosamine removal was primarily studied by operating temperature at room temperature and 120^{o}C with a pressure of more than 300 psi and a constant flow rate of 1 mL min^{-1}. Static and dynamic time were initially kept constant at 5 and 10 min, respectively. A 2.00 mL of dichloromethane (DCM) was used as a trapping organic solvent. After the extraction, the DCM extract was further cleaned up by using SPE under optimal condition. The

fraction was collected, dried with anhydrous sodium sulfate, and then filtered through 0.45 μm Nylon 6,6 membrane filter prior to GC-FID analysis.

3.2.2 Effect of dynamic extraction time

To examine total removal time for quantitative analysis of nitrosamine, the water was pumped at a constant flow rate of 1 mL min^{-1}, under the extraction temperature of 120^{o}C and a static time of 5 min. The collecting vial was changed every 5 min interval for 25 min or 5 fractions. After the extraction, each of DCM extract was further cleaned up by SPE under optimal condition.

3.2.3 Full fractorial design for optimum SWE condition

Full fractorial model designed by Minitab software Release version 14.20 with three replicates was employed for an investigation of the effects of multiple variables on an output variable (response). In this experiment, three factors with their levels were set as follows: 3 levels of temperature at 120, 140 and 170 ^{o}C, 3 levels of static time at 1, 5 and 10 minutes, and 2 levels of flow rate at 0.5 and 1.0 mL min^{-1}. The volume of aqueous extract was kept constant at 10 mL. Each extract was further cleaned up by SPE under optimal condition. A series of experiments assigned by full fractorial design was shown in Table 1.

Table 1 Factor levels and design matrix in the full factorial design for SWE.

Standard order	Run order	Temperature (^{o}C)	Static time (min)	Flow rate (mL/min)
44	1	140	1	1.0
1	2	120	1	0.5
15	3	170	5	0.5
17	4	170	10	0.5
50	5	170	1	1.0
41	6	120	10	0.5
34	7	170	5	1.0
2	8	120	1	1.0
39	9	120	5	0.5
40	10	120	5	1.0
7	11	140	1	0.5
20	12	120	1	1.0
25	13	140	1	0.5
11	14	140	10	0.5
46	15	140	5	1.0
24	16	120	10	1.0
16	17	170	5	1.0
32	18	170	1	1.0
3	19	120	5	0.5
26	20	140	1	1.0
47	21	140	10	0.5
42	22	120	10	1.0
31	23	170	1	0.5
19	24	120	1	0.5
18	25	170	10	1.0
22	26	120	5	1.0
52	27	170	5	1.0

Table 1 (Continued)

Standard order	Run order	Temperature (^{o}C)	Static time (min)	Flow rate (mL)
9	28	140	5	0.5
37	29	120	1	0.5
51	30	170	5	0.5
36	31	170	10	1.0
8	32	140	1	1.0
49	33	170	1	0.5
23	34	120	10	0.5
6	35	120	10	1.0
5	36	120	10	0.5
43	37	140	1	0.5
38	38	120	1	1.0
54	39	170	10	1.0
29	40	140	10	0.5
28	41	140	5	1.0
21	42	120	5	0.5
4	43	120	5	1.0
48	44	140	10	1.0
14	45	170	1	1.0
10	46	140	5	1.0
33	47	170	5	0.5
27	48	140	5	0.5
13	49	170	1	0.5
35	50	170	10	0.5
45	51	140	5	0.5
53	52	170	10	0.5
12	53	1	140	10
30	54	1	140	10

4. Clean-up method

4.1 A conventional method of liquid-liquid extraction (LLE) coupled with centrifugation

Initially, unspiked frankfurter was subjected to the SWE under 120 ^{o}C extraction temperature, 5 and 10 min static and dynamic time, respectively. A 10 mL extract was collected and spiked with 100 μg each of NDEA and NPYR, the mixture was used as a liquid sample in the clean-up step by LLE which was set up following the work of Cox (1973). To facilitate the operation a scale of the experiment was reduced. The liquid sample mixture was added with 2 g sodium chloride, extracted with 2x2 mL dichloromethane, and centifuged at 1,000 rpm for 20 min. The dichloromethane layer was taken and washed with 4 mL of 2 M NaOH. The organic solution was dried with anh. sodium sulfate, filtered through a 0.45 μm Nylon 6,6 menbrane, and evaporated under nitrogen gas to less than 1.0 mL. A 0.5 mL hexane was added and the evaporation was continued to a final volume of 1.00 mL. Quantitation was performed by a 1 μL injection of the solution to GC-FID.

4.2 Liquid-liquid extraction coupled with solid-phase extraction (LLE-SPE)

For the best elimination of fat, pigment and other interferences, both LLE and SPE parameters must be optimized. Unspiked frankfurter was subjected to the SWE under 120 ^{o}C extraction temperature, 5 and 10 min static and dynamic time, respectively. A 10 mL extract was collected and spiked with 100 μg each of NDEA, NPIP and NPYR, giving a cloudy solution which was further used as a liquid sample for optimization in the clean-up step.

4.2.1 Liquid-liquid extraction parameter optimization

4.2.1.1 Kind and volume of organic solvent

Several kinds of trapping organic solvent, namely dichloromethane and ethyl acetate were investigated, as well as their volumes and steps of extraction, in order to optimize between extraction efficiency and their quantities.

After SWE the extract was added with 2 g NaCl and further extracted with dichloromethane or ethyl acetate. The organic layer was collected and transfered into a solid phase column. When using dichloromethane as an extracting solvent, the solid phase column was firstly eluted with 5 mL dichloromethane and the eluent was discarded. Finally, all nitrosamine compounds were eluted from the SPE column with 1% triethylamine in ethyl acetate through the sorbent bed and 15 mL of fraction was collected.

When using ethyl acetate as an extracting solvent, the solid phase column was firstly eluted with 5 ml ethyl acetate and the eluent was discarded. Finally, the nitrosamines were eluted from florisil column with 1% triethylamine in ethyl acetate and 15 mL of fraction was collected.

4.2.1.2 Effect of NaCl addition

To study the effect of NaCl addition on extraction efficiency, nitrosamine extraction with and without an addition of 55% NaCl (2 g NaCl in 10 mL SW extractant). were compared. For both cases, 2 mL of dichloromethane was used in the liquid-liquid extraction step. The organic layer was collected and transferred into a solid phase column. The nitrosamines were eluted from the florisil column with 30 % ethyl ether in dichloromethane and 15 mL of fraction was collected.

4.2.2 SPE parameter optimization

4.2.2.1 Sorbent type

The mixture was further added with 2 g NaCl for salting out and facililating the breaking of emulsion. Afterwards it was simultaneously cleaned up and pre-concentrated by liquid-liquid extraction with 2 mL dichloromethane and the organic layer was subjected to a solid-phase extraction using 4.6 g of either florisil, silica or (1:1 w/w) sulfuric acid-treated silica gel as a packing material. All sorbent columns were firstly eluted with 5 mL dichloromethane, the eluent was discarded and finally nitrosamines were eluted by using pure ethyl acetate. A 15 mL of fraction was collected, dried with anh. sodium sulfate, filtered through a 0.45 μm Nylon 6,6 menbrane, and evaporated to less than 1.0 mL under nitrogen gas. The clear filtrate was then added with 100 μL of 1000 ppm nitrosodipropylamine as an internal standard and the volume was adjusted to 1.00 ml with dichloromethane. Finally, 1 μL of the solution was analysed by gas chromatography to give peak area of each analyte and internal standard. Triplicate extraction was performed (n=3). After GC analysis, the area ratio of each analyte to internal standard were averaged and calculated statistically.

4.2.2.2 Eluent system and volume

The extract after SWE was added with 2 g NaCl and further extracted with 2 mL dichloromethane. The organic layer was collected and transfered into a 4.6 g florisil column. The column was firstly eluted with 5 ml dichloromethane and followed by a further elution with 15 mL of one of the following solvent or its mixture, namely ethyl acetate, 1% acetic acid-ethyl acetate, 1% triethylamine-ethyl acetate, 5% acetone-ethyl ether, 30, 45, 60, 70 and 80% ethyl ether-dichloromethane, and pure ethyl ether.

The eluting solvent volume was also optimized. The nitrosamines were eluted from the SPE column by 60% ethyl ether in dichloromethane. A 5 mL eluent was collected for 5 fractions.

5. Gas Chromatographic Separation and Detection

Quantitative analysis was performed by using GC-FID. The gas chromatograph was equipped with a 0.32 mm. i.d. x 0.52 μm film thickness x 25 m 5% phenyl - 95% dimethylpolysiloxane capillary column (HP-5 column, Hewlette Packard, USA). Both the injector and the flame ionization detector were kept constant at 220 ^{o}C. One microliter of each extract was injected to the separating column with 99.99% oxygen free nitrogen as carrier gas and a split ratio of 1:15. N-nitrosodipropylamine was used as an internal standard for quantification. The oven temperature program was held at 40^{o}C, then ramped from 40 ^{o}C to 80 ^{o}C at 7 ^{o}C min^{-1} and held for 8 min, then raised to 200 ^{o}C at 15 ^{o}C min^{-1} and held for 5 min.

Identification of extracted nitrosamines was confirmed by GC/MS analyses. A spectra for identification of the compounds were obtained by a direct splitless injection of 1 μL of each extract solution into the same column and temperature program applied for the GC/FID. The injector and detector temperature were kept at 220 ^{o}C and 280 ^{o}C, respectively. The mass spectrometric detector was performed in an electron ionization (EI) mode with the electron energy of 70 eV. Helium gas was used as a carrier gas. Temperature of the ion source and of the quadrupole mass analyser were kept at 200 ^{o}C and 100 ^{o}C, respectively. To confirm NDEA, NPYR, NPIP and NMOR in frankfurter a selected ion monitoring (SIM) technique was applied by choosing molecular ions at m/z 102, 100, 114, 116, respectively, while that for NDPA (internal standard) was m/z 70.

6. Efficiency of the method

6.1 Detection limits of by GC-MS

A number of calibration curves were prepared from a low mass range of extracted nitrosamine between 10 to 30 ng injected for both NDEA and NPIP. The mass range between 15-30 ng injected for NPYR and NMOR. The graph was plotted between peak area ratio of analyte and internal standard concentration. Linear regressions, slope, intercept, correlation coefficient, and standard deviation were calculated, followed the reference (Miller, 1988).

6.2 Recoveries

Recoveries of the spiked frankfurter sample under the optimal conditions were also determined. A 1.00 g of sample was spiked with 20 µg each of NDEA, NPIP, NPYR and NMOR and then extracted by SWE under optimal conditions. The SW extract was added with 2 g NaCl for salting out and further clean up and pre-concentrated by liquid-liquid extraction with 2 ml dichloromethane. The organic layer was subjected to a 4.6 g florisil solid-phase extraction which was pre-eluted with 5 ml dichloromethane. The nitrosamines were following eluted with 60% diethyl ether in dichloromethane. Fifteen mL of fraction was collected. A 20 µL 1000 ppm *N*-nitrosodipropylamine was used as an internal standard. Quantitative analysis was performed by using GC-MS. The extraction was performed repeatedly five times (n=5) for each compound. After the GC analysis, the area ratios of each extracted compounds to internal standard were averaged and the recoveries were subsequently calculated.

RESULT AND DISCUSSION

Part I Clean up step

The main purpose of the whole research was to determine nitrosamine compounds in frankfurter by superheated water extraction coupled with GC analysis. However, matrix interference typically exists in the sample, a clean up step is then needed prior to the analysis. The use of the SWE in the extraction of trace nitrosamine compounds then involves in pre-concentration of the compounds present in the extract and a clean up procedure, which also frequently serve as an analyte-enrichment technique. Both cleanup and pre-concentration step are especially necessary when SWE in a dynamic mode is carried out, because high extract volume is obtained and the analytes are diluted in the liquid extract. In addition, the analytes would be quantitatively analysed by GC and the water extractant product from SWE can not be directly injected into an anlytical capillary GC column, an additional step of liquid-liquid extraction (LLE) and solid-phase extraction (SPE) prior to the chromatographic analysis were then investigated for coupling steps.

1. A conventional method of liquid-liquid extraction (LLE) coupled with centrifugation

Initially, the most basic clean up procedure, that is liquid-liquid extraction using dichloromethane was studied. Unspiked frankfurter was subjected to the SWE under 120 ^{o}C extraction temperature with 5 and 10 min static and dynamic time, respectively. A 10 mL extract was collected and spiked with 100 μg each of NDEA and NPYR, the mixture was used as a liquid sample in the clean-up step by dichloromethane extraction. In stead of two separating layers, a cloudy emulsion was occurred in the vial, as a result of a formation of lipid layer, which was more obvious when it was cooled in a fridge. A subsequent experiment was then performed under the same conditions as above, but without cooling, the organic extract containing fat was centrifuged and the dichloromethane lower layer was taken to wash with NaOH and concentrated prior to GC analysis. Table 2 showed the peak area of NDEA and

NPYR and the recovery obtained when using only a clean-up step with dichloromethane. The recoveries in this experiment were calculated by a peak area comparison with an initial amount of nitrosamines spiked into frankfurter. No internal standard was involved. From the result, it was found that dichloromethane extraction coupled with centrifugation applied for the enrichment of analyte did not ensure sufficient enrichment of the analyte. In Table 2 low recoveries of NDEA and NPYR were observed, which occurred from analyte loss in the procedure during the LLE. In addition, an incomplete removal of fat could cause co-extraction of those lipids Therefore, liquid-liquid extraction (LLE) coupled with centrifugation was not suitable to extract lipid-rich sample because it could not perfectly eliminate lipids, pigments and other interferences. The extraction of trace nitrosamine compounds in the presence of extractable major components such as lipids causes several difficulties. In gas chromatography, a large amount of injected fat may cause problems in the injector and at the top of column. When using mass spectrometry detection, the ion source can also be contaminated. In order to solve such problems, an appropriate clean up procedure was developed for nitrosamine extraction from fatty food.

Table 2 Peak area and recovery of NDEA and NPYR obtained from liquid-liquid extraction coupled with centrifugation.

Compounds	Retention time (t_R) (min)	Peak area	% Recovery[1]
NDEA	5.649	6833	61.81
NPYR	11.387	5540	50.12

[1] n = 4

2. Liquid-liquid extraction coupled with solid-phase extraction (LLE-SPE)

Since liquid-liquid extraction (LLE) using dichloromethane coupled with centrifugation was not enough to remove those fats and additives interferences from

the SW extract, two steps for extraction, namely LLE coupled with SPE, were then proposed.

2.1 Liquid-liquid extraction optimization

As mentioned earlier that the LLE step was applied for transference of the analytes to a suitable extractant for GC analysis, as well as being a preconcentration and clean-up step, a couple of parameters in this step, namely type and volume of organic extractant or trapping solvent, and number of extraction, must then be optimized for the best recovery of those analytes.

2.1.1 Kind of trapping organic solvent

In this study type of trapping solvent was also investigated in order to achieve a maximum removal of all of the analytes from the SW extract. Previous studies of other researchers revealed that extraction of nitrosamines from water phase, the analytes could be collected by using dichloromethane as a extracting solvent (Mitch *et al.*, 2003, Byun *et al*., 2004). Liquid–liquid extraction technique by using a trapping solvent was then applied to extract the nitrosamines from the SW aqueous extract.

After superheated water extraction, the water extractant was extracted with organic solvent to remove the extracted nitrosamine prior to GC-FID analysis. A constantly 2 mL of each trapping solvent was investigated in the extraction efficiency of each analyte. Different polarity of organic solvents, namely dichloromethane and ethyl acetate were chosen on the basis of immiscibility with water. Dichloromethane has a polarity less than ethyl acetate. The polarity indexs of dichloromethane and ethyl acetate are 3.4 and 4.3, respectively.

A comparison of each nitrosamine extracted with various solvents from a fortified aqueous sample was reported in Table 3 and showed in Figure 7. From the results, it was seen that by using dichloromethane the yield of total

nitrosamine was highest, achieving the recoveries of each nitrosamine ranging between 75-82 %, whereas using ethyl acetate, all of extracted components were achieved from aqueous layer in lower amount, resulting averagely lower recoveries. The nitrosamine compounds could be partitioned into dichloromethane layer in more amount than into ethyl acetate layer. It was then noted that dichloromethane was the most suitable trapping solvent and had been utilized for all nitrosamines extraction in a further study. Besides, dichloromethane has higher density (1.32 g mL^{-1}) than water, it was in the lower phase so the aqueous upper layer could prevent both dichloromethane from evaporation and analytes loss.

Table 3 Comparison of peak area ratio and recovery of each analyte in SW extract from frankfurter obtained by different trapping solvents.

Compounds	t_R(min)	Area ratio of analyte to internal standard		%Recovery[1]	
		DCM	Ethyl acetate	DCM	Ethyl acetate
NDEA	5.615	0.5376	0.4391	80.64	65.87
NPYR	11.364	0.4353	0.3365	75.74	58.55
NPIP	13.650	0.5373	0.4501	82.21	68.87

[1] n = 3

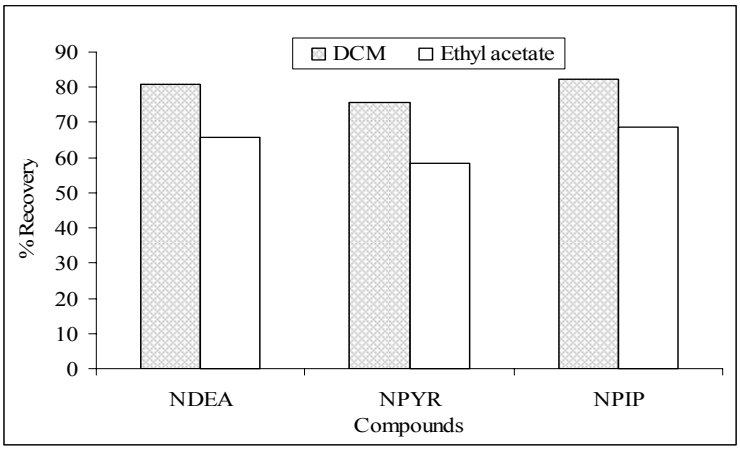


Figure 7 Effect of trapping organic solvent on extraction efficiencies of nitrosamines.

2.1.2 Volume of organic solvent for liquid-liquid extraction

To remove most compounds from the aqueous extractant after SWE, the appropriate volume of dichloromethane was also studied. Quartet extraction of the SW extract with 2 mL dichloromethane was performed and each collected fraction was quantitatively analysed by using a GC-FID. Table 4 showed the area ratio of each analyte to internal standard which was plotted against each fraction as shown in Figure 8. Each time was represented by each number of extraction steps.

Table 4 Peak area ratio obtained from 2 mL each of dichloromethane extraction after SWE.

Compounds	t_R (min)	Area ratio of analyte to internal standard[1]			
		Number of extraction step			
		1	2	3	4
NDEA	5.626	0.5653	0.0000	0.0000	0.0000
NPYR	11.457	0.3731	0.0000	0.0000	0.0000
NPIP	13.713	0.5588	0.0117	0.0000	0.0000

[1] n = 3

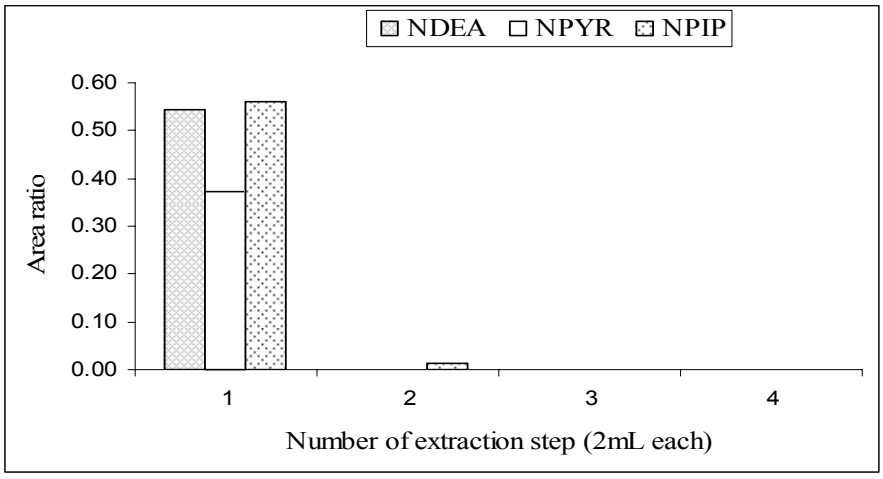


Figure 8 Effect of the volume of dichloromethane trapping solvent on the nitrosamine compounds.

From the above results, the second, third and fourth dichloromethane extraction was performed to ensure that the compounds were not remained in the aqueous phase, showing that NPYR and NDEA were extracted completely in the first extraction, whereas NPIP was completed in the second extraction.

When carefully consider the results, it was found that the second extraction may not have a significant effect to the product yield, since all nitrosamines were almost totally extracted in the first two milliliter or the first dichloromethane extraction. Only 2.1 % of the first 2 mL NPIP fraction was produced in the second one. To maximize the concentration of total compound yields and avoid dilution effect, a single extraction step using 2 mL of the solvent was enough to extract the nitrosamine compounds from the aqueous extract. Besides, it could reduce the cost of organic solvent when using only 2 mL of dichloromethane.

2.1.3 Step of dichloromethane extraction

Generally liquid-liquid extraction method can not complete a removal of all analytes from aqueous phase to organic phase in a single step. In this experiment a double steps using 1 mL each fresh solvent was compared with a single step using 2 mL of dichloromethane for liquid-liquid extraction. The results of a single and multiple steps of liquid extraction by using each 2 mL of dichloromethane are showed in Table 5 and Figure 9.

Table 5 % Recoveries of the nitrosamines extracted from SW extract by using 2 mL dichloromethane trapping solvent in different extraction step.

Compounds	t_R (min)	Area ratio of analyte to internal standard		% Recovery[1]	
		1 step	2 step	1 step	2 step
NDEA	5.540	0.5564	0.5681	83.46	85.22
NPYR	11.208	0.3711	0.3603	64.57	62.70
NPIP	13.710	0.5594	0.5622	85.58	86.02

[1] n = 3

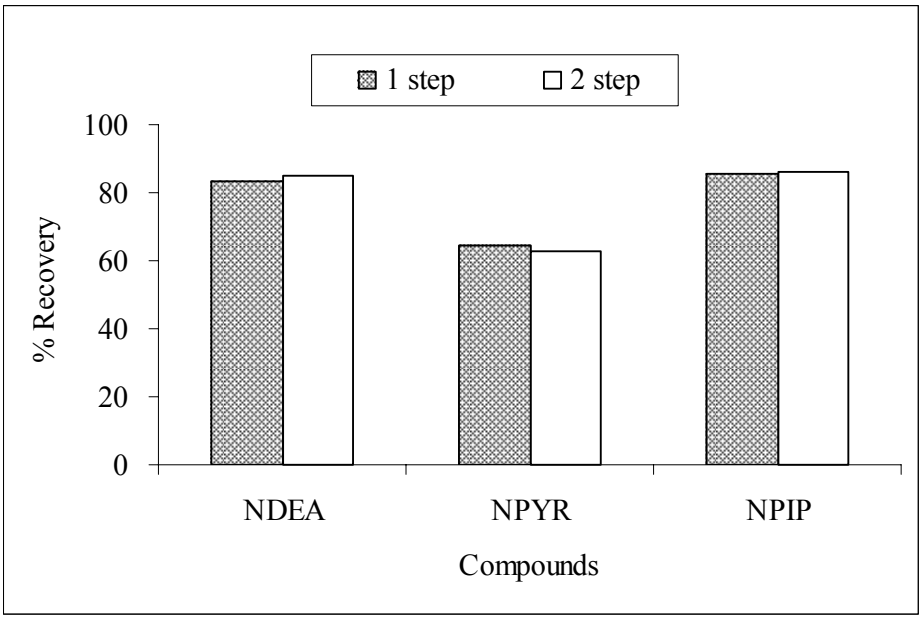


Figure 9 Quantities of analytes extracted in 1 step and 2 step using dichloromethane with a total volume of 2 mL.

It was demonstrated that with the same total volume, higher amount of the extracted NPYR was obtained in a single step. On the other hand, higher recovery was achieved in double step for both NDEA and NPIP. The studies showed that the step of extraction did not significantly affect % recovery. Thus, a

single step using 2 mL fresh dichloromethane was performed for the liquid extraction, which was more beneficial in saving the analysis time**.** In addition, the more extraction step the more lipid could be co-extracted with the analytes in dichloromethane and the high lipid content in DCM extract caused a plug in a SPE column used in the following step.

2.1.4 Effect of NaCl addition

In some procedures nitrosamines in water sample can be extracted in dichloromethane by using a separatory funnel, for example U.S. EPA method 3510 C (US EPA, 1996). Unfortunately, using the method low recoveries were obtained (Mitch., 2003), and may generate difficulty to handle emulsion when applied for wastewater effluent samples. Yoo *et al.* 2000 reported that the extraction efficiency could be improved by adding NaCl into the water up to 100 g L^{-1} for salting out.

Owing to the high water solubility of all nitrosamines, 106 g L^{-1} for NDEA, 1,000 g L^{-1} for NPYR, 860 g L^{-1} for NMOR and 76 g L^{-1} for NPIP at 25^{o}C,. a removal of nitrosamines from aqueous phase was expected not being completed without adding NaCl into the SW extract. In this experiment, NaCl was then tested as a salting out reagent. Salt was added to the SW extractant to decrease the solubility of analytes in the aqueous phase, shifting equilibrium towards the organic phase. To determine the effect of NaCl addition on extraction efficiency, an addition of salt was examined by adding 2.0 g NaCl into a 10 mL extractant after superheated water extraction. Such amount was correspond to 55% saturated salt solution. The results were compared with those without NaCl addition and summarized in Table 6 and Figure 10.

Table 6 Effect of NaCl addition on extraction efficiencies of nitrosamines.

Compounds	t_R (min)	Area ratio of analyte to internal standard		%Recovery[1]	
		Without NaCl addition	55% saturated NaCl	Without NaCl addition	55% saturated NaCl
NDEA	5.601	0.4314	0.5476	64.71	82.13
NPYR	11.360	0.2654	0.3986	46.18	69.35
NPIP	13.655	0.4442	0.5374	67.97	82.22

[1] n = 3

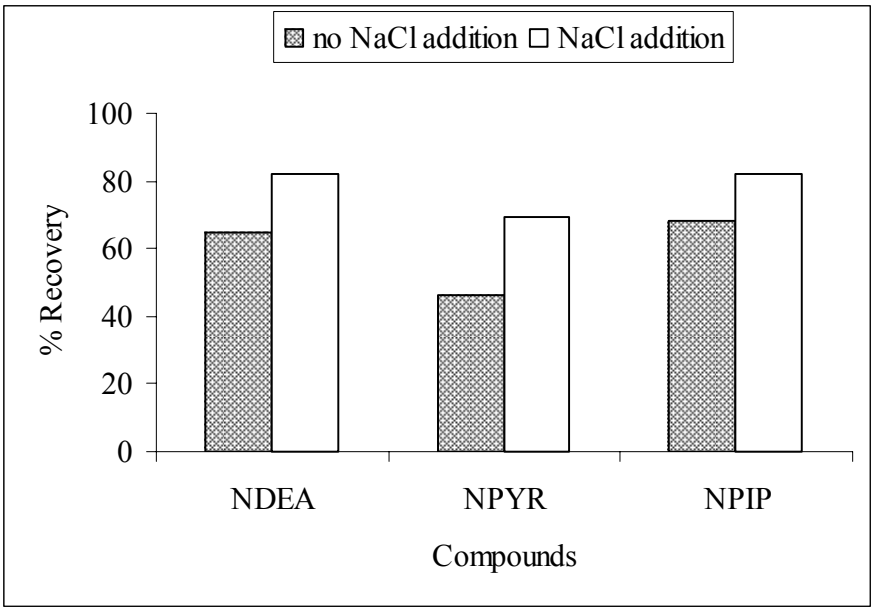


Figure 10 Effect of NaCl addition on extraction efficiencies of nitrosamines.

By comparison of recoveries of nitrosamines (Figure 10) between with and without 2.0 g NaCl addition, differences in recoveries obtained with and without the salt addition were observed. The recoveries of all nitrosamine compounds with a further salting out step by adding NaCl were higher than those without the addition. All compounds were significantly improved the recovery from averagely

60% to 78%. The low recovery obtained for the nitrosamine compounds without NaCl addition could be explained by a strong interaction with the aqueous matrix and these compounds with high polarity remained in the aqueous layer after extraction. NaCl addition gave salting out effect, releasing the analyte from aqueous to the organic phase, hence promoting the recoveries.

2.2 Solid-phase extraction optimization

After the liquid-liquid extraction with dichloromethane, high molecular weight matrix components such as lipids and pigments are frequently present in the extract and need to be eliminated to permit a more definitive identification of lower limit residues and to minimize adverse effects on the detection instruments and compounds of interest. Thus, the removal of co-extracted matrix components is necessary and for this, different clean-up procedures have been developed (Encarnacion *et al*., 2001, Maxwell *et al*., 1993, Andrade *et al*., 2005). Co-extractives are frequently removed during the post extraction clean-up steps. Commonly, the post-clean-up procedures were used including adsorption columns or solid-phase extraction. In this research, a solid phase extraction was chosen to support as a clean up step. Parameters affecting solid-phase extraction efficiency, such as sorbent type, eluting solvent system and solvent volume were then studied.

2.2.1 Sorbent type

A cleaning-up of fatty samples is very tedious and time consuming, and sometimes more than one step is required to remove lipids. In order to avoid the exhaustive clean-up of extracts prior to analysis, this study was focused on a development of clean-up method. In case of fatty samples, in situ elimination of lipids can be achieved by using fat retaining sorbents preventing lipids and other co-extractable materials from coming out in the extract. A possibility of in situ removal of the lipids from fatty samples was investigated including several fat-retaining sorbents to prevent lipids and pigments. A number of sorbents, such as florisil, silica

gel and sulphuric acid-impregnated silica gel were tested for their fat-retaining properties in high-lipid content samples.

Selection of an appropriate SPE extraction sorbent depends on a mechanism of interaction between the sorbent and the nitrosamine. Florisil and silica were polar absorbing materials. Normal phase SPE procedures typically involve a polar analyte interacted with polar adsorption media. Retention of analyte under normal phase conditions is primarily due to interactions between polar functional groups of the nitrosamine and polar groups on the sorbent surface. These include dipole-dipole interactions, and dipole-induced dipole interactions. A compound adsorbed by these mechanisms was eluted by passing a solvent that disrupts the binding mechanism, usually the solvent must be more polar than the sample's original matrix. In this study, elution solvent and original sample solvent were ethyl acetate and dichloromethane, respectively. SPE glass column configuration was in a flow-through mode that the sample can be passed vertically through the column. While elution, the present contaminant was retained while the analyte of interest was allowed to pass through.

Florisil, a registered trade name of US Silica Co., is a magnesium silicate with slightly basic surface for adsorption of low to moderate polarity. It was used to separate analyte from lipid prior to sample analysis by a chromatographic method and was used for clean up of nitrosamines. After solid-phase extraction, a yellow segment or lipid deposit was observed on the top of packed florisil and red color of frankfurter pigment was seen on the anh. sodium sulfate segment in solid phase column. From the observation, it was concluded that the florisil column could be used to trap lipid and pigment which was co-eluted in the dichloromethane layer.

Although clear and clean fraction of eluent was observed for florisil and sulphuric acid-impregnated silica gel sorbents, the highest recovery was obtained using florisil. Sulphuric acid-impregnated silica gel has also been used successfully as a lipid-retainer with complex fatty materials. However, in this work no nitrosamine peak was detected because the nitrosamines are unstable under strong

acidic condition. When using silica gel sorbent, the lipid was co-extracted into a collected fraction and low recovery was obtained. The recovery of NDEA when using silica gel and florisil with ethyl acetate eluting solvent were 53.5 and 58.93, respectively (n = 3). Therefore florisil was the most appropriate sorbent for frankfurter in this work and was employed in further study.

In addition, the sorbent mass between 3.0 g and 4.6 g were investigated. With 3.0 g florisil the lipid was co-extracted in the collected fraction, but none were observed when using 4.6 g of the same material. A mass of 4.6 g was therefore chosen as optimum to minimize the lipid content.

2.2.2 Solvent system and solvent volume

Choice of elution solvent was critical to the success of solid-phase extraction. For selectively removed the analytes of interest, the adsorbent was then washed and the adsorbed material was extracted with a small amount of suitable solvent(s) or their mixtures in order to minimize any contamination that may be present in the system and ultimately interfere with GC analysis. Prior to the elution, activation of the solid sorbent was critical in order to effectively extract organic analytes from the sample. Without this step, solvent could not penetrate the pores and wet the surface. Thus, only a small fraction of the surface area was available for interaction with the nitrosamine. In addition, if the florisil column was not conditioned properly, the solid phase particles may not be solvated, causing problems with sample flow through the column and can ultimately result in low recoveries of nitrosamine. Therefore the florisil column was conditioned with 5 mL dichloromethane before applying the dichloromethane extract on the top.

Many types of solvent often combined to achieve a desired viscosity and solvent strength for the particular extraction. Those studied solvents and solvent mixtures were ethyl acetate, 1%acetic acid-ethyl acetate, 1%triethylamine-ethyl acetate, 5% acetone-ethyl ether, 30, 45, 60, 70 and 80% ethyl ether-DCM, and pure ethyl ether.

All the results obtained from different organic solvents gave similar trend for all nitrosamine compounds (Table 7 and Figure 11). With an increase portion of diethyl ether, nitrosamine compounds could be extracted in larger amount. A decrease in the extracted nitrosamine amount was found for more than 60% diethyl ether eluent. A graph of extraction efficient (presented as %recovery) was plotted versus % diethyl ether in dichloromethane is illustrated in Figure 12 and the GC chromatograms of the collected extract of different eluting solvent system are showed in Figure 13. The retention time of NDEA, NPYR and NPIP were 5.68, 11.36 and 14.0 min, respectively. The retention time of NDPA internal standard was 11.83 min.

The results from Table 7 and Figure 11 showed that 60% ethyl ether in dichloromethane was the most suitable eluent because it provided highest recoveries of NDEA**,** NPYR and NPIP of 99.09, 81.07, and 109.59 %, respectively. In addition, among other solvents tested, diethyl ether was proved to be sufficient for rapid evaporation so it required less analysis time. Co-evaporation of the nitrosamines with diethyl ether was unlikely occurred because of the high boiling point difference between the analytes and the solvent. The average boiling temperature of the studied nitrosamines was 208 ^{o}C, but that of diethyl ether was 34.6 ^{o}C. A variation in recovery observed between each nitrosamine was explained by difference in polarities of both the analytes and solvents, and their interactions with florisil sorbent, which affected their elutions from the column. Loss of nitrosamine could occur during the final concentration step, where the solvent was reduced to achieve an appropriate volume of 1.00 mL.

Table 7 Effect of type of solvent system for SPE on the yield of extracted nitrosamines.

Solvent systems	%Recovery[1]		
	NDEA	NPYR	NPIP
ethyl acetate	58.93	-	-
1% acetic acid-ethyl acetate	73.67	-	-
1% triethylamine-ethyl acetate	79.75	80.99	82.94
acetone/ethyl ether(5:95)	91.70	81.50	106.78
30% ethyl ether-DCM	83.46	64.57	85.58
45% ethyl ether-DCM	92.76	75.07	103.59
60% ethyl ether-DCM	99.09	81.07	109.59
70% ethyl ether-DCM	94.11	76.15	104.15
80% ethyl ether-DCM	92.04	70.99	103.60
100% ethyl ether	87.78	57.19	101.07

[1] n = 3

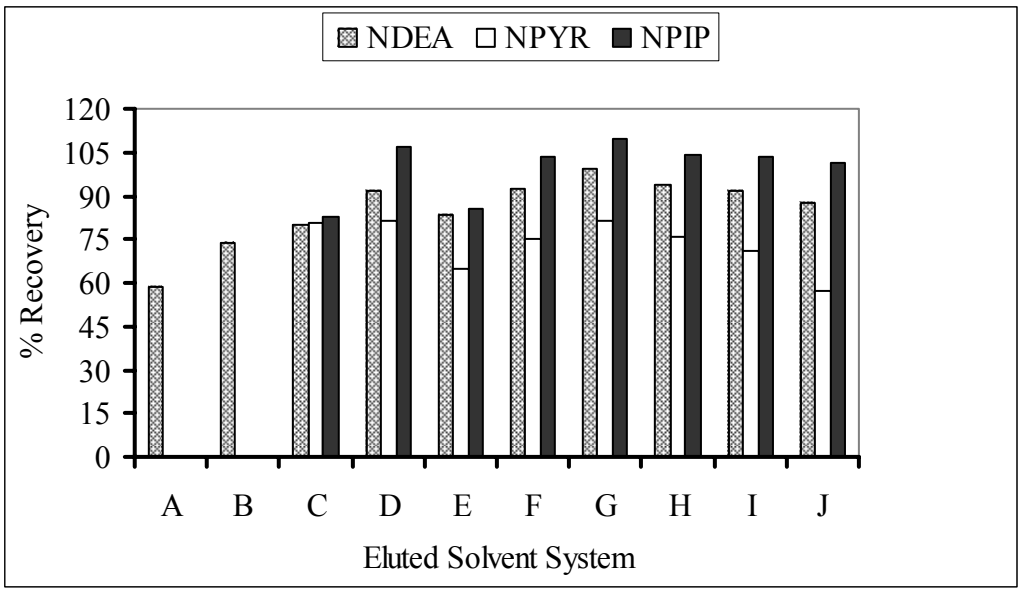


Figure 11 Comparison of different eluting solvent systems. A: ethyl acetate, B: 1%acetic acid-ethyl acetate, C: 1%triethylamine-ethyl acetate, D: acetone-ethyl ether(5:95), E: 30% ethyl ether-DCM, F: 45% ethyl ether-DCM, G: 60% ethyl ether-DCM, H: 70% ethyl ether-DCM, I: 80% ethyl ether-DCM and J: pure ethyl ether.

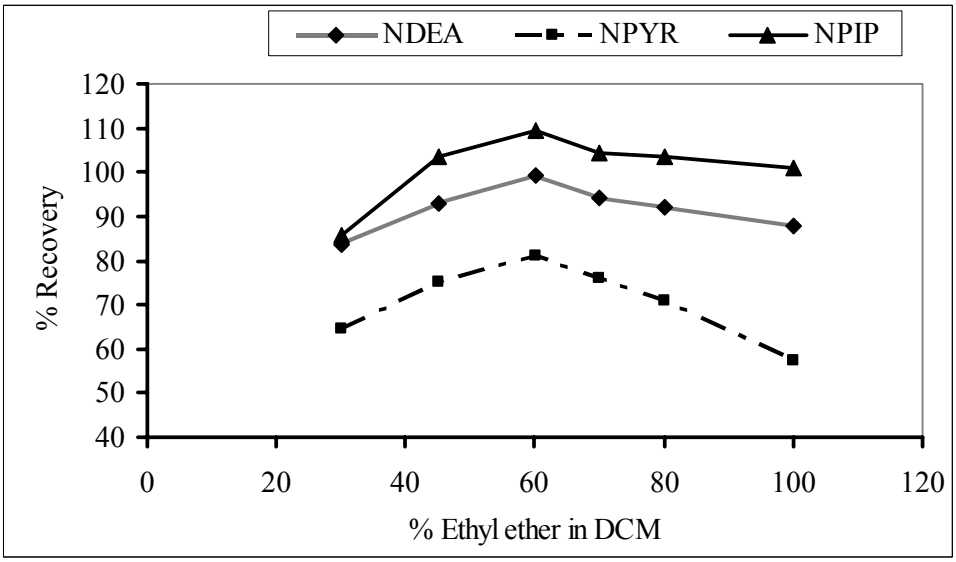


Figure 12 A comparison between each analytes extracted by SPE at different percentage of ethyl ether in DCM.

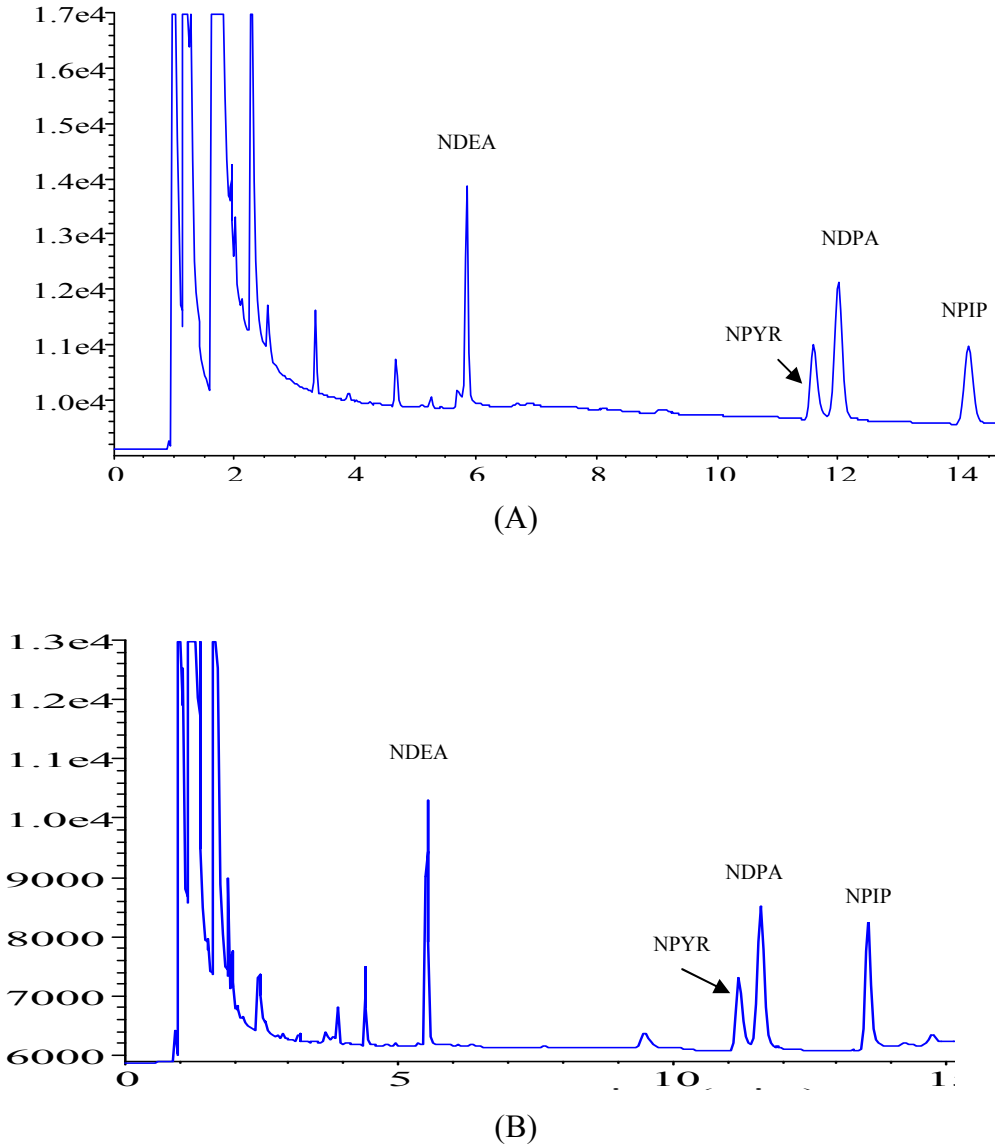
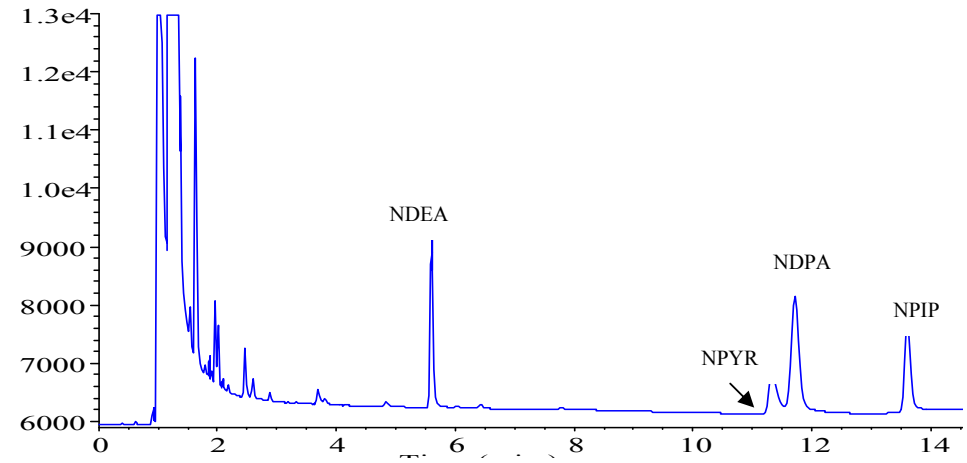


Figure 13 GC chromatogram of the extracted nitrosamine obtained by using different types of eluting solvent: (A) 1%triethylamine-ethyl acetate (B) acetone-ethyl ether (5:95); (C) 30% ethyl ether-DCM; (D) 45% ethyl ether-DCM; (E) 60% ethyl ether-DCM; (F) 70% ethyl ether-DCM; (G) 80% ethyl ether-DCM and (H) pure ethyl ether.



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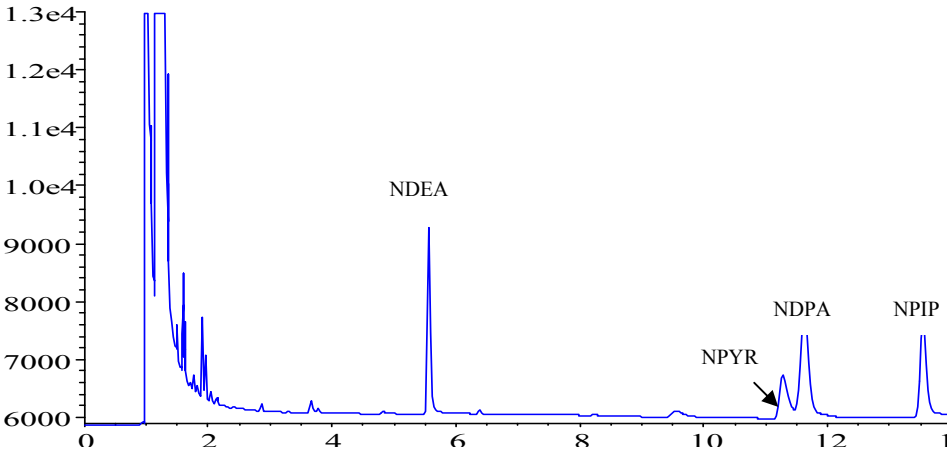

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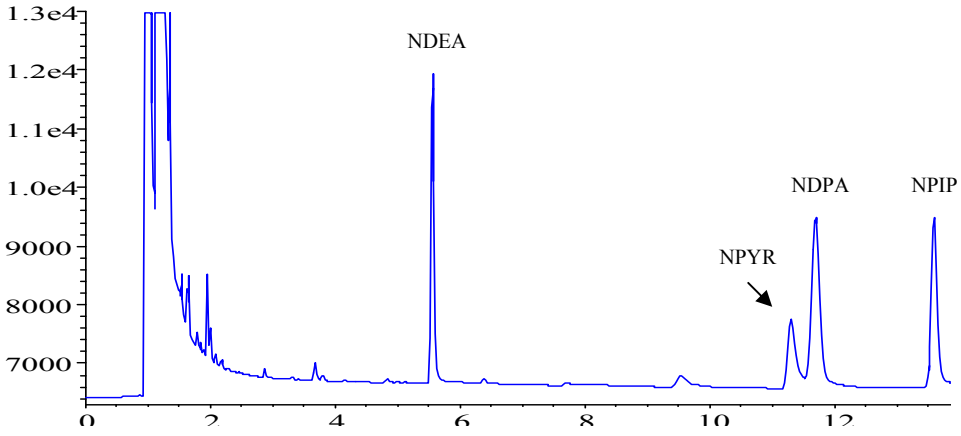
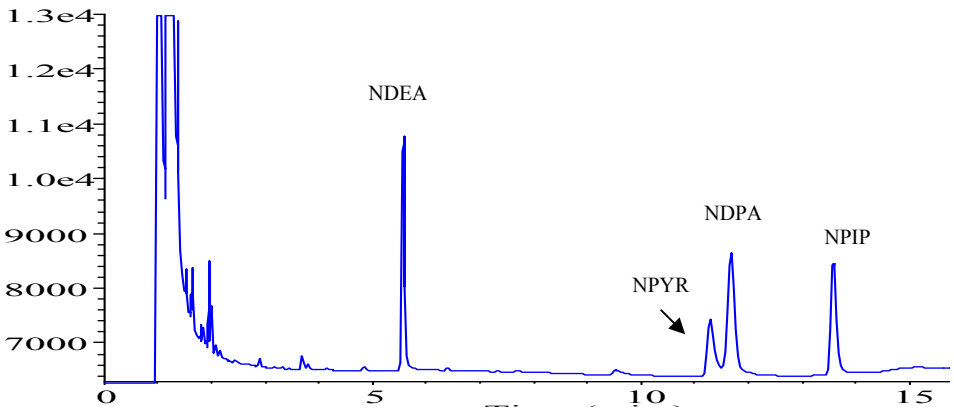


(E)

Figure 13 (Continued)



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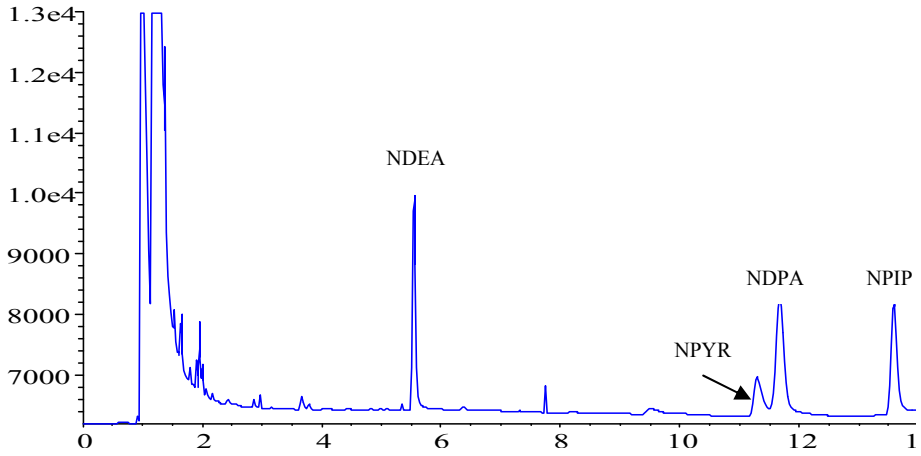
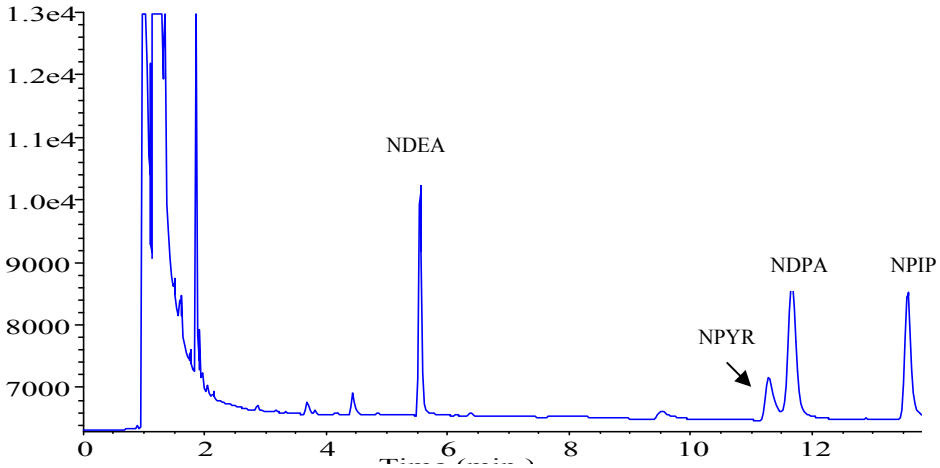


(G)



(H)

Figure 13 (Continued)

The amount of solvent required for optimum elution was determined by eluting with different volumes of 60% ethyl ether in dichloromethane and changing the collecting vial every 5 mL for 5 fractions. The eluent was collected and analysed by GC. The results of the study are showed in Table 8 and Figure 14 showed the plots of area ratio of each analyte to internal standard versus elution volume.

The results demonstrated that 15 mL was the minimum elution volume used to sufficiently extract analytes since no trace amount was obtained for higher volumes. Changing elution volume resulted in a change in the yields in each fraction. The yield of the extracted NPYR improved with increasing the solvent volume from 5 to 10 mL but for a higher elution volume, the amount of NPYR was decreased. The maximum yield was obtained when collecting 10 mL elutent. Meanwhile, the amounts of NPIP and NDEA were decreased with elution volume from 5 to 10 mL and greatly reduced from 10 to 15 mL, but no trace of the nitrosamine was detected in the fourth collection or 20 mL. Therefore all analyte removals from the SPE column were achieved with 15 mL collection volume.

Table 8 Effect of volume of 60% ethyl ether in dichloromethane on extraction efficiencies of nitrosamine compounds.

Compounds	Area ratio of analyte to internal standard [1]				
	5 mL	10 mL	15 mL	20 mL	25 mL
NDEA	0.3499	0.2509	0.0347	0.0000	0.0000
NPYR	0.0072	0.2912	0.0497	0.0000	0.0000
NPIP	0.3597	0.2798	0.0341	0.0000	0.0000

[1] n = 3

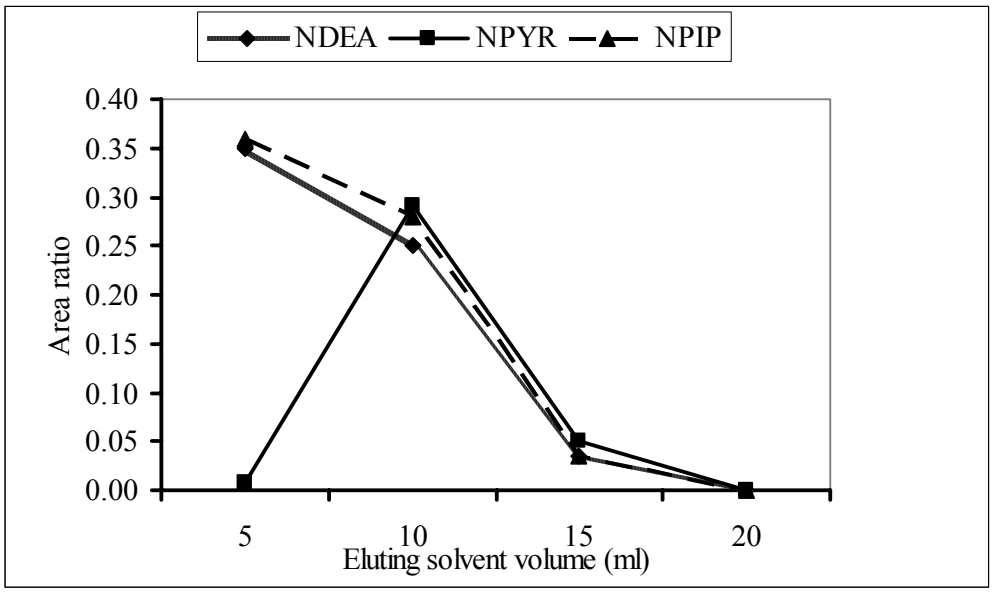


Figure 14 Effect of volume of 60% ethyl ether-DCM on the yield of nitrosamines.

From all above experiments, the studied parameters affecting the liquid-liquid extraction and solid-phase extraction are shown in Table 9.

Table 9 Optimization of some conditions of clean up and preconcentration step.

Parameters	Range studied	Optimum value
Liquid-liquid extraction		
Type of trapping solvent	Ethyl acetate, dichloromethane	Dichloromethane
Volume of solvent (mL)	2-8	2
Number of extraction steps	1-2	1
NaCl addition	Without, 55% saturated NaCl	55% saturated NaCl
Solid-phase extraction		
Sorbent type	Florisil, silica gel, sulphuric acid-impregnated silica gel	Florisil
Sorbent mass (g)	3.0, 4.6	4.6
Solvent system	ethyl acetate, 1%acetic acid-ethyl acetate, 1%triethylamine-ethyl acetate, acetone-ethyl ether(5:95), 30, 45, 60, 70, 80% ethyl ether-DCM, pure ethyl ether.	60% ethyl ether-DCM
Eluting volume (mL)	5-25	15

Part II Superheated water extraction

A number of parameters of superheated water extraction of nitrosamine from frankfurter were optimized to obtain maximum yield and to minimize the extraction time. Those parameters affecting SWE are temperature, dynamic extraction time, static time and flow rate of the water extractant. From previous reports (Basile *et al*.,1998, Lou *et al*., 2000 and Richter *et al*., 2003), the temperature of water in extraction cell was a key parameter of superheated water extraction. Thus, in this experiment the temperature was primarily investigated for the extraction of nitrosamine compounds.

1. A primary study of temperature effect

Since the dielectric constant, viscosity and surface tension of water are decreased by increasing temperature (Smith, 2002, Martinez *et al*., 2005 and Ramos *et al.*, 2002). An initial study was performed to primarily examine if high temperature had an effect to the extraction. The experiment was performed for water at a room temperature comparing with superheated water. In this work, fortified frankfurter sample packed in the extraction cell was extracted by water at room temperature and at a higher temperature of 120^{o}C to compare the yield of the compounds. The test was started by using a constant flow rate of 1 mL min^{-1}, the static and dynamic time for each extraction was 5 and 10 min, respectively. The end of tubing was crimped to give enough pressure of more than 300 psi to maintain the water in liquid state. For the clean up step, the optimal conditions from those previous experiments were used.

The results of the effect of extraction temperature on the product yields of nitrosamine, namely NDEA, NPIP and NPYR are given in Table 10 and in Figure 15 the extraction temperature was plotted against the recoveries for each compound.

As the temperature was increased from 30 ^{o}C to 120 ^{o}C, the extraction efficiencies of all nitrosamine compounds were obviously increased. The solubility of an organic compound in superheated water is often many orders of magnitude higher

than its solubility in water at ambient temperature for two reasons (Martinez *et al*., 2005). The first is due to a change in dielectric constant as previously described. The second is that solubilities typically increase with temperature, particularly for a compound with low solubility at ambient temperature, which has a high positive enthalpy of solution. In addition, in SWE, thermal energy was used to break an interaction between solute and its matrix. Raising the temperature increases the diffusion rates, solubility, and mass transfer of the analytes. These changes improved the contact of the analytes with the solvent and enhanced the extraction.

At room temperature (30^{o}C), the analytes can slightly dissolve in water and be eluted from the extraction cell. Compared the extraction temperature at ambient with those at 120^{o}C, the yields of all analytes were improved. It was obvious that temperature had a significant effect on nitrosamine extraction from frankfurter.

Table 10 A comparison of nitrosamine extraction efficiencies from fortified frankfurter by using typical water and superheated water at 120 ^{o}C.

Compounds	t_R(min)	Area ratio of analyte to internal standard		% Recovery[1]	
		30^{o}C	120 ^{o}C	30 ^{o}C	120 ^{o}C
NDEA	5.600	0.4075	0.5520	64.80	87.78
NPYR	11.347	0.2750	0.3799	48.13	66.50
NPIP	13.647	0.4900	0.6407	65.66	85.86

[1]n = 3

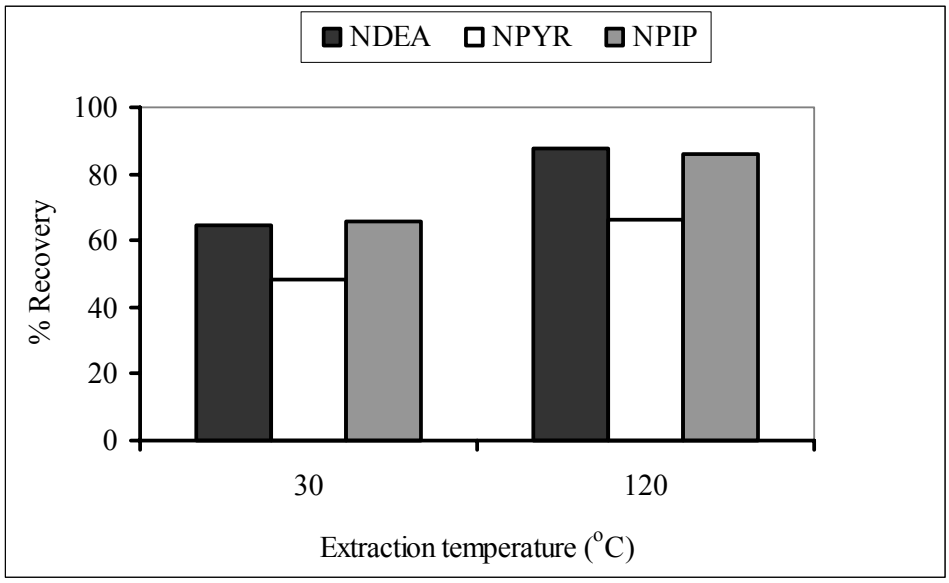


Figure 15 Effect of extraction temperature on the yield of the extracted compounds from frankfurters by SWE; SWE conditions; flow rate, 1mL/min; time for extraction, 10 min; static time, 5 min.

The chromatograms obtained from superheated water extraction under the selected conditions are shown in Figure 16 and 17, showing NDEA, NPYR, NDPA and NPIP peak corresponding to their retention times at 5.606, 11.366, 11.763, and 13.666 min, respectively. A completion time of one run was approximately 14 mins per one injection.

The chromatogram showed an increase in peak area of all components extracted under superheated water conditions compared with those under ambient conditions. Under room temperature water extraction, the area of each peak, except for the internal standard peak, was very low, relating with small amounts of compounds that were extracted. It was explained that the compounds were less soluble in water at room temperature than at 120°C. In other words, ambient temperature water could hardly dissolve the analytes, but superheated water promoted the extraction capability.

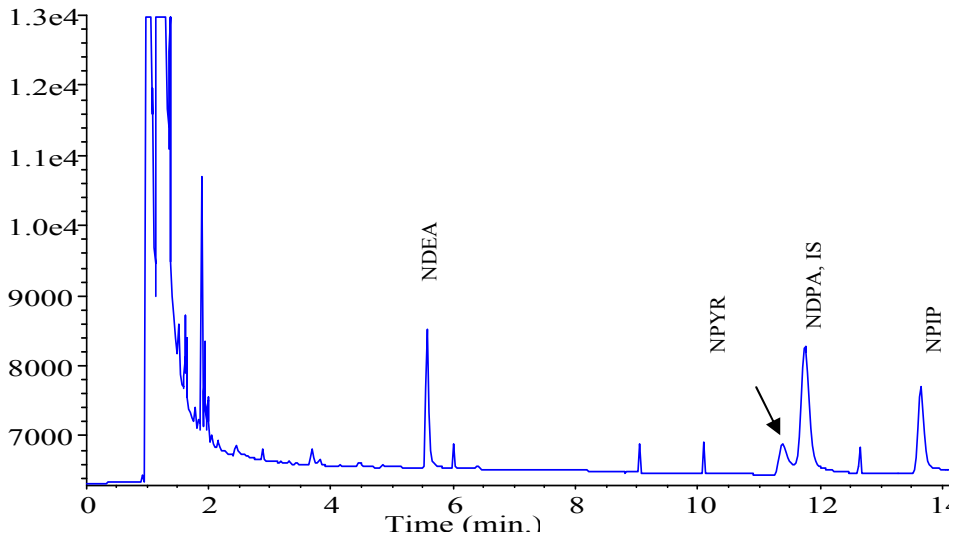


Figure 16 GC chromatogram of nitrosamine extract obtained at room temperature. Extraction conditions: temperature, 30 °C; flow rate, 1 mL min^{-1}; static time, 5 min; dynamic time, 10 min.

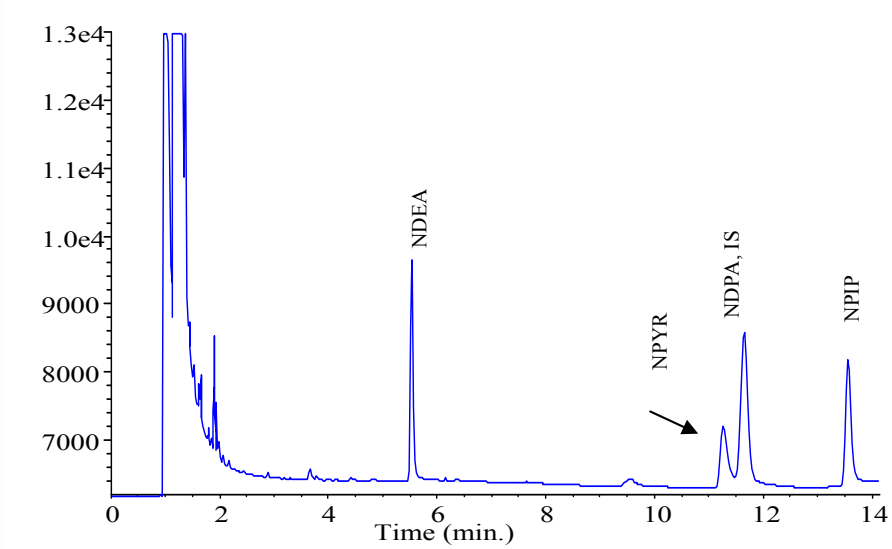


Figure 17 GC chromatogram obtained from SWE. Extraction conditions; temperature, 120 °C; flow rate, 1 mL min^{-1}; static time, 5 min; dynamic time,10 min.

2. Effect of dynamic time on SWE efficiency

The next parameter to study was dynamic extraction time or elution time. The dynamic time was studied to ensure that all analytes in the sample were totally eluted from the extraction cell and was determined to maximally remove the spiked nitrosamines from the frankfurter sample. In this experiment, the time that fresh extractant was passed continuously through the extraction cell is called the dynamic extraction time. Volume of pure water flowing through the extraction cell depended on the dynamic time interval. It must be sufficient to remove the analytes but not to give a dilution effect. The effect of dynamic time was examined by changing the collecting vial every 5 min for 25 min or 5 fractions, while the temperature, static time and flow rate were kept at 120^{o}C, 5 min and 1 mL min^{-1}, respectively. The other liquid-liquid and solid-phase extraction parameters were at their optimal values as previously observed. Quantitative determination of the extracted analytes for each vial was achieved by using GC-FID. The peak area ratios of each extracted component to the internal standard for every 5 mL of collected extract were reported in Table 11 and plotted a graph as shown in Figure 18.

Table 11 Peak area ratio of each analyte to its internal standard for every 5 mL of aqueous extract.

Compound	t_R(min)	Area ratio of analyte to internal standard [1]				
		Number of collection				
		First, 5 mL	Second, 5 mL	Thrid, 5 mL	Fourth, 5 mL	Fifth, 5 mL
NDEA	5.624	0.5484	0.0404	0.0000	0.0000	0.0000
NPYR	11.445	0.3875	0.0000	0.0000	0.0000	0.0000
NPIP	13.701	0.5903	0.0306	0.0000	0.0000	0.0000

[1] n = 3

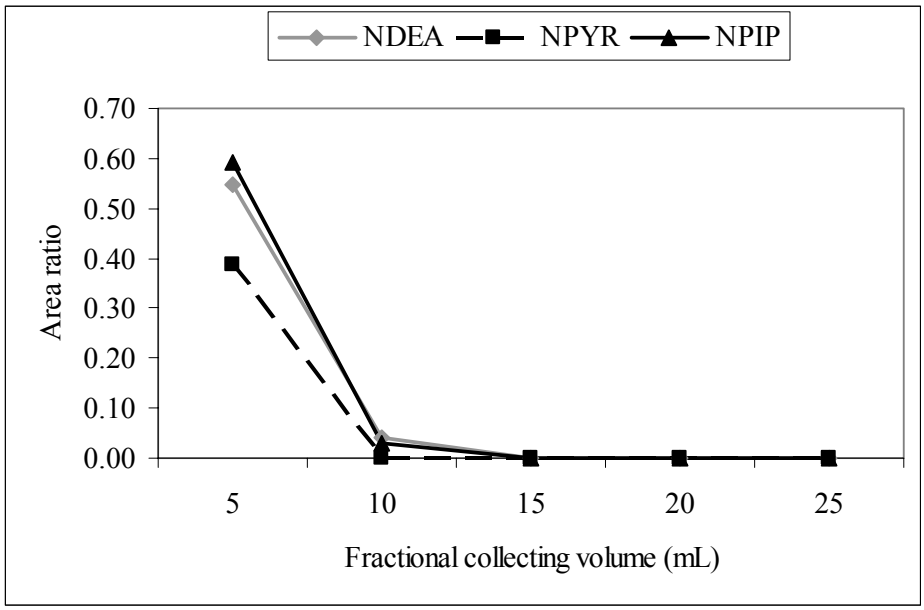


Figure 18 Relationship between fractional collecting volume against the peak area ratio for each nitrosamine.

The extraction time had a significant effect to the yield of analytes. From Table 11 and Figure 18, it was obviously shown that at the dynamic extraction period of 5 min, all nitrosamines were rapidly eluted, while at 10 min, low amount of NPIP and NDEA were extracted and NPYR could not be detected. NPIP and NDEA were completely extracted in 10 min, whereas NPYR was in 5 min. So the dynamic time of 10 min or extraction volume of 10 mL was sufficient to simultaneous remove all the nitrosamine compounds from frankfurter.

The result of elution volume was also confirmed by continuously collecting the analytes at difference volume at a constant flow rate of 1 mL min^{-1} without changing a vial. The area ratio for each nitrosamine compound with increased dynamic time between 5-20 min were given in Table 12 and Figure 19.

Table 12 Effect of dynamic time for extraction on the product yields of nitrosamine.

Compound	t_R(min)	Area ratio of analyte to internal standard [1]			
		Dynamic time (min)			
		5	10	15	20
NDEA	5.626	0.5344	0.5564	0.5211	0.4687
NPYR	11.457	0.3832	0.3854	0.3058	0.2510
NPIP	13.713	0.5798	0.6141	0.5639	0.5101

[1] n = 3

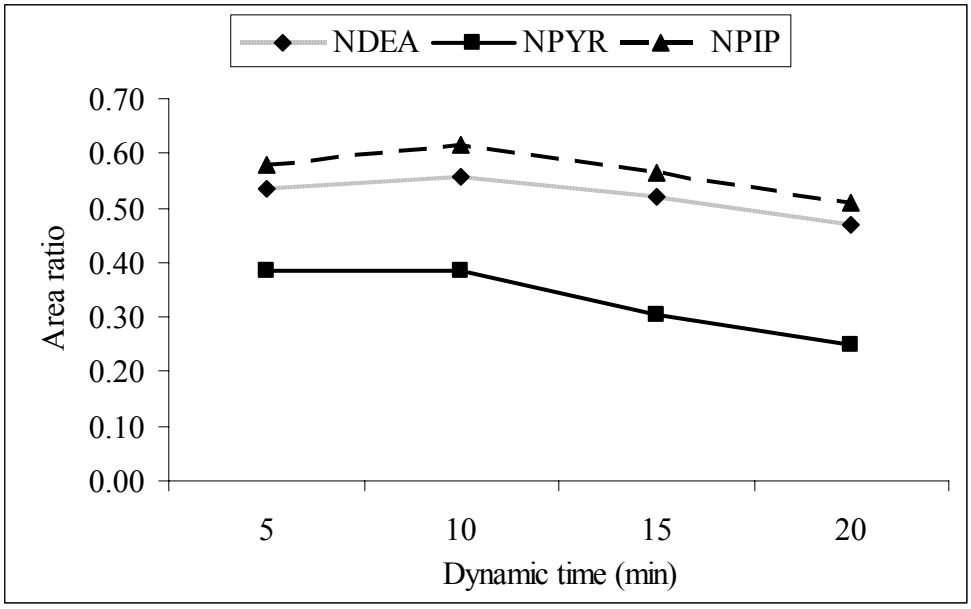


Figure 19 Graphs of relationship between dynamic extraction times against the peak area ratio for all nitrosamine compounds.

It can be seen in Figure 19 that the highest yields of all analytes were extracted in a high quantity at the 10 min and decreased with prolonging the time. The yield was decreased with longer dynamic time (10-20 min).

Since the water solubility of nitrosamine is high, increasing the dynamic time means increasing the volume of water extractant to help the elution. When increasing the dynamic extraction time to 20 min, while keeping the flow constantly at 1 mL min^{-1}, the total volume of the aqueous extract collected in the vial was equal to 20 mL. The increasing volume resulted the concentration of analyte to be more diluted, as well as promoting nitrosamine to be in the aqueous more than organic layer. The more volume of aqueous solution, the lower yield of nitrosamines, especially for NPYR since water solubility of NPYR was higher (1,000 g L^{-1}) than other nitrosamines. Therefore an opportunity of NPYR was partitioned into dichloromethane layer was low, resulting low peak area ratio. From overall results, in order to avoid the dilution problem and reduce extraction time, the dynamic time of 10 min or the volume of aqueous extractant only 10 mL which gave maximum yield was performed through out the following experiments.

Some attributes of an ideal extraction method are short extraction time, low energy requirement and low toxicity solvent consumption. The experiment was demonstrated that a dynamic mode of SWE could not only minimize extraction volume of extractant but also perform faster extraction than a standard extraction method of nitrosamines from frankfurter, namely distillation.

3. Full factorial design for optimising SWE condition

Regarding determination of interactions between parameters, finding the most suitable experimental conditions, and minimizing a number of experiment, some studies have recently been reported the use of "design of experiment (DOE)" (Martinez *et al*., 2005, Cárdenes *et al.*, 2002, Andrade *et al*., 2005 and Reche *et al*., 2002). A full factorial design is one of DOE that allows a simultaneous study of the effects that several factors may have on an output response. When performing an experiment, varying levels of factors simultaneously rather than one at a time is more efficient in terms of time and cost. It also allows a study of interactions between those factors, thus possible to detect the influencing factors while the number of trials can be kept to a minimum.

3.1 Design of experiment of nitrosamine extraction

Before starting the DOE, a model containing factors and their levels must be set up. Since the boiling point of NDEA are 177^{o}C, lower than the other nitrosamine compounds. In order to simultaneous extract all nitrosamines, the hightest temperature of this model was set at 170 ^{o}C, just below its boiling point. According to the instrumental limitation, the lowest temperature of the oven that could be set was 120 ^{o}C, therefore the lowest temperature of this model was 120 ^{o}C. The extraction temperature, which was the most important factor, was then varied from 120-170 ^{o}C. The other factor was a static time, which was defined as a period in which the temperature was hold before restarting the flow for a dynamic time. For a long static time and high temperature, e.g. 15 min at 170 ^{o}C, the lipid and pigment were more co-extracted and plugged the SPE column. So the static time was carried out at 1, 5 and 10 min. Since an effective flow rate for a general dynamic SWE is usually in a range of 0.5–2.0 mL min^{-1} for most applications, in this study it was carried out at 0.5 and 1.0 mL min^{-1}. A higher flow rate than 1.0 mL min^{-1} was not performed because excessively high pressure occurred and it sometimes caused the system leak.

In a full factorial experiment, responses were measured at all combinations of the experimental factor levels. The investigated factors were extraction temperature with 3 levels of 120, 140, and 170 ^{o}C; static time with 3 levels of 1, 5 and 10 min; and flow rate with 2 levels of 0.5 and 1.0 mL min^{-1}. Three replicates were run in order to estimate the standard error of the design. All in all, the three-factor design required 54 experiments, which were run in a randomized manner. Table 13 lists the design matrix as well as the values given to each factor and the response was expressed as % nitrosamine recovery for each run.

Table 13 A comparison between % recovery of all nitrosamine compounds for each batch experiment designed by full factorial design.

Run order	Factor			% Recovery (%RSD)		
	Temperature	Static time	Flow rate	NDEA	NPYR	NPIP
1	140	1	1.0	72.51 (3.9)	68.69 (3.8)	76.04 (3.7)
2	120	1	0.5	67.55 (4.6)	56.47 (3.4)	63.09 (4.0)
3	170	5	0.5	68.99 (4.2)	60.13 (5.2)	70.85 (4.3)
4	170	10	0.5	59.94 (4.8)	53.68 (4.6)	57.64 (3.8)
5	170	1	1.0	71.56 (4.1)	58.12 (4.1)	67.43 (3.7)
6	120	10	0.5	68.64 (4.4)	61.05 (3.7)	69.15 (2.7)
7	170	5	1.0	66.98 (4.7)	57.86 (4.3)	67.22 (3.0)
8	120	1	1.0	75.44 (3.6)	64.90 (3.6)	74.54 (3.0)
9	120	5	0.5	70.94 (3.4)	60.51 (3.9)	67.84 (2.8)
10	120	5	1.0	87.24 (4.2)	67.86 (4.7)	85.36 (3.3)
11	140	1	0.5	69.59 (4.1)	56.84 (3.7)	66.36 (4.4)
12	120	1	1.0	70.21 (3.6)	60.96 (3.6)	70.65 (3.0)
13	140	1	0.5	74.21 (4.1)	59.66 (3.7)	67.26 (4.4)
14	140	10	0.5	89.07 (4.6)	81.64 (3.1)	92.98 (3.0)
15	140	5	1.0	96.54 (2.6)	84.98 (3.7)	98.65 (3.0)
16	120	10	1.0	78.45 (2.9)	61.69 (3.0)	80.93 (3.8)
17	170	5	1.0	70.65 (4.7)	59.72 (4.3)	69.47 (3.0)
18	170	1	1.0	67.69 (4.1)	57.46 (4.1)	64.05 (3.7)
19	120	5	0.5	73.55 (3.4)	62.98 (3.9)	71.25 (2.8)
20	140	1	1.0	78.44 (3.9)	73.95 (3.8)	80.91 (3.7)
21	140	10	0.5	81.64 (4.6)	76.98 (3.1)	87.54 (3.0)
22	120	10	1.0	81.59 (2.9)	62.89 (3.0)	81.67 (3.8)
23	170	1	0.5	63.41 (4.2)	52.94 (4.9)	59.31 (5.2)
24	120	1	0.5	70.03 (4.6)	59.08 (3.4)	66.54 (4.0)
25	170	10	1.0	60.12 (4.0)	56.36 (4.0)	65.99 (4.7)

Table 13 (Continued)

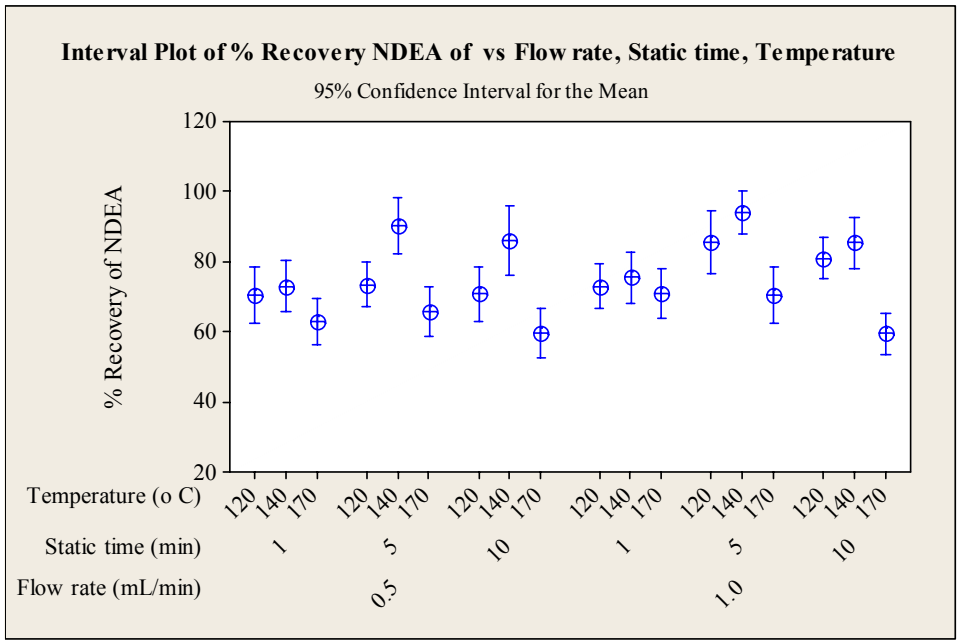
Run order	Factor			% Recovery (%RSD)		
	Temperature	Static time	Flow rate	NDEA	NPYR	NPIP
26	120	5	1.0	81.56 (4.2)	63.07 (4.7)	80.98 (3.3)
27	170	5	1.0	73.53 (4.7)	62.98 (4.3)	71.36 (3.0)
28	140	5	0.5	93.45 (3.6)	84.36 (4.2)	92.65 (3.8)
29	120	1	0.5	74.05 (4.6)	60.45 (3.4)	68.25 (4.0)
30	170	5	0.5	64.01 (4.2)	60.07 (5.2)	70.66 (4.3)
31	170	10	1.0	56.85 (4.0)	52.13 (4.0)	60.21 (4.7)
32	140	1	1.0	75.66 (3.9)	70.57 (3.8)	81.28 (3.7)
33	170	1	0.5	65.35 (4.2)	53.87 (4.9)	63.04 (5.2)
34	120	10	0.5	74.35 (4.4)	64.57 (3.7)	72.34 (2.7)
35	120	10	1.0	83.01 (2.9)	65.46 (3.0)	86.66 (3.8)
36	120	10	0.5	69.28 (4.4)	60.29 (3.7)	69.07 (2.7)
37	140	1	0.5	75.19 (4.1)	61.20 (3.7)	72.02 (4.4)
38	120	1	1.0	73.36 (3.6)	61.08 (3.6)	71.05 (3.0)
39	170	10	1.0	61.45 (4.0)	53.50 (4.0)	64.46 (4.7)
40	140	10	0.5	87.88 (4.6)	80.71 (3.1)	90.67 (3.0)
41	140	5	1.0	91.69 (2.6)	79.95 (3.7)	93.45 (3.0)
42	120	5	0.5	75.94 (3.4)	65.47 (3.9)	71.22 (2.8)
43	120	5	1.0	88.22 (4.2)	68.96 (4.7)	86.02 (3.3)
44	140	10	1.0	82.55 (3.4)	77.33 (3.6)	88.54 (4.2)
45	170	1	1.0	73.33 (4.1)	61.91 (4.1)	68.92 (3.7)
46	140	5	1.0	93.35 (2.6)	85.53 (3.7)	98.10 (3.0)
47	170	5	0.5	64.35 (4.2)	54.82 (5.2)	65.65 (4.3)
48	140	5	0.5	90.12 (3.6)	82.23 (4.2)	89.90 (3.8)
49	170	1	0.5	60.07 (4.2)	49.05 (4.9)	56.83 (5.2)

Table 13 (Continued)

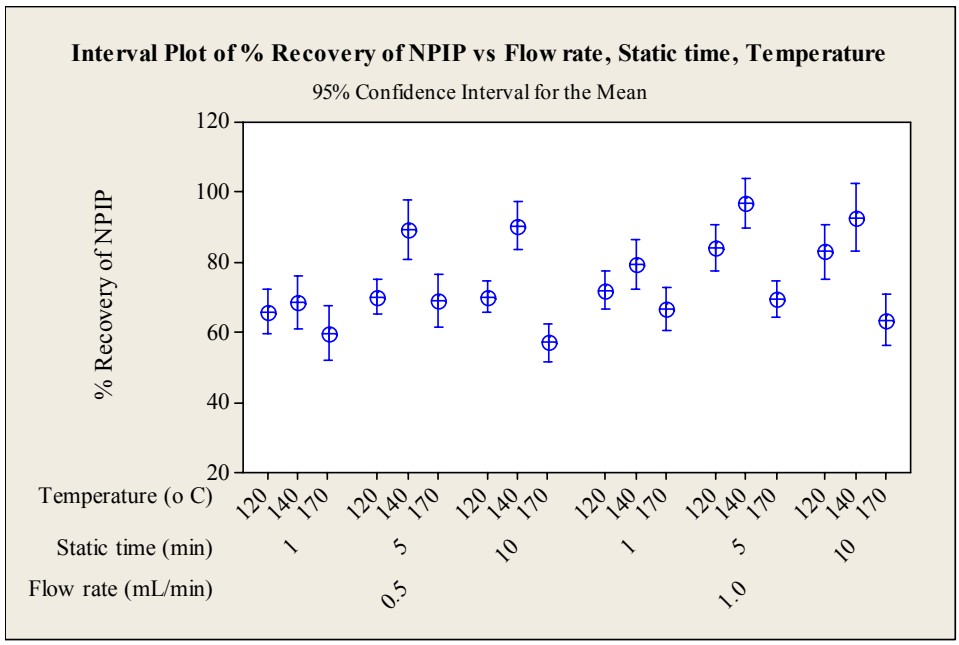
Run order	Factor			% Recovery (%RSD)		
	Temperature	Static time	Flow rate	NDEA	NPYR	NPIP
50	170	10	0.5	62.47 (4.8)	53.64 (4.6)	59.10 (3.8)
51	140	5	0.5	86.91 (3.6)	77.70 (4.2)	85.88 (3.8)
52	170	10	0.5	56.80 (4.8)	49.46 (4.6)	54.78 (3.8)
53	140	10	1.0	88.41 (3.4)	83.03 (3.6)	96.16 (4.2)
54	140	10	1.0	85.26 (3.4)	80.28 (3.6)	93.84 (4.2)

From Table 13, it was noticed that the highest recovery for all extracted components and for every experiment did not exceed 100%. Maximum recoveries for NDEA, NPYR and NPIP were found for the experiment number of 15, 46 and 15, respectively, which was related to the extraction temperature of 140 ^{o}C, 5 min static time, and flow rate of 1.0 mL min^{-1}. It can be seen that high recoveries obtained for NDEA, NPYR and NPIP were 96.54%, 85.53% and 98.65%, respectively.

Figure 20 shows the interval plot of % recovery of nitrosamine compounds versus flow rate, static time and temperature obtained from three replicates at each value of all factors. An interval plot was used to assess and compare means and confidence intervals. The confidence interval was a range of values which was likely to include the true value. The extreme value of the range was called the confidence limits. For example, the mean for % recovery of NDEA at an extraction temperature of 120 ^{o}C, 1 min static time, and a flow rate of 0.5 mL min^{-1} was 70.54% and the 95% confidence interval extends from 62.39 to 78.69%. From the figure, rather good repeatability and precision were obtained, owing to the proximity of three replicate results. However the suitable method for comparing means was available with ANOVA.

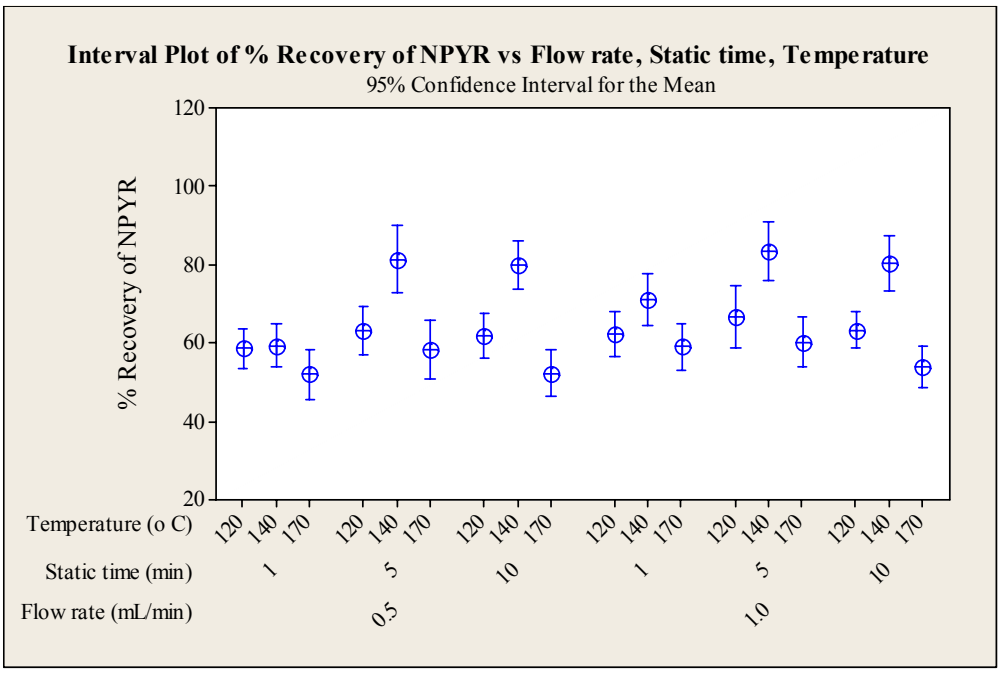


A



B

Figure 20 Interval plot of % recovery of nitrosamine compounds versus flow rate, static time and temperature. A: NDEA, B: NPIP, C: NPYR



C

Figure 20 (Continued)

3.2 Analysis of Variance (ANOVA) of the full factorial design

The results obtained from the factorial design were analyzed by using three-way ANOVA. This test was used to detect whether there were any statistically significant differences between the means of these levels of factor. In addition, ANOVA can be used to test significance of main effects and their interactions. The results of the ANOVA produced by MINITAB software are shown in Table 14. The ANOVA is an exact test of a null hypothesis of no difference in level means, or in this work no significant effect of temperature, static time and flow rate and its interaction to the recovery of each nitrosamine. In this study, the Fisher's statistical test (F-test) was used on the basis of statistical method to compare between each data. In general, if the calculated F-value was higher than the critical F value from the table, the null hypothesis was rejected, meaning that the main or interaction effect was significant or the factor did give a significant difference in the mean of recovery.

Table 14 Three way analysis of variance (3-way ANOVA) for analyzing three factors: flow rate, static time and temperature. A: NDEA, B: NPIP, C: NPYR

A: Analysis of variance for NDEA recovery

Source	Degree of freedom	Sum of squares	Mean square	F	Significance (P)
Temperature	2	3325.00	1662.50	188.45	0.000
Static time	2	747.90	373.95	42.39	0.000
Flow rate	1	302.79	302.79	34.32	0.000
Temperature*Static time	4	660.83	165.21	18.73	0.000
Temperature*Flow rate	2	97.59	48.80	5.53	0.008
Static time*Flow rate	2	33.14	16.57	1.88	0.168
Temperature*Static time*Flow rate	4	113.65	28.41	3.22	0.023
Error	36	317.59	8.82		
Total	53	5598.49			

B: Analysis of variance for recovery of NPIP

Source	Degree of freedom	Sum of squares	Mean square	F	Significance (P)
Temperature	2	4351.72	2175.86	281.60	0.000
Static time	2	1143.93	571.97	74.03	0.000
Flow rate	1	756.15	756.15	97.86	0.000
Temperature*Static time	4	705.71	176.43	22.83	0.000
Temperature*Flow rate	2	95.65	47.83	6.19	0.005
Static time*Flow rate	2	1.94	0.97	0.13	0.883
Temperature*Static time*Flow rate	4	148.00	37.00	4.79	0.003
Error	36	278.16	7.73		
Total	53	7481.27			

Table 14 (Continued)

C: Analysis of variance for recovery of NPYR

Source	Degree of freedom	Sum of squares	Mean square	F	Significance (P)
Temperature	2	3687.32	1843.66	276.38	0.000
Static time	2	646.51	323.25	48.46	0.000
Flow rate	1	190.29	190.29	28.53	0.000
Temperature*Static time	4	575.80	143.95	21.58	0.000
Temperature*Flow rate	2	8.18	4.09	0.61	0.547
Static time*Flow rate	2	101.98	50.99	7.64	0.002
Temperature*Static time*Flow rate	4	46.65	11.66	1.75	0.161
Error	36	240.15	6.67		
Total	53	5496.88			

In three way ANOVA test, there are three main effects, three two-way interactions, and one three-way interaction. A degree of freedom for a main effect equals to the number of levels of the factor minus one. An interaction degree of freedom is always equal to a multiplied product of two or more degrees of freedom of the component parts. Degree of freedom of the error is equal to the number of observations minus the number of groups which was clarified as the number of experiment without replicates. In this work the number of group was 18, and the replicates was 3, the number of observations was 18 x 3 equal to 54. The degree of freedom was therefore 54-18 equal to 36. The F ratio is computed by dividing the mean square (MS) of the factor by the MS of the error.

From Table 14, all of three main effects had an important influence in nitrosamine extraction, this can be noticed from the high F-values of all main factors. The critical F-values from a table of temperature, static time and flow rate factor for 5% level of confidence were 3.266, 3.266 and 4.116, respectively. Since the

calculated F-values of temperature, static time and flow rate for all compounds were much higher than critical F-values, meaning that the null hypothesis would be rejected at the 5% level of significance. For example, the calculated F-value of temperature for NDEA was 188.45, which was much higher than the critical F-value, 3.266. Temperature therefore played an important role to the extraction. The higher calculated F-value than the critical value, the more significant the factor is. So temperature, static time and flow rate gave highly significant effects to % recovery of all nitrosamine compounds. The extraction temperature obtained the highest F-value of all nitrosamines, so this factor was the most important in improving extraction efficiency. When comparing between the calculated F value of static time and flow rate among the three compounds, static time affected the SWE more than the flow rate for NDEA and NPYR. Meanwhile, the flow rate was more important than the static time for NPIP extraction.

In this example we were able to reject the null hypothesis, even at the 0.05 significance level. The obtained p value was 0.000, which was extremely low and therefore the null hypothesis was rejected. The lower P-value, the more reliable result obtained and the more influence of the factor to the experiment. There was very strong evidence to suggest that the means of recovery at different levels or conditions were not all equal. Therefore, the main factor (temperature, static time and flow rate) accounted for a significant amount of variation in the response variable.

A statistical analysis of results was performed considering all three possible interactions, namely temperature*flow rate, temperature*static time, and static time*flow rate. From the calculated F-values and P-values of temperature*static time effect in Table 14A-C, it was deduced that a combination between the temperature and static time gave rise a high effect to all nitrosamines. These were noticed from the higher calculated F-values of NDEA(18.73), NPYR(22.83) and NPIP(21.58) than the critical value (3.266) and a very low P-value of 0.000 for all compounds.

Temperature*flow rate interaction had an important influence on only NDEA and NPIP extraction, but not for NPYR, since the p-values of both NDEA and NPIP extraction was lower than the acceptable P-value (0.05). The interaction was not significant for NPYR experiments, since its P-value was approximately ten times higher than the critical value.

No significant evidence for a static time*flow rate interaction effect was given for NDEA and NPIP because their calculated F-values, 1.88 for NDEA and 0.13 for NPIP, were lower than the critical F-value (3.266), and the calculated P-values, 0.168 for NDEA and 0.883 for NPIP, were higher than the acceptable P-value (0.05) at 95% significant level. So the recoveries of both NDEA and NPIP did not depend upon the combination of static time and flow rate interaction, in contrast with that for NPYR.

When considering the three-factor interaction, temperature*static time*Flow rate, it showed a p-value of 0.023, 0.003 and 0.161 for NDEA, NPIP and NPYR, respectively. Since the critical value for the interaction was 0.05, this indicated that the SWE of NDEA and NPIP depended on overall combination of temperature, static time and flow rate, but no such effect was significant for NPYR extraction. Or in other word, temperature*static time*flow rate interaction could not improve the extraction efficiency of NPYR from frankfurters.

The effect of those factors and their interactions to the extraction of each nitrosamine by using superheated water could be summarized in Table 15.

Table 15 Main effects and their interaction effects to the recovery of nitrosamine.

Source	Compounds*		
	NDEA	NPIP	NPYR
Main effect			
Temperature	+++	+++	+++
Static time	++	+	++
Flow rate	+	++	+
Two-way interaction			
Temperature*Static time	++	++	++
Temperature*Flow rate	+	+	-
Static time*Flow rate	-	-	+
Three-way interaction			
Temperature*Static time*Flow rate	+	+	-

* +++ highly significant + low significant
++ significant - insignificant

Table 15 shows significance of main effect and interaction effects to the mean recovery of each nitrosamines. From the table, it was concluded that the three main effects were significant. NDEA and NPYR presented similar tendencies versus the three main factors. Meanwhile, the interaction effects to NDEA and NPIP recovery were similar but that for NPYR recovery was different.

3.3 Analysis of factor effects in factorial design

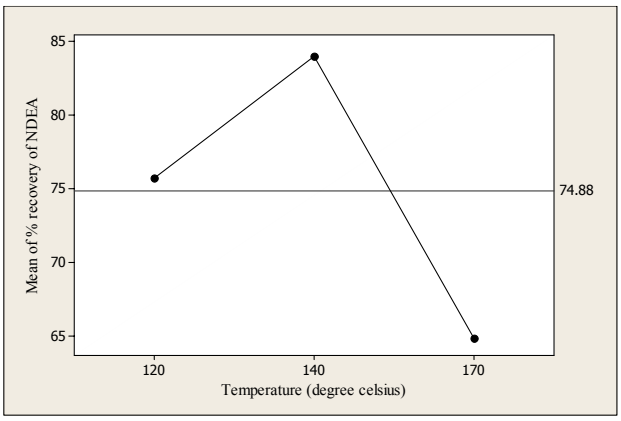
3.3.1 Main effects

In this study, main effect plot was used to visualize effect of the factors to the response (% recovery). The response means for each level was plotted, then drew a connection line between the data points. A reference line was drawn at the overall (grand) mean. A main effect plot should only be viewed the plots for

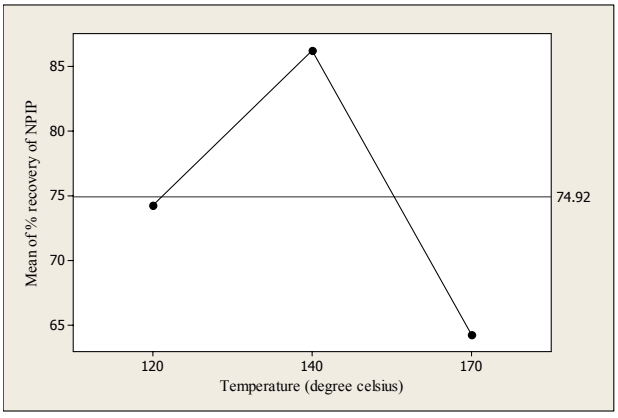
significant factors according to the effects and coefficient table from ANOVA test. The main effect was present when a change in a mean recovery across the levels of a factor was significant. In this experiment, the significant factors are temperature, flow rate and dynamic time.

3.3.1.1 Effect of extraction temperature

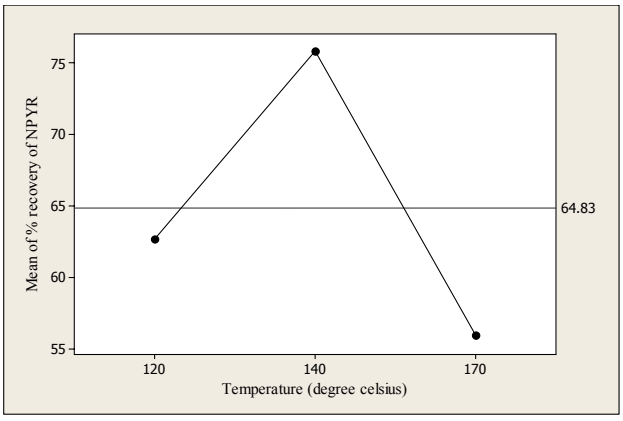
When giving a rank to those factors, it was found that temperature was the most significant factor. Figure 21 show temperature effect plots for % mean recovery of nitrosamine compounds. It was found that all nitrosamines, namely NDEA, NPIP and NPYR, had similar tendencies of temperature factor. The figures showed that the yields were significantly depended on the range of studied temperature. When the temperature was raised, the recoveries of all nitrosamines were increased to maximum at 140 °C then slightly decreased after the temperature 140 °C. For example, the mean recovery was improved from 75.75% at 120°C to 83.97% at 140°C for NDEA. From the results obtained, the best efficiency was found at 140°C, obtaining the optimized temperature for all nitrosamines. The yield increased as a result of a decrease in water dielectric constant to be close to those of the extracted compounds. The polarity of water was sufficiently reduced at 140 °C (ε= 46, Appendix C), which was comparable to that of a mixture of 45 % methanol-water (see Appendix F). In addition, higher water temperature caused higher diffusion and desorption rate of the analytes from the matrix, while high pressure allowed sample wetting and better penetration of the water extractant, thus increasing the SWE efficiency. On the other hand, when the temperature was increased to 170 °C, it appeared strong smell and lipid co-extracted and plugged the filter and the yield of analytes decreased. For example, the mean recovery was decreased from 83.97% at 140°C to 64.86% at 170°C for NDEA. At the highest temperature, the more co-extracted lipid might hinder the nitrosamines removal from the SPE column during a cleaning up step. Again, the excessive heat could promote the nitrosamines to be co-vaporized with high temperature water at the end of tubing, provided that the outlet was not sufficiently cooled. Therefore, the yield was lower.



NDEA



NPIP



NPYR

Figure 21 Temperature effect for % recovery of nitrosamines.

3.3.1.2 Effect of static time

In this experiment, a static extraction time was defined as a time period during the extraction cell placed in the oven until the flow began. During the period the water extractant was expected to diffuse thoroughly in the sample and the analytes were solvated and desorbed from the matrix. In the experiment, the static extraction times were performed from 1 to 10 minutes. Figure 22 show the static time effect plot for % mean recovery of nitrosamine compounds. It was found that all nitrosamines, namely NDEA, NPIP and NPYR present similar tendencies of %recovery versus the static time. The results showed that the static time affected the yield of the extraction. At 5 min, the yields of the extracted compounds were higher than those obtained from 1 min and 10 min, since, 1 min static time was too fast to allow a thermal equilibration between the oven and extraction cell and 10 min gave lower recoveries. Thus, 5 min static time was chosen as optimum.

3.3.1.3 Effect of flow rate

The flow rate of water eluent was investigated in order to optimize between the extraction time and efficiency. It was studied in a range of 0.5-1.0 mL min^{-1} and the final collected volume was 10 mL. However, the flow rate of more than 1.0 mL min^{-1} was not performed because high pressure occurred and caused system leak. Figure 23 show the flow rate effect plot for % mean recovery of nitrosamine compounds. It was found that all nitrosamines, namely NDEA, NPIP and NPYR present similar tendencies of %recovery versus the flow rate. When the flow was altered from 0.5 to 1.0 mL min^{-1}, all components were extracted at higher amount. The flow of 0.5 mL min^{-1} gave too long extraction time and lower recovery. Hence the flow rate of 1 mL min^{-1} was chosen as optimum.

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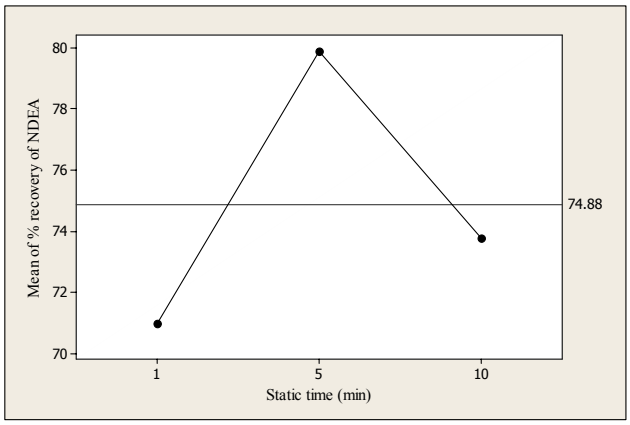
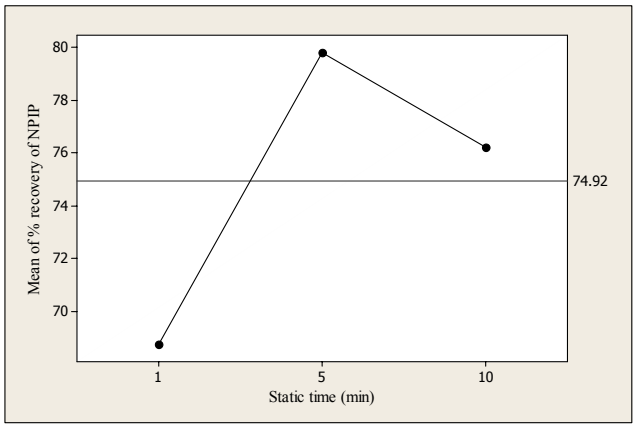
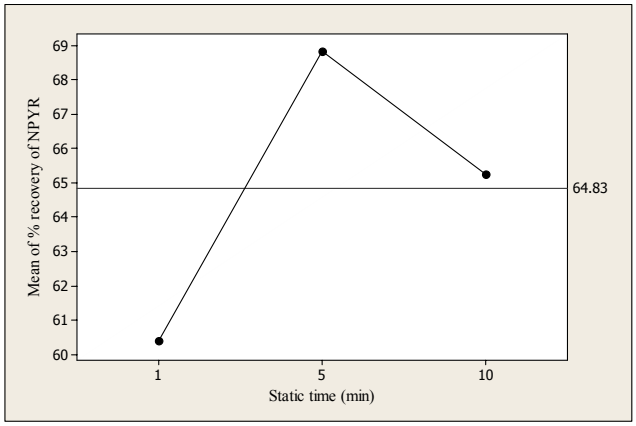


NDEA

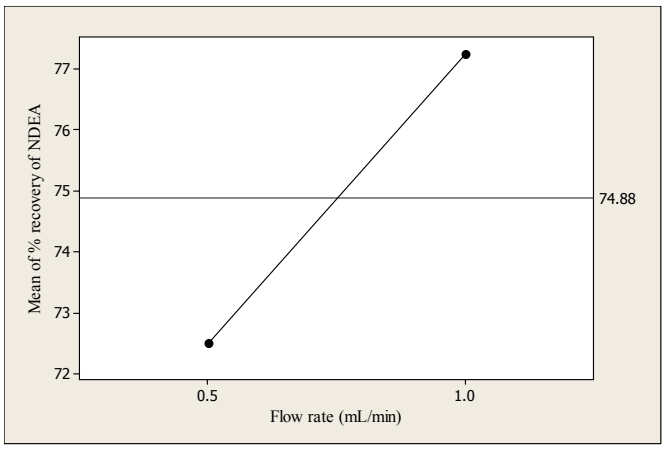


NPIP

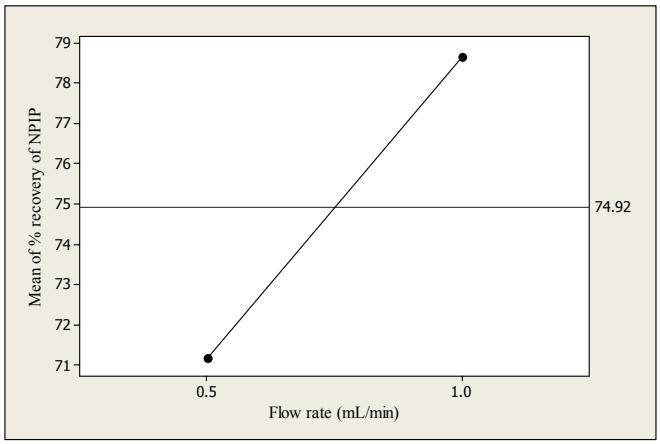


NPYR

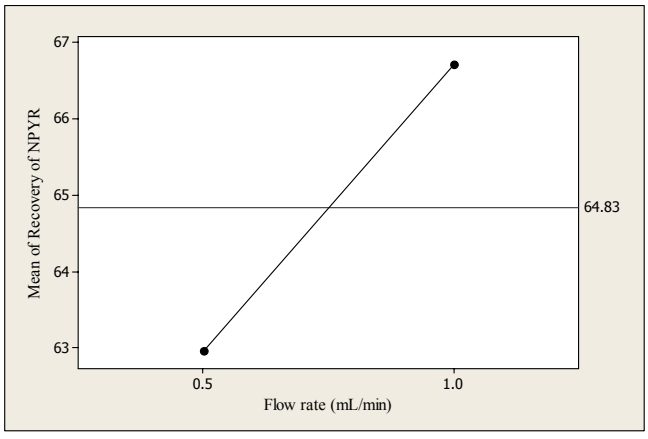
Figure 22 Static time effect for % recovery of nitrosamines.



NDEA



NPIP



NPYR

Figure 23 Flow rate effect for % recovery of nitrosamines.

3.3.2 Interaction effect

Interactions plot was used to visualize the interaction effect of two factors on the response. An interaction effect plot should only be viewed the plot for interactions of significant factors according to the effects and coefficient table from ANOVA test. The interaction is present when a change in the response mean from a low to a high level of a factor depends on the level of a second factor. If the lines were not parallel to each other, there may present an interaction. As the change in the response mean from the low to the high level of a factor depends on the level of a second factor, the greater the degree of departure from being parallel, the stronger the effect. Large interactive effects can make the main effect insignificant, such that it becomes more important to pay attention to the interaction of the involved factors than to investigate them individually (Minitab handbook version 14.20).

3.3.2.1 Temperature*static time interaction effect

From Table 15 the temperature*static time interaction effect was significant for mean recoveries of NDEA, NPYR and NPIP. In Figure 24 it was noticed that the lines of the temperature*static time interaction plot were not parallel to each other so there may be an interaction present. It also showed the greatest degree of departure of those lines from being parallel, so the strongest effect was obtained and this was agreed with the ANOVA test. The highest recovery of all nitrosamine compounds was obtained at temperature of 140^{o}C and static time of 5 min, which corresponded to the main effect plot. As both interaction and main effects gave the same conclusion that the highest recovery was obtained at 140 ^{o}C and 5 min static time, this meant that the interaction effect had no affect to the main effects, or in other words, the interaction effect could not make the main effects insignificant.

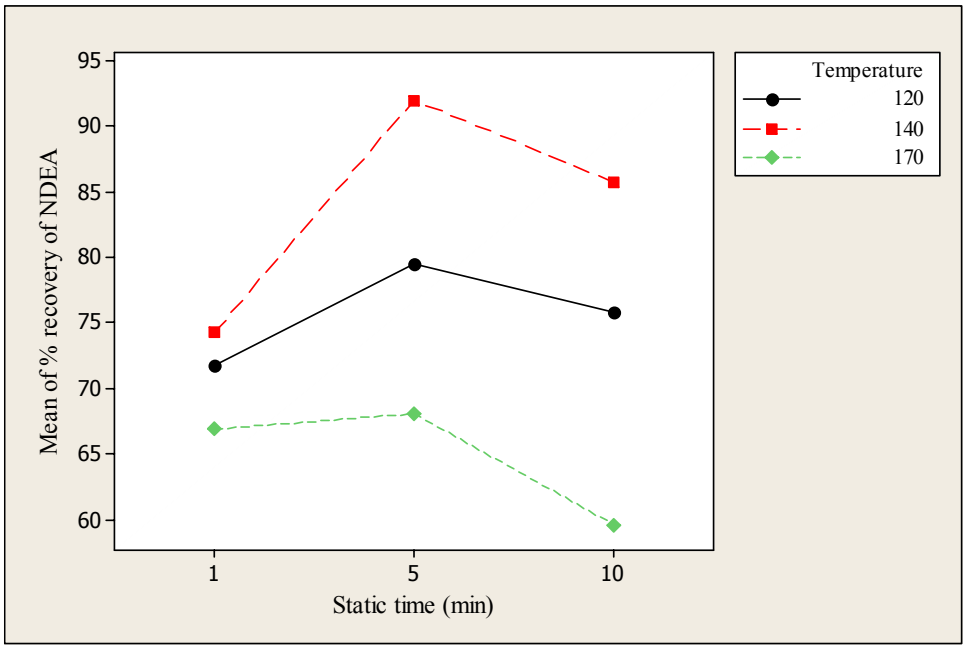
In case that the interaction effect gives different result of temperature and static time, the results obtained from the interaction effect are more critical than those of main effects (Minitab handbook version 14.20).

3.3.2.2 Temperature*flow rate interaction effect

From Table 15 the temperature*flow rate interaction effect was significant for the mean recovery of NDEA and NPIP. In Figure 25 the lines of the temperature*flow rate interaction plot of NDEA and NPIP were not parallel to each other, so the interaction was present. On the other hand, the lines of the temperature*flow rate interaction plot of NPYR were parallel to each other, so no interaction between temperature and flow rate was present. The highest recovery of NDEA, NPIP and NPYR was obtained at temperature of 140^{o}C and flow rate of 1.0 mL min^{-1}, which corresponded to the main effect plot. As the interaction effect had no effect to the main effects, it meaned that interaction effect could not make the main effects insignificant.

3.3.2.3 Static time*flow rate interaction effect

From Table 15 the static time*flow rate interaction effect was significant for only mean recovery of NPYR. In Figure 26, the lines of the static time*flow rate interaction plot of NPYR were not parallel to each other, so there may be the interaction present. On the other hand, parallel lines of the static time*flow rate interaction plot of NPIP were observed and the lines for NDEA were parallel to some degree, meaning that no interaction between static time and flow rate of both compound extractions was presented. The highest recovery of all components was obtained at static time of 5 min and flow rate of 1.0 mL min^{-1}, that was agreed with the main effect plot. As the interaction effect had no effect to the main effects, interaction effect could not make the main effects insignificant.



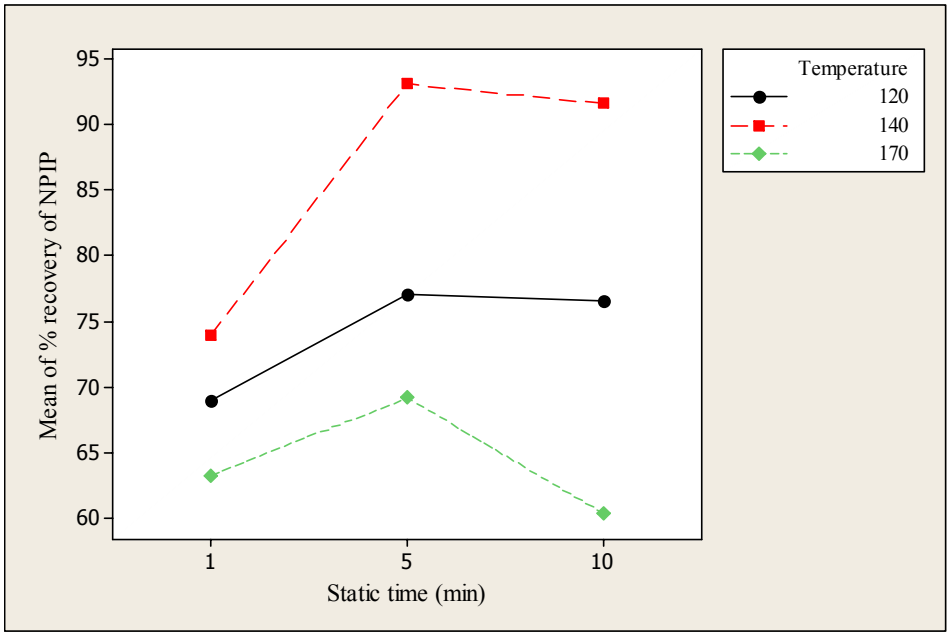


Figure 24 Temperature*static time interaction plot for % recovery of nitrosamines.

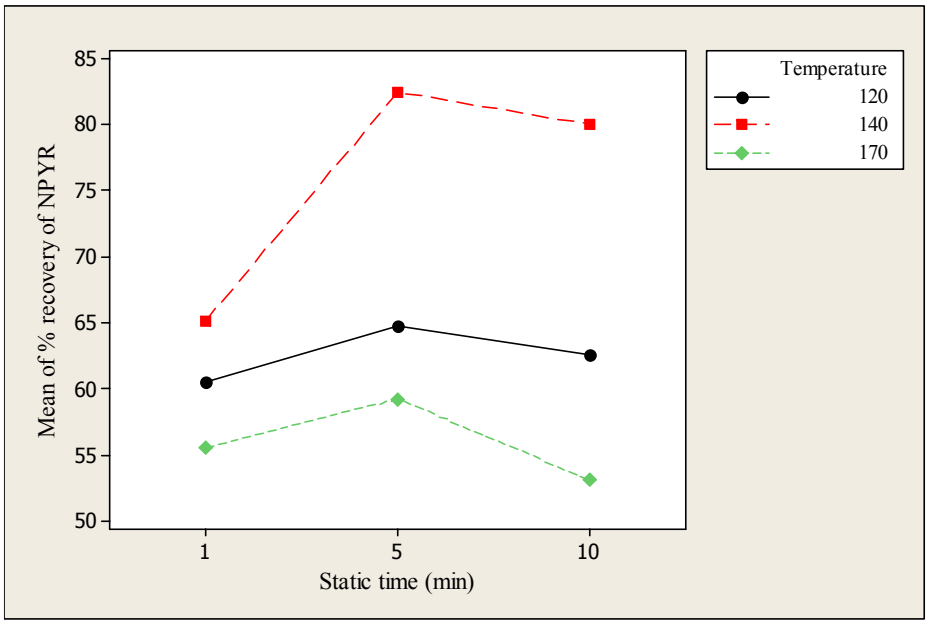


Figure 24 (Continued)

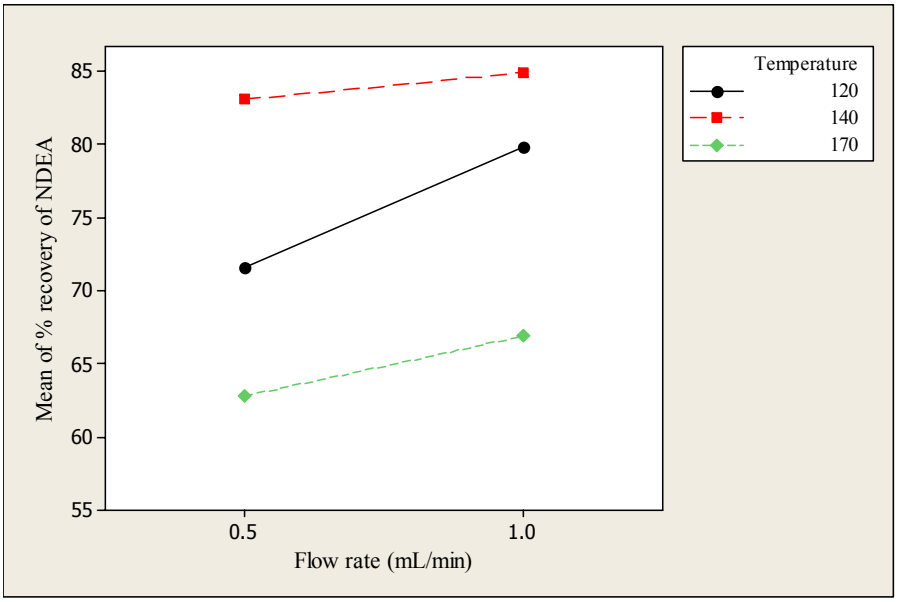
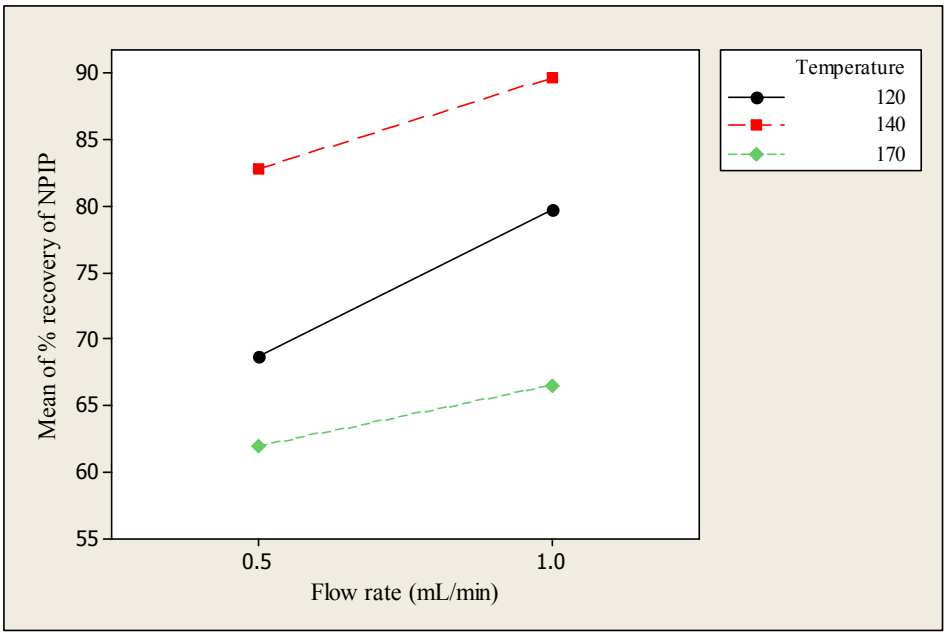


Figure 25 Temperature*flow rate interaction plot for % recovery of nitrosamines.



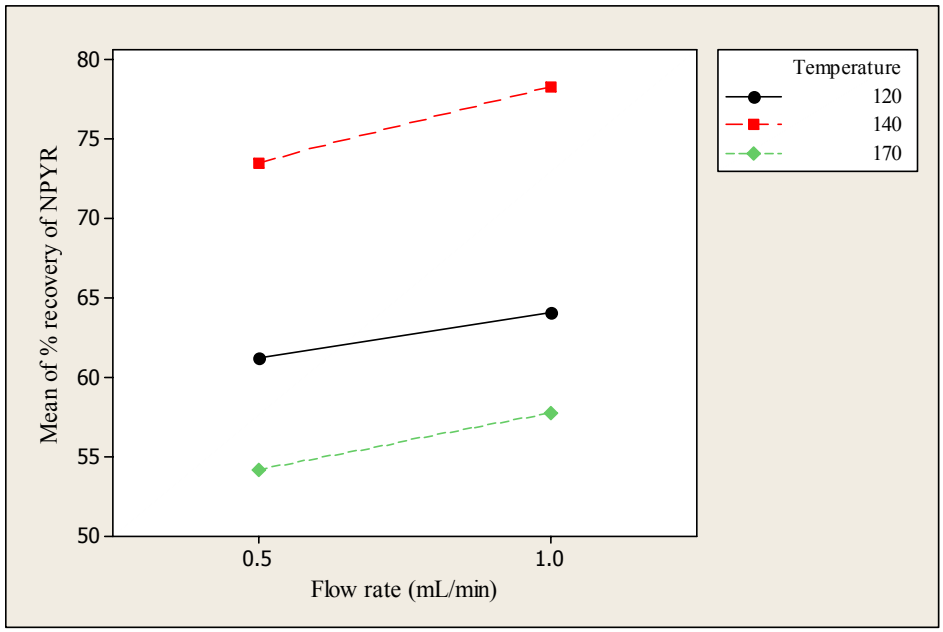


Figure 25 (Continued)

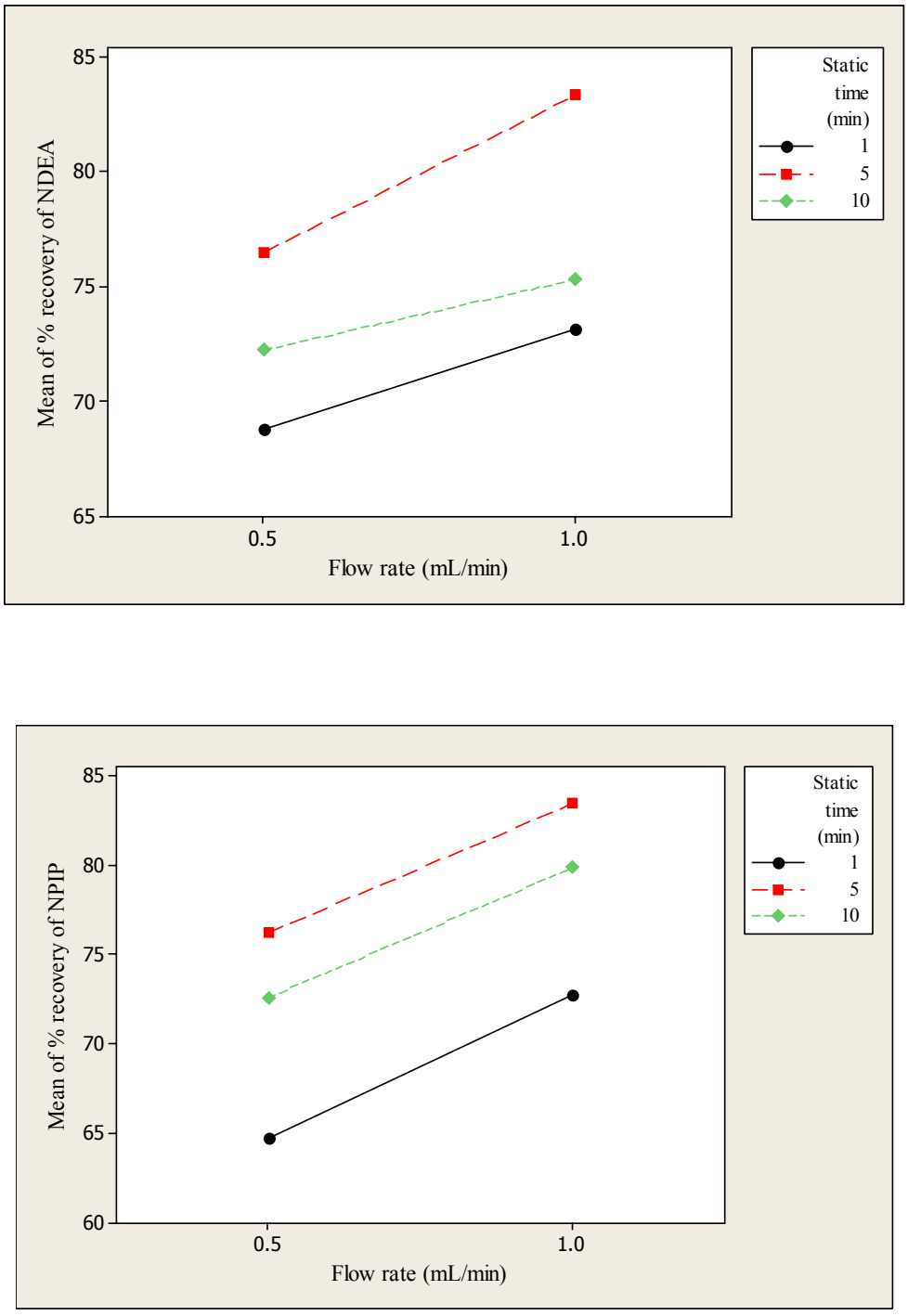


Figure 26 Static time*flow rate interaction plot for % recovery of nitrosamines.

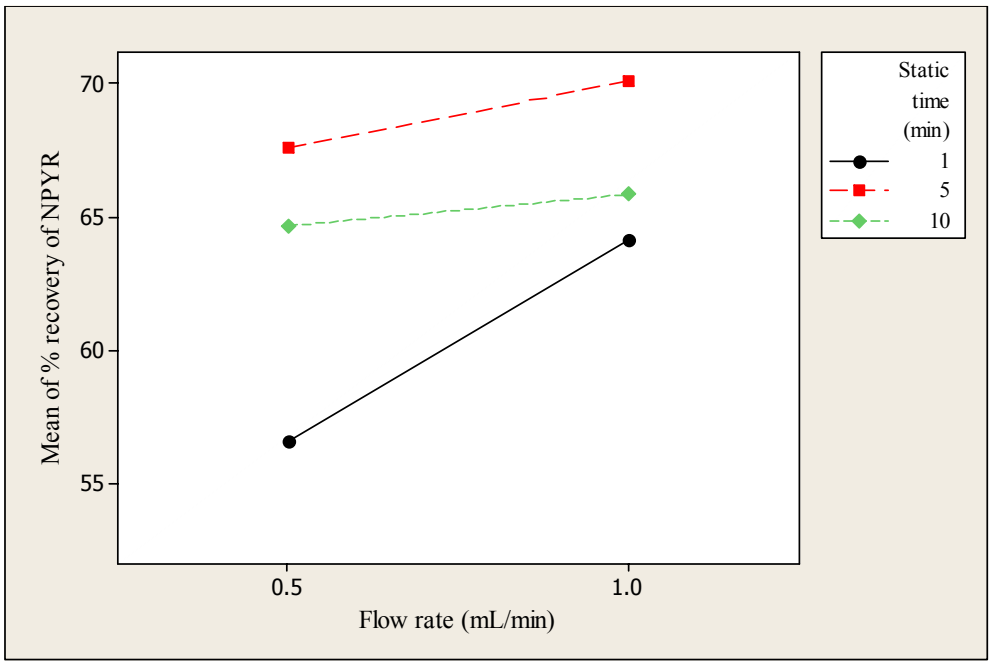


Figure 26 (Continued)

3.3.3 Surface plot

Surface plots show how a response variable (the z-variable) relates to two factors (the x- and y-variables). In this study the response variable was % recovery.

3.3.3.1 Surface plot of temperature and static time interaction

A combination of temperature and static time factors could improve the extraction efficiency of NDEA, NPIP and NPYR. The Figure 27-29 show that the obtained yield depended on both static time and extraction temperature. The surface plots also illustrated that an increase in the yield of NDEA, NPIP and NPYR from 1 to 5 min static time was greater at the temperature of 140 ^{o}C. The optimum extraction condition were 5 min static time and extraction temperature of 140 ^{o}C.

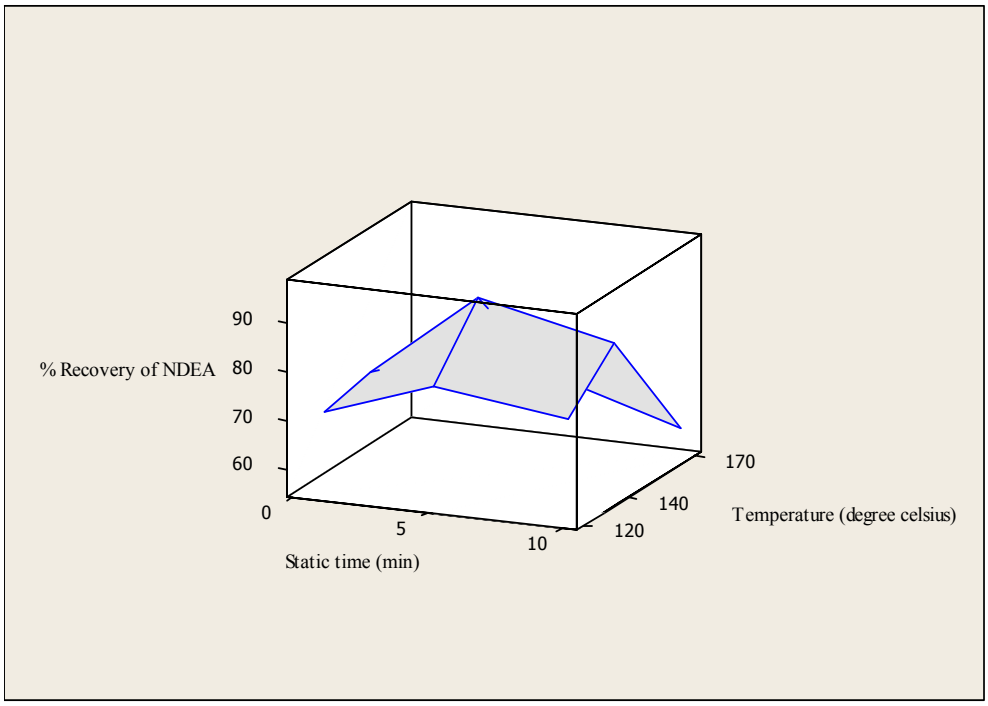


Figure 27 Surface plot of % recovery of NDEA versus temperature and static time.

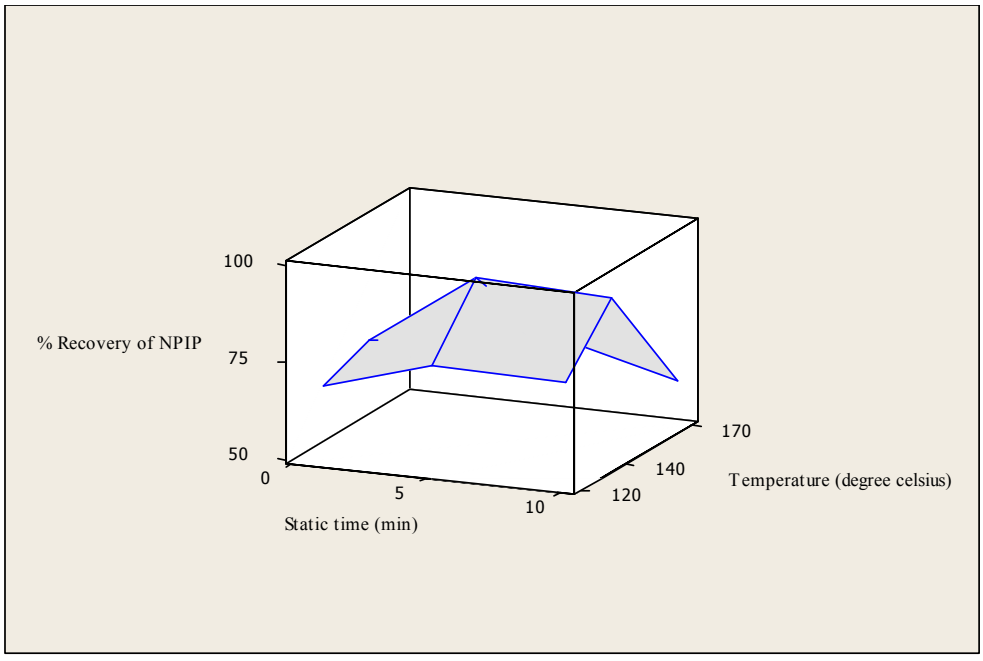


Figure 28 Surface plot of % recovery of NPIP versus temperature and static time.

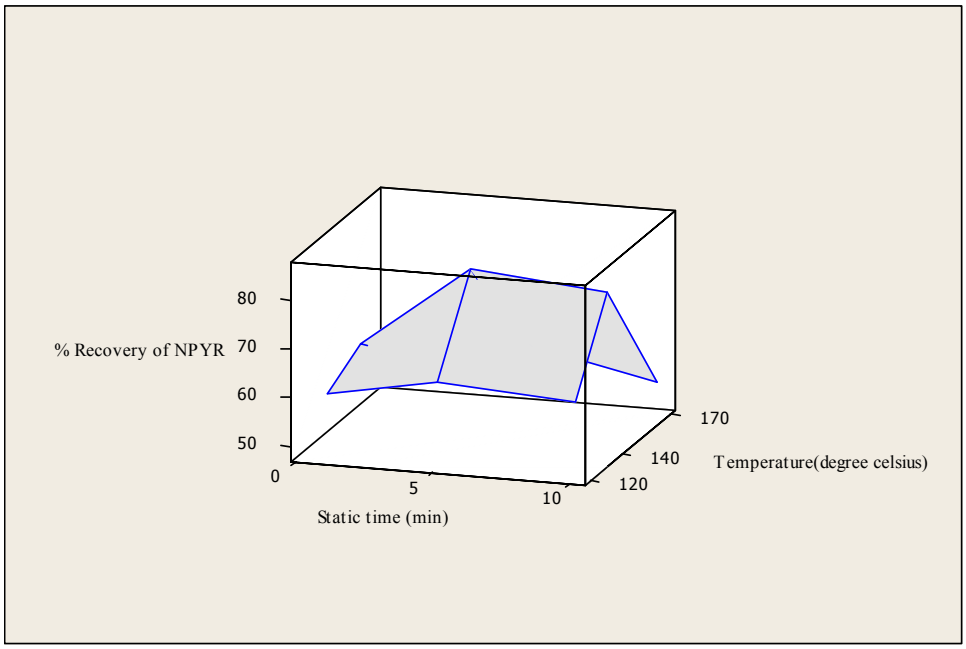


Figure 29 Surface plot of % recovery of NPYR versus temperature and static time.

3.3.3.2 Surface plot of temperature and flow rate interaction

A combination of temperature and flow rate could improve the extraction efficiency of NDEA and NPIP. Figure 30-31 show that the obtained yield depended on both flow rate and extraction temperature and both analytes gave similar trend. The surface plot also shows that the highest yield was obtained when the flow rate was high and the temperature was equal to140 ^{o}C. In addition, from the plot it could be seen the shape of the response surface and got a general idea of yield at various temperature and flow rate. The optimum extraction conditions were the flow rate of 1.0 mL min^{-1} and the extraction temperature of 140 ^{o}C.

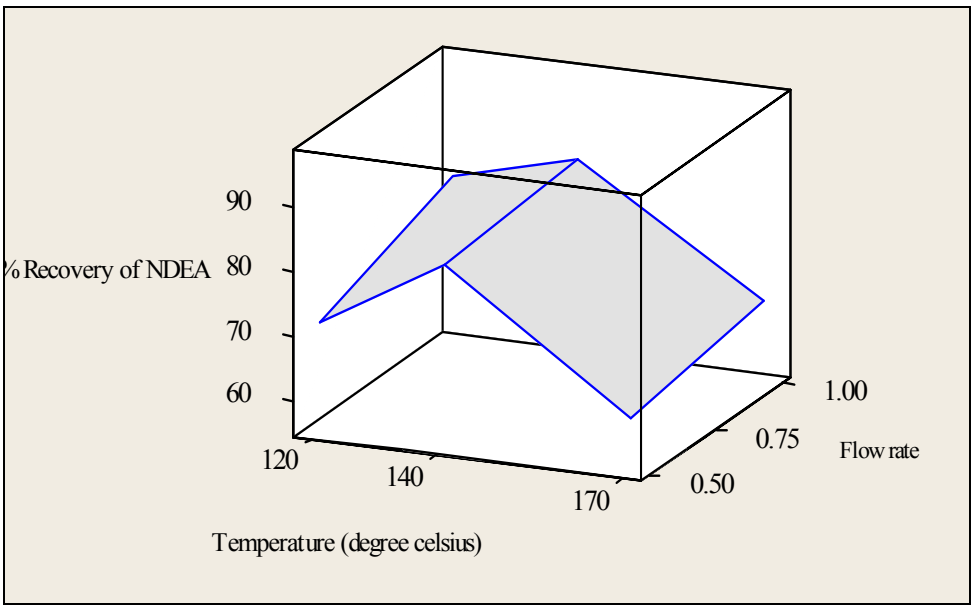


Figure 30 Surface plot of % recovery of NDEA versus temperature and flow rate.

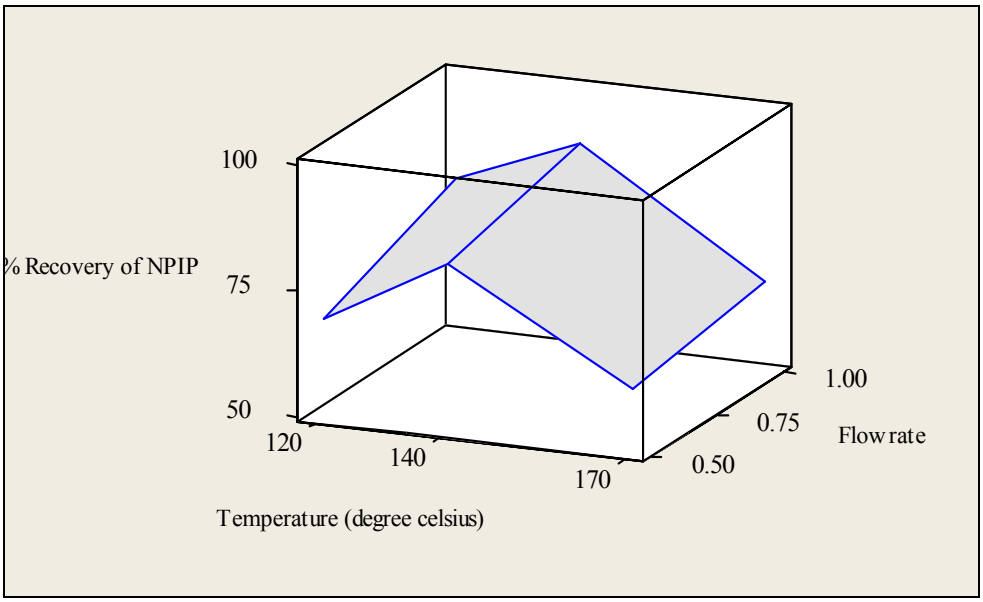


Figure 31 Surface plot of % recovery of NPIP versus temperature and flow rate.

3.3.3.3 Surface plot of static time and flow rate interaction

The combination of static time and flow rate could improve only the extraction efficiency of NPYR. Figure 32 shows that the obtained yield

depended on both static time and flow rate. The surface plot also shows that the highest recovery of NPYR was obtained when the flow rate was 1.0 mL min^{-1} and the static time was equal to 5 min.

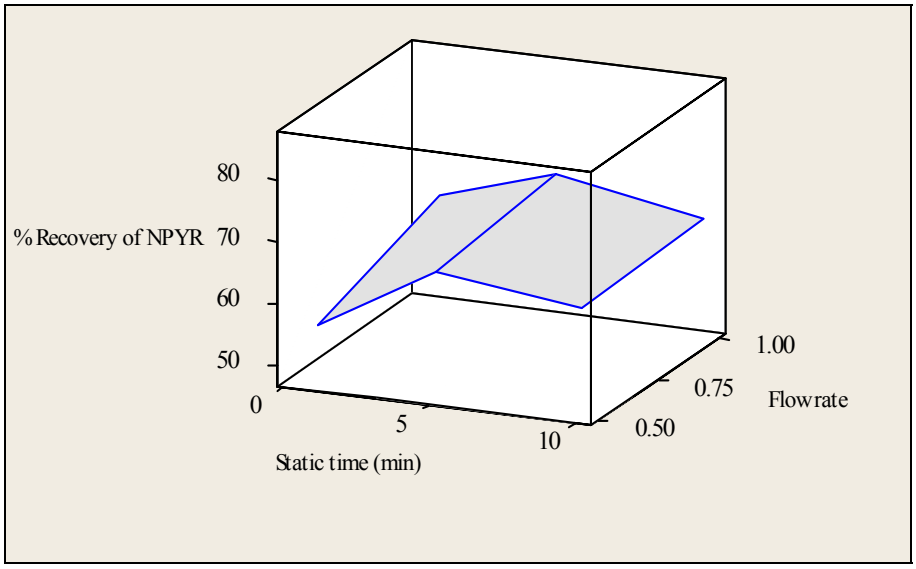


Figure 32 Surface plot of % recovery of NPYR versus static time and flow rate.

For all above experiments for optimization the parameter, Table 16 shows a summary of each SWE factor influence the extraction efficiency, presented as % recovery, of all nitrosamines.

Table 16 Optimized conditions of superheated water extraction**.**

Parameters	Range studied	Optimum value
Dynamic time (min)	5-25	10
Temperature (^{o}C)	30, 120-170	140
Static time (min)	1-10	5
Flow rate (mL min^{-1})	0.5-1.0	1.0

4. Application of the SWE method to real sample

The objective of this experiment was to demonstrate an efficiency of a coupling method using superheated water and solid-phase extraction for the determination of nitrosamine compounds in frankfurter sample. The sample used in the experiment was the same as that in the previous experiment and the extraction conditions for all nitrosamine compounds were performed under the optimal conditions for superheated water extraction as shown in Table 16 and followed by a clean up step under the optimal SPE conditions shown in Table 9. To study the recovery of the method, 1.0 g frankfurter was fortified with 20 μg (for MS analysis) or 100 μg (for FID analysis) of each nitrosamine and used for extraction. A lately received NMOR or *N*-nitrosomorpholine was also added into the fortified sample and subjected to both extractions under the same optimal conditions, but for MS analysis only. Since the tendency of the optimized factors for all above analytes were very similar, the recovery of NMOR was then expected to fall in the same range of those for the other nitrosamines. Quantitative analysis of the other nitrosamines were performed by either GC-FID or GC-MS. The results in term of %recovery of each nitrosamine were compared in Table 17.

From the Table 17 the extraction efficiencies of all analytes were considerably high, obtaining more than 80 %recovery under the optimized conditions. The recoveries were within an acceptable range of 70% to 130%, required by the California Department of Health Services (Grebel *et al*, 2006). This was confirmed that the SWE method was a remarkable alternative for extraction the nitrosamines from food sample. Loss of analytes in the procedure may be caused by human error during extraction procedure, as well as a solvent evaporation.

In analytical chemistry, it is usually necessary to express analytical accuracy and precision of a new method in term of recovery and repeatability in order to reveal random errors. Thus statistics were applied for calculation of experimental data and utilized in the comparison of precision of results. A popular term widely used to measure data distribution is the coefficient of variation (CV), also known as the

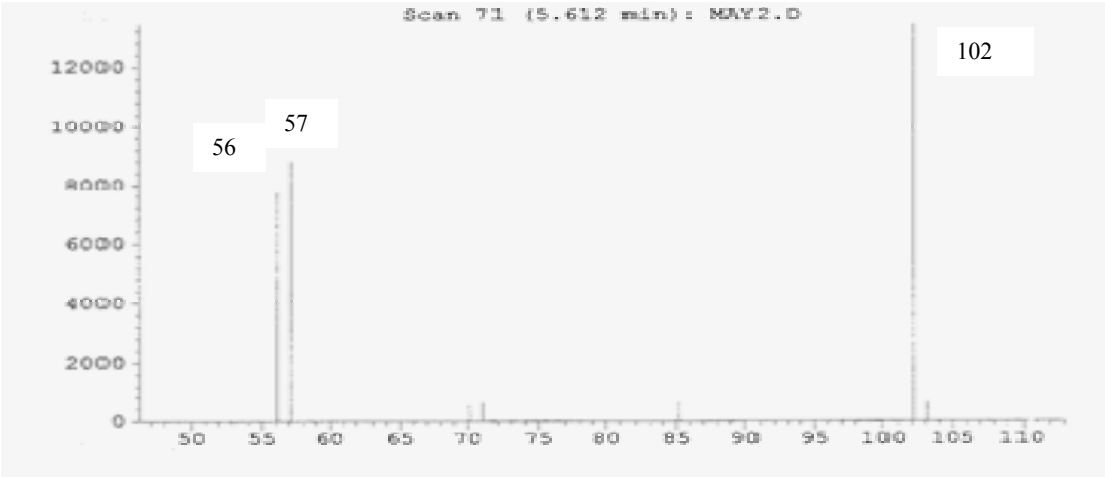
relative standard deviation (RSD), which is obtained from (SD/mean) x 100. A high RSD value means a large distribution of information. Table 17 shows the recovery of nitrosamines in frankfurter subjected to the extraction by superheated water and solid phase under the optimized conditions. From the results, the precision of the coupling method was satisfactory, varying between 1.9-7.2%.

Table 17 % Recovery of nitrosamines obtained from a SWE-SPE under the optimum conditions.

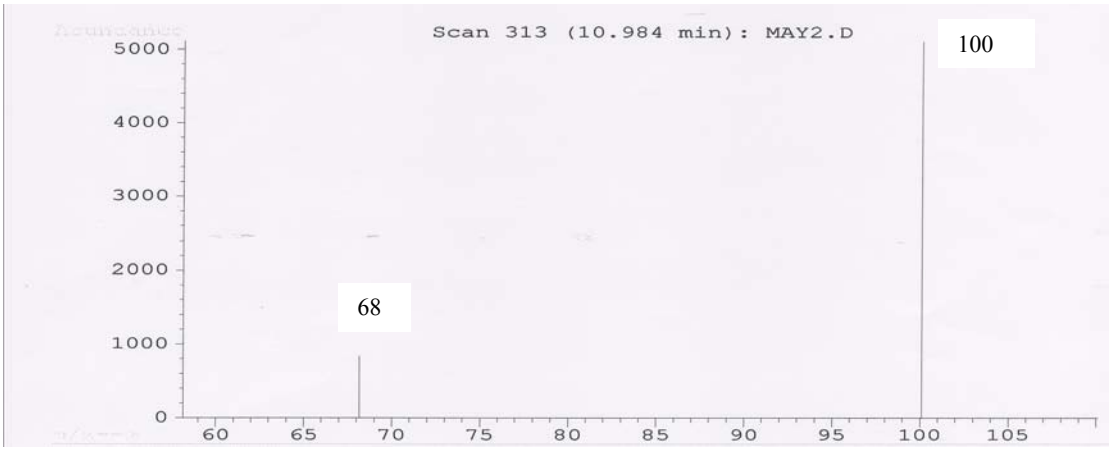
Compounds	%Recovery ± SD (RSD)	
	GC-FID[1]	GC-MS[2]
NDEA	93.53 ± 3.9 (4.2)	95.05 ± 5.9 (6.2)
NPYR	83.15 ± 1.6 (1.9)	81.82 ± 4.9 (6.0)
NPIP	96.40 ± 2.7 (2.8)	106.93 ± 7.7 (7.2)
NMOR	-	87.34 ± 5.7 (6.5)

[1]n = 3, [2]n = 5

Figure 33 shows the mass spectrum acquired from an injection of 100 µg mL^{-1} concentration of NDEA in the organic extract after the SPE step (Figure 33-A), 100 µg mL^{-1} concentration of NPYR (Figure 33-B), 13µg mL^{-1} concentration of NMOR (Figure 33-C) and 8 µg mL^{-1} concentration of NPIP (Figure 33-D). All spectrum obtained with a 1 µL injection. The NDEA spectrum showed m/z 102, 57 and 56, while those of NPYR, NMOR and NPIP spectrum showed m/z at 100 and 68; 116 and 56; and 114 and 55, respectively. No additional ions of significant relative abundance were detected in those spectrum of the extract, indicating that the extract was very clean with no interference compound having similar retention.

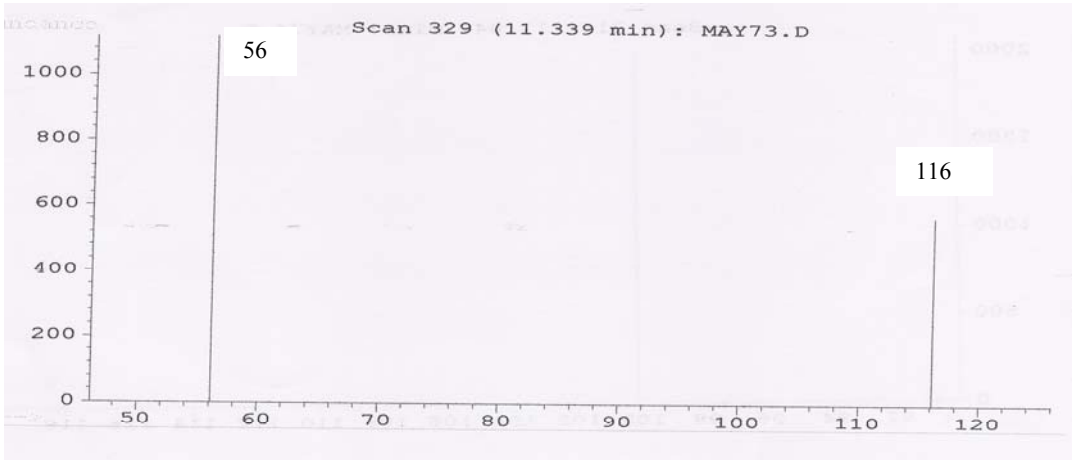


(A)

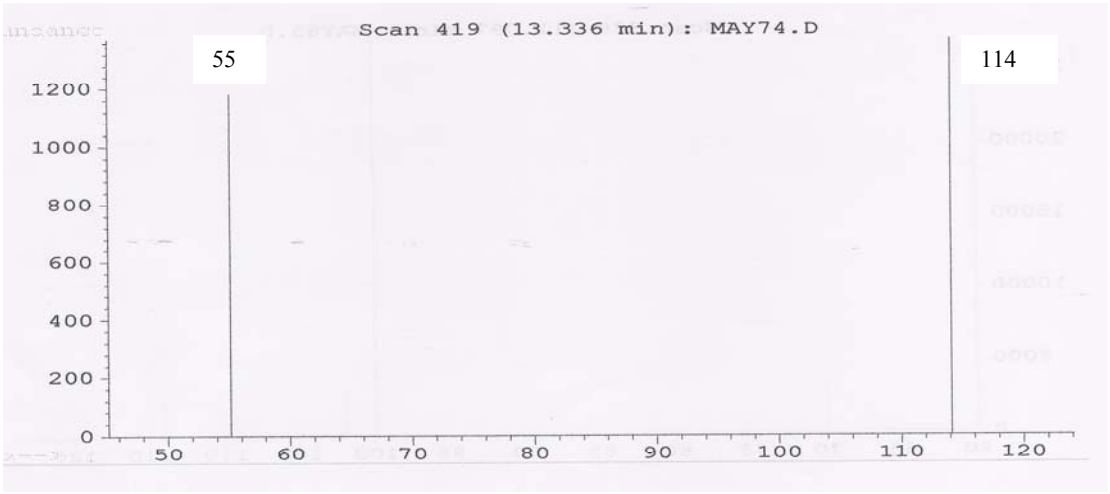


(B)

Figure 33 Mass spectrum of extracted nitrosamines by using superheated water coupled with solid-phase extraction under optimal conditions.



(C)



(D)

Figure 33 (Continued)

5. Detection limit of the extraction method examined by GC-MS detection

This experiment was aimed to investigate detection limits of all analytes, which was simultaneously extracted by superheated water and cleaned up by solid-phase extraction under optimal conditions. The organic extract was then quantitatively analyzed by using GC-MS instrument. The fortified frankfurter was extracted and a

mass range of NDEA and NPIP between 10-30 ng injected, and NPYR and NMOR between 15-30 ng injected were prepared. When NDEA, NPIP and NPYR were simultaneously extracted, NDPA was used as the internal standard. The detection limit of NMOR was examined by using NPIP as the internal standard. One microlitre of each solution was applied to GC-MS. The result of peak area ratio of each extracted analyte to its internal standard for each concentration were calculated and averaged the value as given in Table 18 and calibration curves for all analytes are shown in Figure 34-37 .

Detection and determination by using instrumental method, i.e. gas chromatography particularly coupled with mass spectrometric detection, can provide much smaller quantities of analyte than several classical methods. In analytical chemistry, a definition of limit of detection (LOD) is an analyte concentration giving a signal equal to the blank signal, y_B, plus three times of a standard deviation of blank, s_B, as shown in equation (1).

$$y - y_B = 3\, s_B \quad \text{----------} \quad (1)$$

In practice, a limit of detection can be obtained from a calibration graph plotted between the extracted analyte mass (x-axis) and its signal (y-axis), where the slope (b) and intercept (a or y_B) are given, according to the linear equation, $y = a + bx$. Finally, the limit of detection can be calculated from $y_B + 3s_B$ (Miller and Miller, 1988)

In general to estimate the limit of detection, peak height should be obtained from a chromatogram. However, in the work in stead of the peak height, the peak area was utilized because all nitrosamine peaks were slightly tailing (see appendix I), thus probably resulted in not only an error in measurement of the analyte peaks but also a systematic error in the linear calibration curve.

Table 18 Peak area ratio of analytes to internal standard in a mass range of 10-30 ng injected for NDEA and NPIP and 15-30 ng injected for NMOR and NPYR.

Compounds	Peak area compound / internal standard[1]				
	Mass (ng injected)				
	10	15	20	25	30
NDEA	0.2576	0.3836	0.5305	0.6243	0.7498
NPIP	0.1902	0.2737	0.3467	0.4116	0.4705
NPYR	-	0.1475	0.2334	0.2989	0.3630
NMOR	-	0.8825	1.1892	1.8460	2.4512

[1]n = 3

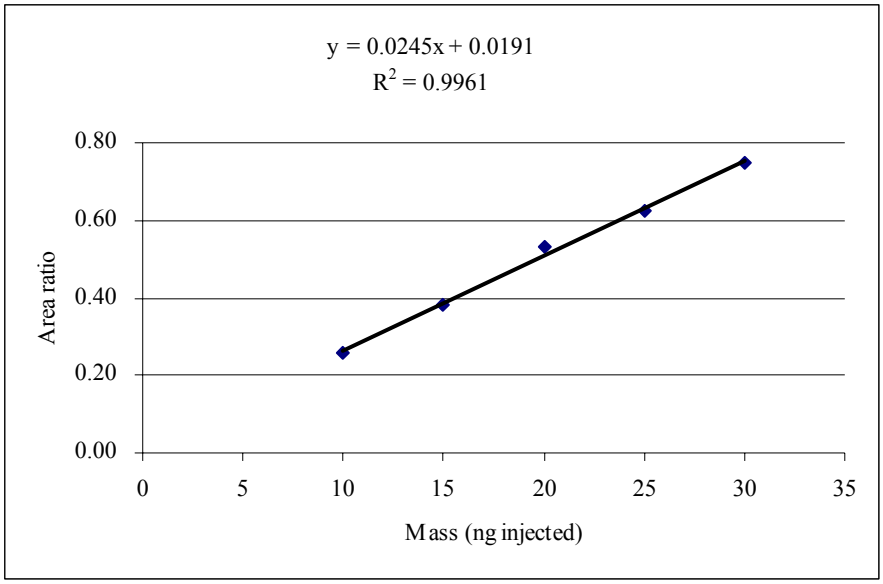


Figure 34 Calibration curve of NDEA.

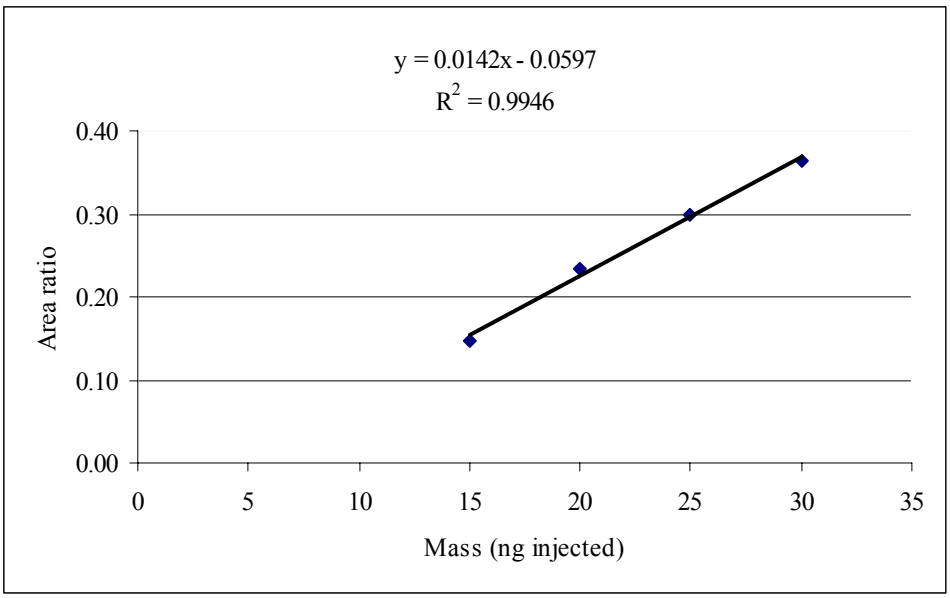


Figure 35 Calibration curve of NPYR.

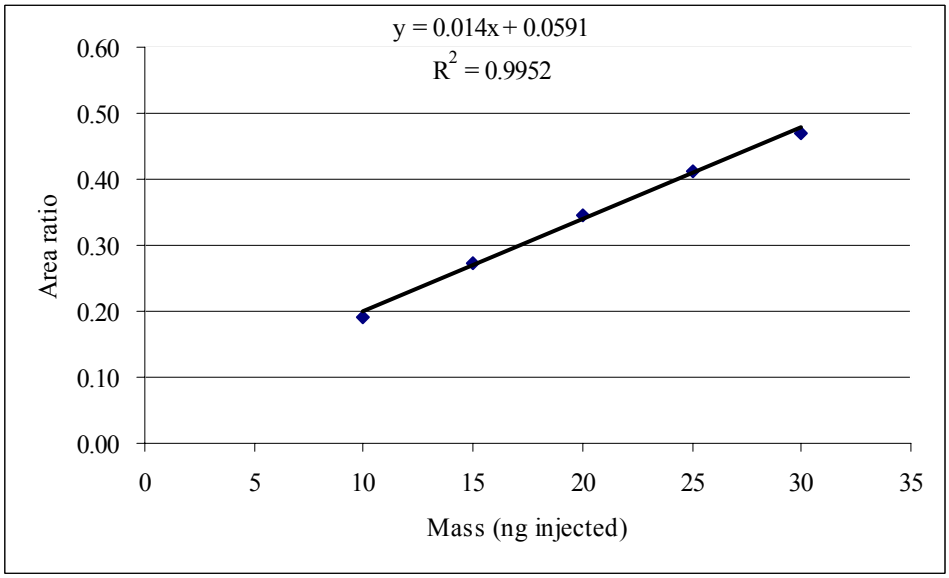


Figure 36 Calibration curve of NPIP.

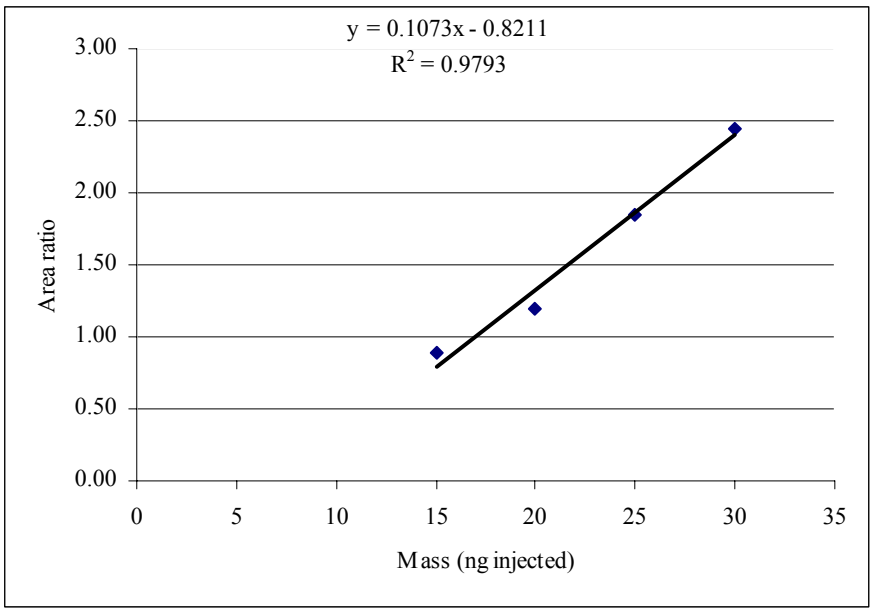


Figure 37 Calibration curve of NMOR.

In Figure 34-37, the average peak area ratio of each compound was plotted against the analyte mass, showing a good linearity with a slope, intercept, and least square of correlation coefficient (r^2). All of the result obtained from the calibration curves were summarized in Table 20.

For a standard deviation of the blank (s_B) estimation, 8 µg NDEA, 8 µg NPIP, 13 µg NMOR and 14 µg NPYR were mixed with 1.0 g frankfurter. The sample was extracted for 10 times and the standard deviation (s_B) of peak area ratio of each analyte was calculated and given in Table 19. The y_B and s_B were substituted in equation (1), resulting y that was the analytical signal at the limit of detection. Subsequently, y was substituted in the regression equation (y = a + bx), obtaining x, the true limit of detection, which was added in the last column of Table 20.

Table 19 Peak area ratio of each analyte obtained from ten times extraction for measuring the standard deviation (S_B).

No.of extraction	Peak area compound / internal standard			
	NDEA	NPYR	NPIP	NMOR
1	0.1678	0.1056	0.0666	0.5648
2	0.1714	0.1057	0.0714	0.5587
3	0.1587	0.1300	0.0589	0.5339
4	0.1578	0.1159	0.0776	0.5532
5	0.1653	0.1147	0.0731	0.5711
6	0.1687	0.1155	0.0658	0.5476
7	0.1583	0.1098	0.0594	0.5874
8	0.1702	0.1145	0.0645	0.5860
9	0.1691	0.1097	0.0624	0.5510
10	0.1592	0.1110	0.0588	0.5583
S_B	0.0055	0.0070	0.0065	0.0168

Table 20 Statistical values from each regression line of analyte and their calculated detection limits.

Compounds	Intercept, a, y_B	Slope, b	r^2	Calculated detection limits (ng injected / 1 g sample)
NDEA	+ 0.0191	0.0245	0.9961	0.68
NPYR	- 0.0597	0.0142	0.9946	1.48
NPIP	+ 0.0591	0.0140	0.9952	1.38
NMOR	- 0.8211	0.1073	0.9793	0.47

From Table 20, the limit of detection (of extraction) of nitrosamine compounds in the fortified frankfurter was found in a range of 0.47-1.48 ng injected per 1 g sample. The detection limits of all analytes obtained were not very low as expected, probably due to insufficiently high sensitivity of the HP5989 mass spectrometric detector used in the work. It was then postulated that the detection

limits could be lowered by using more sensitive and selective detectors, namely thermal energy analyzer and tandem mass spectrometric detector.

6. Comparison of our method with the others method

Because the nitrosamines are mutagenic compounds that can be found in meat, the development of extraction method was desired. In our work, nitrosamine was extracted with superheated water and then was clean up by using liquid-liquid extraction coupled with solid-phase extraction. Comparisons in terms of preparation method, analytical method, sample mass, %recovery and detection limit between the proposed method and other methods was shown in Table 21.

Although the extraction efficiency, represented by %recovery, of proposed method was quite close to those of some methods, more advantages were obtained from the proposed method. For example, in the proposed SPE method, the amount of solvent used as an eluent was drastically reduced relative to the other SPE methods. In this work, the amount of dichloromethane was less than 30 mL. For SWE technique, only 1 g of food sample was required and the amount was reduced relative to the vacuum distillation method. In addition, the extraction time of SWE technique was much shorter, for example the SWE time were less than 20 min per sample, while it took approximately one hour to finish the steam distillation. In the proposed SWE the water solvent was also environmental friendly.

In the reference method 1, 5 and 10, high efficiencies of the nitrosamine extraction were not obtained. In the method 10, the recovery of NDEA was only 63 %. Higher amount of food sample and many steps of sample preparation were required in method 1. In the reference method 3 and 6, too large volume of organic solvent was needed, particularly for the method 3, the aqueous filtrate was extracted four times with 300 mL dichloromethane and the organic extract were subjected to alumina SPE with 200 mL DCM, so the total volume of dichloromethane in this experiment was 1400 mL. In the method 6, the distillate was collected and extracted with 60 and 3 x 180 mL of dichloromethane, so too much volume of 600 mL dichloromethane was

consumed for each extraction. In the reference method 5, long extraction time was required, as the vacuum steam distillation in the method took approximately 80 min to complete the removal, in addition with the low recovery of the method. Additional step was also introduced to improve the method sensitivity of determination, but sometimes it could be complicated. For example in the method 11 the denitrosation step needed several and large volume of reagents, such as dansyl chloride in buffer solution, glacial acetic acid and hydrobromic acid.

In this work, the LOD results were not as low as expected, which were postulated that it occurred from the very low amount of frankfurters packed in the extraction cell and the low sensitivity of the MS detector used in this work. However, the LOD quantification can be improved by using more sensitive and specific detectors.

Table 21 Comparison between various methods for determination of nitrosamines

Ref.	1	2	3	4	5	6
Sample matrix	Pork luncheon meat, pig liver	Frankfurters	Fresh and preserved food	Cured ham, bacon and sausages	Canned sausages	Fermented sausage
Preparation method	Steam distillation (alkali,acid), electrochemical reduction and amine derivative method	Supercritical CO_2 coupled with SPE (Florisil)	Liquid-liquid extraction with DCM and SPE with basic alumina	Flow-injection method (based on photochemical cleavage of N-NO bond), then nitrite was detected	Steam distillation (rotary vacuum evaporator) and SPE with active carbon	Steam distillation on a steam generator and extraction with DCM
Analytical method	HPLC	GC-TEA	GC-TEA	Diode-array spectrophotometer	MEKC	GC-TEA
Sample mass	200 g	2.5 g	10.0 g	2.0 g	150 g	20 g
% recovery	% yield of derivative was 42-77 %	89-100 %	60-90 %	91.6-105.8 %	38.2-55.0 %	87-93 %
Detection limit	1-10 µg/kg	-	0.1-0.5 µg/kg	6.0 ng/mL	-	0.1 µg/kg

Table 21 (Continued)

Ref.	7	8	9	10	11	Proposed method
Sample matrix	Dried seafood	Sausages	Fish sample	Sausages	Meat products	Frankfurter
Preparation method	Steam distillation and extraction with DCM	Headspace solid-phase micro-extraction	Two-step SPE with Extrelut and Florisil	Two-step SPE with Extrelut and Florisil	Steam distillation, extraction with DCM and the denitrosation	SWE, LLE couple with SPE (florisil)
Analytical method	GC-TEA	GC-TEA	GC-MSD	GC-TEA	HPLC with fluorescence	GC-MS
Sample mass	25 g	2.5 g	6.0 g	6.0 g	80-100 g	1.0 g
% recovery	83.2-102.2 %	105-110 %	79-88 %	40-100 %	72-76 %	81-106 %
Detection limit	0.1 µg/kg	3 µg/kg	0.10 µg/kg	0.3 ppb	0.7-2.2 ng	0.47-1.48 ng

CONCLUSION

In this study a laboratory-made superheated water system with combination of static and dynamic mode was applied to extract volatile nitrosamine from fatty food. A number of superheated water extraction (SWE) and solid-phase extraction (SPE) parameters were optimised to obtain highest efficiencies. In SWE experiment, extraction temperature, water flow rate, and static time were expected to be major factors affecting the extraction efficiency, which were investigated their effects and correlations by using a full fractorial design produced by Minitab software version 14.2. Sample preparation and pre-concentration were achieved by a liquid-liquid extraction coupled with solid-phase extraction (LLE-SPE). Various LLE parameters such as, kind and volume of trapping solvent, step of extraction and NaCl addition, affecting the recovery of nitrosamine were investigated. Besides, some SPE factors influencing the extraction efficiencies, namely sorbent type, sorbent mass, solvent system and volume were also optimized.

An optimization of all SWE parameters by using the factorial design was revealed that the optimum conditions for extraction of nitrosamines from frankfurter were a temperature of 140 ^{o}C and a static time of 5 min. The minimum time to complete extraction of all nitrosamine was totally 10 min with a flow rate of 1 mL min^{-1}. Analysis of the results by three-way ANOVA from the Minitab showed significances of temperature, static time and flow rate. F-value showed that temperature was the most important parameter for improving extraction efficiency of all nitrosamines. The recoveries of NDEA and NPIP were depended on the combination of temperature, flow rate and static time. In the ANOVA test for the recovery of NPYR, the p-values indicated that only two two-way interactions, temperature*static time and static time*flow rate were significant, in contrast with the insignificant three way interaction of temperature*static time*flow rate.

The nitrosamines were maximal removed from the aqueous extract by using 2 mL of dichloromethane with NaCl addition. Without NaCl addition, low recovery of all nitrosamines were obtained. The experimental results showed that optimum

conditions for SPE were 4.6 g florisil sorbent eluted with 60% ethyl ether-dichloromethane, which allowed the efficient and fast extraction to avoid lipid and emulsion. Separation and identification of components were carried out using both GC-FID and GC-MS.

By coupling the SWE and SPE technique under the optimized extraction conditions, the percent recoveries of nitrosamine extraction from frankfurter were in a range of 81 to 106 %. The detection limit determination was performed by GC-MS. Calibration graphs obtained for calculating limit of detection gave excellent linear correlation coefficients. The detection limit of the coupling method was ranged between 0.47-1.48 ng injected of analytes from frankfurter sample.

The proposed reserach work gained benefits more than other published methods for nitrosamine determination in the following ways: firstly, the sample preparation steps are simple, efficient, and relatively inexpensive while using only small amounts of toxic solvents. Secondly, the period of time necessary for both extraction and pre-concentration was not too long, ranging from 45 min to 1 hour. Thirdly, factorial designs can be considered as an effective tool to study the influence of parameter affecting SWE, and they permit acquisition of more robust results with a reduced number of experiments when compared with the classical one-to-one parameter approach. Forthly, the use of mass spectrometric detector provided identification of all analytes without using other confirmatory techniques.

From all of results, it was concluded that the coupling of superheated water extraction and solid-phase extraction are an interesting alternative effective extraction technique, providing several advantages, i.e. low extraction cost, reduced analysis time, low organic solvent consumption, thus generating low amount of waste. This method may be an alternative for a routine determination of nitrosamines in real sample.

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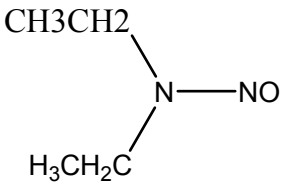
APPENDIX

Appendix Table 1 Physical properties of selected nitrosamines.

Analytes	NDEA	NPIP	NPYR	NMOR
formula	$(C_2H_5)_2N_2O$	$C_5H_{10}N_2O$	$C_4H_8N_2O$	$C_4H_8N_2O_2$
molecular weight	102.14	114.15	100.2	116.12
boiling point (^oC)	177	217	214	224
density (g/mL)	0.94	1.06	1.09	-
refractive index	1.4386	1.4933	-	-
physical appearance	yellow liquid	yellow liquid	yellow liquid	yellow solid
water solubility (mg/L)	106,000	76,480	1,000,000	861,527.50
polarity (log Koctanol-water)	0.48	0.36	-0.19	-0.44
vapour pressure (mm Hg)	0.86	0.14	0.06	0.036
UV absorption (λ max, nm)	231	366	230	237

Appendix 1 Chemical information of analyte.

N-nitrosodiethylamine (NDEA)



$(C_2H_5)_2NNO$

CAS no. 55-18-5

Synonyms: diaethylnitrosamin (German); *N,N*-diethylnitrosamine; diethylnitrosamine; diethylnitrosoamine; ethylamine,*N*-nitrosodi-; *N*-ethyl-*N*-nitrosoethanamine; nitrosodiethylamine; DANA; DEN; DENA; NDEA.

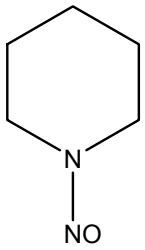
Use information: NDEA has been suggested for use as a solvent, a softener for copolymers, a lubricant additive, in electrical condensers and chemical synthesis.

Toxicology: LD50 for NDEA is 280 mg/kg after oral administration to the rat.

It is carcinogenic to many different animal species which include sub-human primates. NDEA induces cancer following prenatal exposure and in single dose experiments. NDEA was administered to rats in drinking water in a chronic exposure experiment. Total dose, until death occurred, was between 64 and 965 mg/kg. The tumor induction time was between 68 and 840 days. All daily doses > 0.15 mg/kg gave a tumor incidence of 100%. When a dose of 0.15 mg/kg per day was administered, a tumor yield of 90% was obtained. At 0.075 mg/kg per day, 20 rats survived for > 600 days and 11 of 20 animals had tumors of liver, esophagus, or nasal cavity. All 4 animals that lived > 940 days at this dose level had tumors. The location of the tumors was dependent on the dose given, the non-liver tumors evolved only if cancer of the liver had not caused death. NDEA causes cancer in rat, African white-tailed rat, mouse, Syrian Golden, Chinese and European hamsters, guinea pig, rabbit, parakeet and monkey. The principal affected organs were liver, esophagus, nasal cavities, kidney, forestomach, lung and larynx.

Appendix 1 (Continued)

N-nitrosopiperidine (NPIP)



$C_5H_{10}N_2O$

CAS no. 100-75-4

Synonyms: *N*-nitroso-piperidin(German); 1-nitrospiperidine; NO-Pip; NPIP.

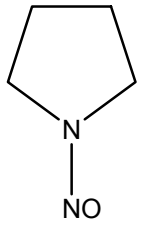
Use information: It is used as a research chemical and is found in some foods and tobacco smoke.

Toxicology: LD50 for NPIP, after oral administration to the rat, is 200 mg/kg.

When 20 mg/kg was given to rats in daily drinking water, 17 of 20 rats died prematurely. Three rats died after 186, 232 and 289 days of liver cancer with metastases in the lungs, the last animal also had cancer in the esophagus. Because the 20 mg/kg daily dose was not tolerated well, the experiment was repeated at 5 mg/kg. Of nine animals, one died with papillomas, the other eight died of esophageal cancer. Three rats also had liver cancer. The mean carcinogen dose was 1.4 g/kg. and the average induction period was 280 days. NPIP has produced cancer in the rat, mouse, Syrian Golden hamster and monkey. The principal affected organs were the esophagus, liver, nasal cavities, larynx, trachea and forestomach.

Appendix 1 (Continued)

N-nitrosopyrrolidine (NPYR)



$C_4H_8N_2O$

CAS no. 930-55-2

Synonyms: *N*-nitrosopyrrolidin (German); pyrrolidine, 1-nitroso-; NO-Pyr; NPYR.

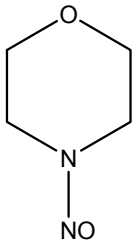
Use information: It is used as a research chemical.

Toxicology: LD50 for NPYR, administered orally to the rat, is 900 mg/kg.

In chronic experiments, 10 mg/kg and 5 mg/kg were administered in daily drinking water to two groups of five and 20 rats. Because the carcinogenic effect was weak, the dose was doubled after 150 days. All the animals, except two died without tumors, developed liver cancer. The average carcinogenic doses (D50) were 4.2 and 3.9 g/kg and the mean induction periods (t50) were 290 and 470 days. The agent was determined to be a weak but relatively certain carcinogen. NPYR has produced cancer in the rat, mouse and Syrian Golden hamster. The principal affected areas were the liver, nasal cavities, testes, lung and trachea.

Appendix 1 (Continued)

N-nitrosomorpholine (NMOR)



$C_4H_8N_2O_2$

CAS no. 59-89-2

Synonyms: 4-nitroso- *N*-nitrosomorpholin (German); 4 nitrosomorpholine; morpholine, NMOR.

Use information: NMOR was used as a solvent for polyacrylonitrile and as a intermediate for the synthesis of *N*-aminomorpholine. NMOR has been found to be an effective agent to combat microbial infections.

Toxicology: LD50 for NMOR, after oral administration to the rat, was 320 mg/kg. When 16 rats were given 8 mg/kg NMOR daily in drinking water, 14 animals died of liver cancer. The mean induction period (t50) was only 165 days. A dose of 16 mg/kg caused liver tumors in two of four rats after only 45 and 65 days, respectively and after 115 days liver cancer was observed. NMOR was clearly the most rapidly acting liver carcinogen of the 65 *N*-nitroso compounds tested. NMOR has produced cancer in the rat, mouse, and Syrian Golden hamster. The principal affected organs were liver, nasal cavities, kidney, esophagus, ovary, trachea, larynx and bronchi.

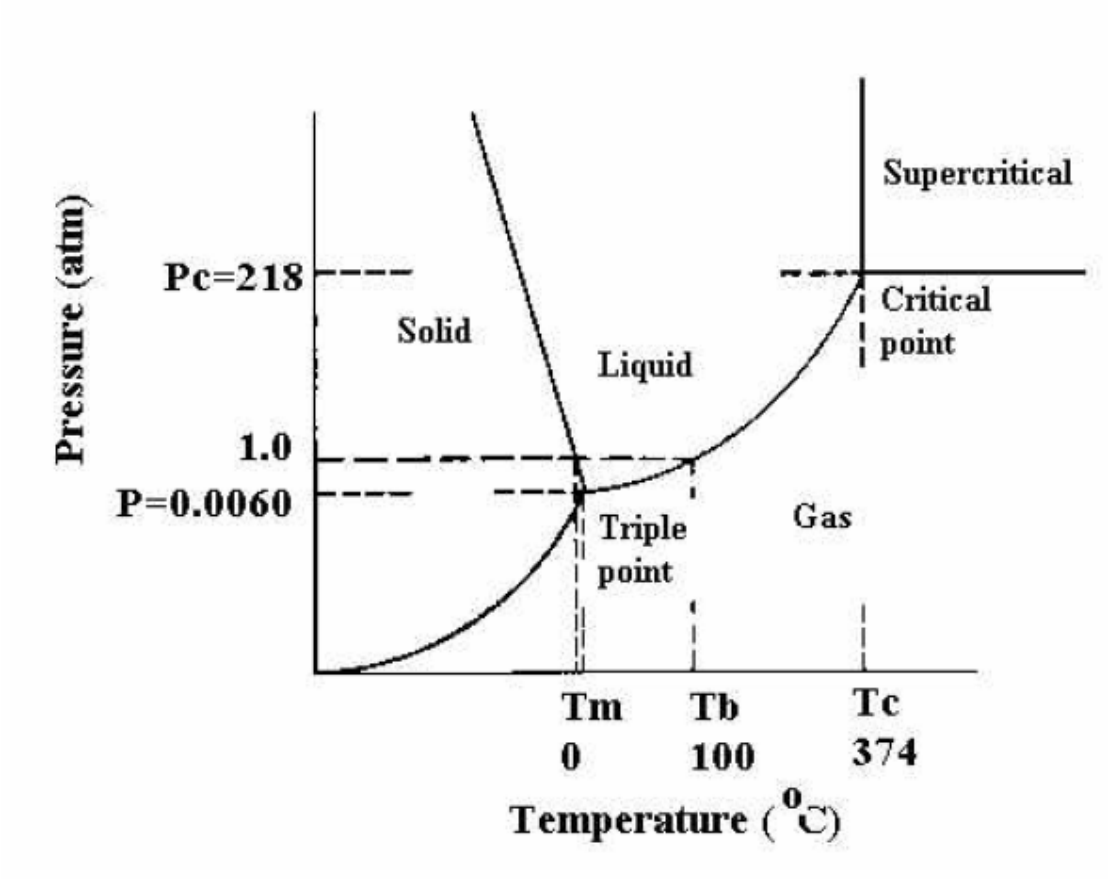
Appendix Table 2 Chemicals list

Chemicals	Formula	Molecular weight	Company
Acetone	C_3H_6O	58.08	Ajak Finechem
Dichloromethane	CH_2Cl_2	84.93	BDH
Diethyl ether	$C_4H_{10}O$	74.12	BDH
Ethyl acetate	C_4H_8O2	88.10	BDH
Florisil	MgO_3Si	100.39	Sigma
Glacial acetic acid	CH_3COOH	60.05	BDH
Methanol	CH_3OH	32.04	Merck
N-nitrosodiethylamine	$C_4H_{10}N_2O$	102.14	Fluka
N-nitrosomorpholine	$C_4H_8N_2O_2$	1 16.12	Supelco
N-nitrosopiperidine	$C_5H_{10}N_2O$	114.15	Sigma
N-nitrosodipropylamine	$C_6H_{14}N_2O$	130.19	Supelco
N-nitrosopyrrolidine	$C_4H_8N_2O$	100.12	Aldrich
Silica gel	SiO_2	60.08	Merck
Sodium chloride	NaCl	58.44	BDH
Sodium sulphate anhydrous	Na_2SO_4	142.04	Ajak Finechem
Triethylamine	$C_6H_{15}N$	101.19	Aldrich

Appendix Table 3 Computed values for the dielectric constant of water at temperature between 100 and 370 ^{o}C.

Temperature, ^{o}C	Dielectric constant	Temperature, ^{o}C	Dielectric constant
100	55.39	240	28.24
110	52.89	250	26.75
120	50.48	260	25.29
130	48.19	270	23.86
140	46.00	280	22.45
150	43.89	290	21.05
160	41.87	300	19.66
170	39.96	310	18.27
180	38.10	320	16.88
190	36.32	330	15.51
200	34.59	340	14.10
210	32.93	350	12.61
220	32.32	360	11.22
230	29.75	370	9.74

Source: Akerlof and Oshry (1950)



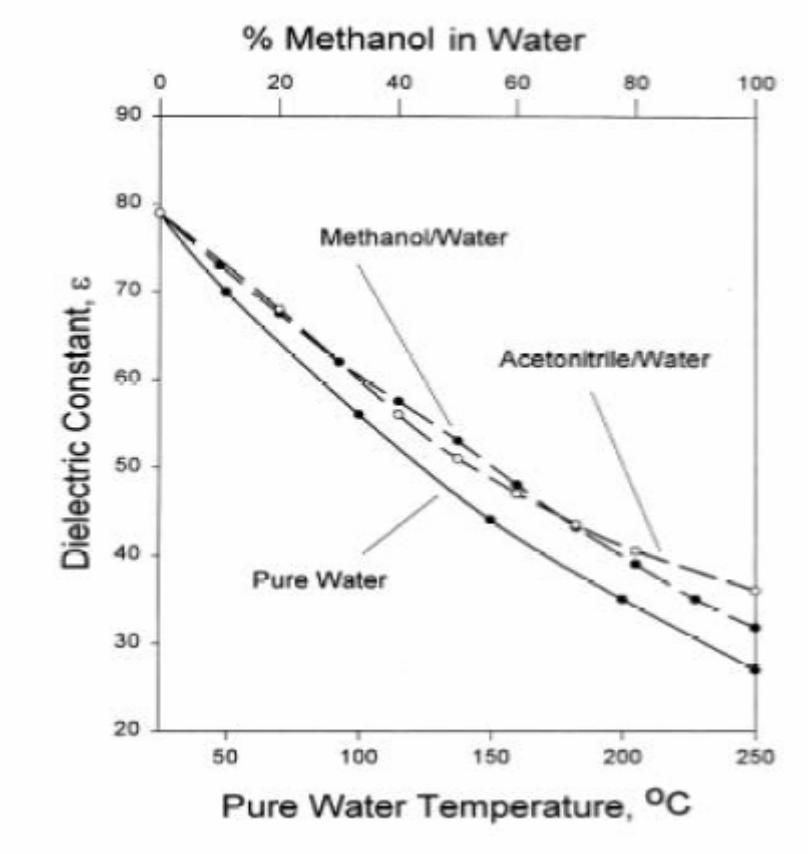
Appendix Figure 1 The phase diagram of water as a fuction of the temperature and pressure showing critical temperature 374^{o}C and critical pressure 218 atm.

Appendix Table 4 Vapor pressure of water from boiling point, 100 to critical point, 373^{o}C.

Temperature, ^{o}C	Pressure, kPa	Temperature, ^{o}C	Pressure, kPa
100	101.32	250	3973.6
110	143.24	260	4689.4
120	198.48	270	5499.9
130	270.02	280	6413.2
140	361.19	290	7438.0
150	475.72	300	8583.8
160	617.66	310	9860.5
170	791.47	320	11279
180	1001.9	330	12852
190	1254.2	340	14594
200	1553.6	350	16521
210	1906.2	360	18655
220	2317.8	370	21038
230	2795.1	373	21803
240	3344.7		

Appendix Table 5 Pressure conversion_factors.

Pressure	PSI	atm (std.)	kg/cm^2	Torr (mmHg)	Inch (Hg)	kPa	Bar
PSI	1	0.068	0.0703	51.813	2.0359	6.8948	0.06895
atm (std.)	14.695	1	1.0332	760	29.921	101.3	1.0133
kg/cm^2	14.223	0.9678	1	735.56	28.958	98.06	0.9806
Torr (mmHg)	0.0193	0.00132	0.0013	1	0.00394	0.133	0.00133
Inch (Hg)	0.4912	0.0345	0.0345	25.4	1	3.386	0.03386
kPa	0.145	0.00987	0.0102	7.52	0.2953	1	0.01
Bar	14.5033	0.9869	1.0197	751.88	29.53	100	1



Appendix Figure 2 The dielectric constant of pure water comparing to mixing water with methanol or acetonitrile by changing temperature. (Yang *et al*., 1998)

The dielectric constant of water at 150 °C was 43.89, which was comparable to a mixture of 70 % methanol-water.

Appendix Figure 3 Photograph of superheated water apparatus.

Appendix 2 Method of calculation of the nitrosamines recovery from chromatographic data

Supposed that a standard solution for calculating the response factor was a mixture of 100 µg of the nitrosamine analyte and 50 µg of NDPA internal standard in 1.00 mL methanol. The solution was injected for chromatographic analysis, giving the result as in the table below.

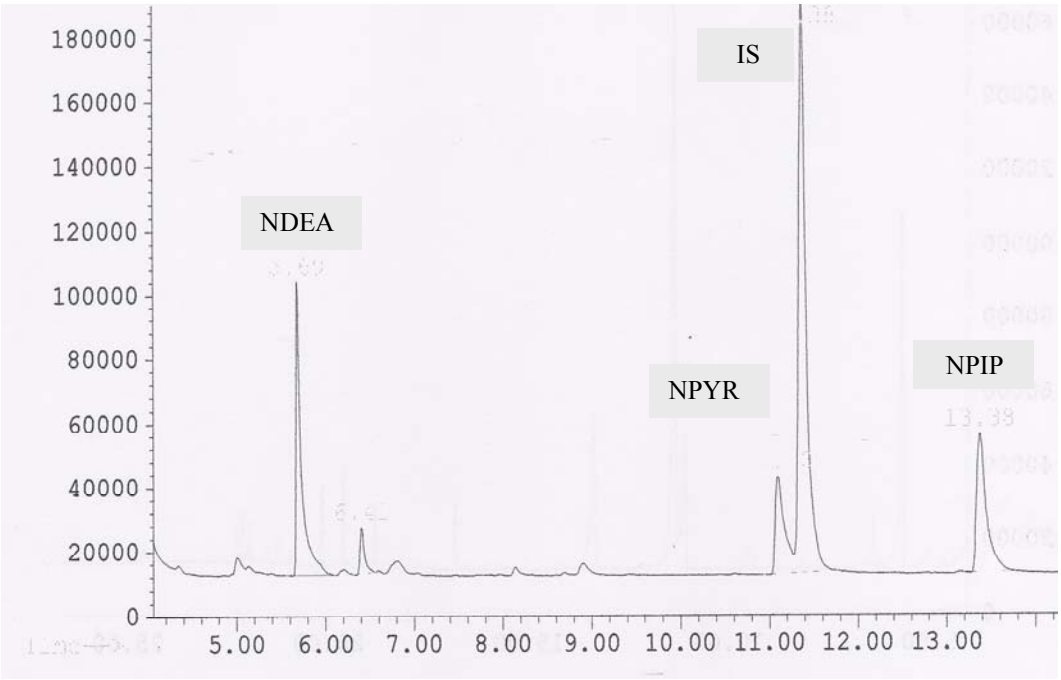
The frankfurter sample was also spiked with 100 µg nitrosamine compounds and extracted. 50 µg internal standard was used and then the solution was applied to GC, resulting the peak area as following example data.

Compound	Peak area	
	Analytes	Internal standard
Standard	16728	12546
Frankfurter sample	29654	23442

$$\text{Response factor} = \frac{100\ \mu g \times 12546}{50\ \mu g \times 16728} = 1.50$$

$$\% \text{ Recovery} = \frac{29654 \times 50\ \mu g \times 1.50 \times 100}{23442 \times 100\ \mu g}$$

$$= 94.87\%$$



Appendix Figure 4 GC-MS chromatogram (SIM mode) of extracted nitrosamine compounds by using superheated water coupled with solid-phase extraction under optimal condition.