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THESIS

INVESTIGATION OF SILICA MONOLITH AS A NON-POLAR MATRIX RETAINER FOR NITROSAMINES DETERMINATION

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Narumol Ramnut 2008: Investigation of Silica Monolith as a Non-Polar Matrix Retainer for Nitrosamines Determination. Master of Science (Chemistry), Major Field: Chemistry, Department of Chemistry. Thesis Advisor: Assistant Professor Orapin Chienthavorn, Ph.D. 120 pages

A novel, simple, rapid and inexpensive sorbent trap for extraction and clean-up of nitrosamines from frankfurters was developed. In a capillary the trap was fabricated to form monolithic polystyrene-co-divinylbenzene (PS-DVB), polydivinylbenzene (poly-DVB) and silica material and they were morphological characterized by a scanning electron microscope (SEM). To be used as an on-line sorbent trap, various factors, such as capability in trapping non-polar matrix and column length of monolithic column were preliminarily studied. Silica monolith provided the best result and it was chosen as the most suitable trap for nitrosamine determination. With a coupling between superheated water extraction (SWE) and on-line silica monolithic sorbent column prior to an analytical column of gas chromatograph (GC), various factors affecting the extraction and determination of nitrosamines, such as sensitivity with and without the monolith, reusability, injection-to-injection precision, column-to-column reproducibility and chromatographic separation, were investigated. Optimal column length of silica monolith was found to be 30.0 mm that offered 27 times reuse. Separation and quantification of selected volatile nitrosamines, namely nitrosodiethylamine (NDEA), nitrosopiperidine (NPIP) nitrosopyrrolidine (NPYR), and nitrosodi-n-propylamine (NDPA), were carried out using GC-FID and GC-MS in a selected ion monitoring (SIM) mode. Using GC-MS the overall extraction and determination method allowed recoveries of *N*-nitrosamines in a range of 75-88% with a relative standard deviation of less than 5%. The detection limit of the coupled method ranged from 2.10 to 5.40 ng nitrosamine injected.

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Student's signature Thesis Advisor's signature

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Narumol Ramnut

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LIST OF ABBREVIATIONS

AIBN	=	azobisizobutyronitryle
anh.	=	Anhydrous
AR	=	analytical reagent
BMA	=	butylmethacrylate
°C	=	degree Celsius
C	=	Carbon
CAR	=	Carboxen
CEC	=	capillary electrochromatography
CLC	=	capillary liquid chromatography
cm	=	Centimeter
CO_2	=	carbon dioxide
CRM	=	certified reference material
CW	=	Carbowax
DMF	=	N,N-dimethylformamide
DPPH	=	2,2-diphenyl-1-picrylhydrazyl hydrate
DVB	=	divinylbenzene
EDMA	=	ethylenedimethacrylate
EGFR	=	epidermal growth factor receptor
EI	=	electron ionization
EPA	=	environmental protection agency
ESI	=	Electrospray ionization
FID	=	flame ionization detector
fmol	=	Famtomole
γ-MAPS	=	γ-(trimethoxysilyl)propyl methacrylate
g	=	Gram
GC	=	gas chromatography
GMF	=	glass microfiber
h	=	Hour
HETP	=	height equivalent to theoretical plate

LIST OF ABBREVIATIONS (Continued)

HNO_2	=	nitrous acid
HP-5	=	5%-phenyl-methylpolysiloxane (Hewlette Packard)
HPLC	=	high performance liquid chromatography
HS	=	head space
i.d.	=	inner diameter
IGC	=	inverse gas chromatography
IP-RP-HPLC	=	gradient elution ion pairing reversed-phase
IS	=	internal standard
kg	=	Kilogram
L	=	Litre
LC	=	liquid chromatography
LLE	=	liquid-liquid extraction
LOD	=	limit of detection
LTQ	=	linear ion trap MS
M	=	Molar
m	=	Metre
m/z	=	mass-to-charge ratio
MEKC	=	micellar electrokinetic chromatography
µ-HPLC	=	micro high-performance liquid chromatography
µL	=	Microlitre
µm	=	Micrometer
mg	=	Milligram
min	=	Minute
mL	=	Milliliter
mm	=	Millimeter
MPa	=	Megapascal
MS	=	mass spectrometry
MSD	=	mass selective detector
MW	=	molecular weight

LIST OF ABBREVIATIONS (Continued)

MΩ	=	mega-ohm
N_2	=	nitrogen
N_2O	=	nitrous oxide
Na_2SO_4	=	sodium sulphate
NaCl	=	sodium chloride
NaOH	=	sodium hydroxide
NDEA	=	N-nitrosodiethylamine
NDMA	=	N-nitrosodimethylamine
NDPA	=	N-nitrosopropylamine
ng	=	Nanogram
NPIP	=	N-nitrosopiperidine
NPYR	=	N-nitroso-pyrrolidine
OFN	=	oxygen free nitrogen gas
PA	=	Polyacrylate
PAHs	=	Polynuclear aromatic hydrocarbons
PCI	=	positive-ion chemical ionization
PDMS	=	polydimethylsiloxane
P-DVB	=	porous polydivinylbenzene
PEEK	=	polyether ether ketone
PEG	=	polyethylenglycol
PEG	=	polyethyleneglycol
pH	=	power of hydrogen
PLOT	=	porous layer open tubular
ppm	=	part per million
PS-DVB	=	polystyrene-co-divinylbenzene (PS-DVB)
PS-DVB-C_{18}	=	octadecylated PS-DVB
psi	=	pounds per square inch
RP-18e	=	reverse phase C18-bonded silica
RSD	=	relative standard deviation

LIST OF ABBREVIATIONS (Continued)

SD	=	standard deviation
SEC	=	size exclusion chromatography
SEM	=	scanning electron micrograph
SFE	=	supercritical fluid extraction
SIM	=	single ion monitoring
SPE	=	solid phase extraction
SPME	=	solid phase microextraction
SW	=	subcritical water
SWE	=	super heated water extraction
TEA	=	thermal energy analyzer
TGA	=	thermal gravimetric analysis
TMOS	=	tetramethoxysilane
t_R	=	retention time
ug	=	Microgram
UV	=	Ultraviolet
v/v	=	volume to volume
wt	=	Weight
ZB-5	=	5%-phenyl-methylpolysiloxane (Phenomenex Zebron)

INVESTIGATION OF SILICA MONOLITH AS A NON-POLAR MATRIX RETAINER FOR NITROSAMINES DETERMINATION

INTRODUCTION

Monolithic material was emerged about 20 years ago and best known as a new category of stationary phases in liquid chromatography. It defined as a column consisting of a single piece of solid that had an interconnected skeleton and interconnected flow paths (or so-called through-pores). Interconnected skeleton or the sorbent domain contains relatively small pores, which provides high specific surface area for adsorption capacity of the column and can be reached only by molecular diffusion as illustrated schematically in Figure 1.

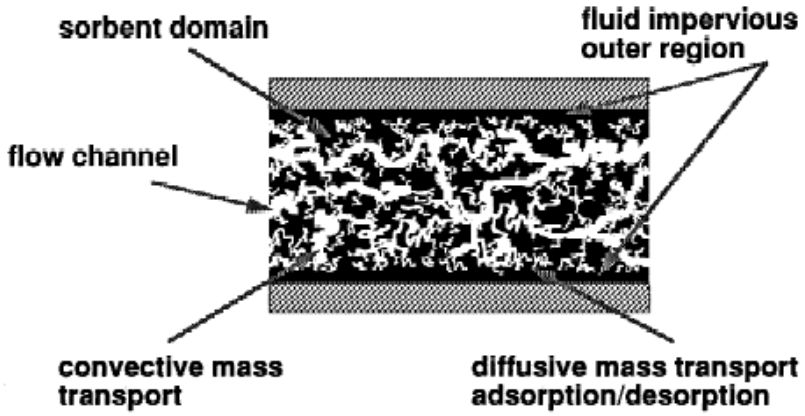


Figure 1 Schematic diagram of porous polymer monolith.

Nowadays monolithic materials can be divided into two categories: polymer-based monolithic column and silica-based monolithic column. An ease of preparation of monolithic materials is of particular importance compared with particles packed column. Preparation of the packed columns includes two key steps: (i) the fabrication of retaining frits within a capillary and (ii) the packing of small diameter particles into the narrow-bore tubes. Both steps require considerable experimental skill and experience in order to obtain stable columns with reproducible properties. In contrast, the production of monolithic columns is often a single-step process amounting to

polymerization in a mold or in a capillary tubing that avoids the problems of both frit formation and packing.

Organic polymer monoliths are synthesized by a thermally initiated one-step radical polymerization process in the presence of a mixture of monomer, porogen and initiator that induces phase separation and creates the interskeleton macropore. The reaction temperature and composition of porogenic mixture determine the macropore, as well as the microglobule diameter and the development of micropores in the microglobules.

Silica-based monoliths, in contrast, are prepared through a two-step process involving a sol-gel mechanism. The first step is a phase transition which determines domain size and macropore diameter, followed by a solvent exchange leaching out the silica skeleton to create the intraskeleton mesopore space. Consequently, the resulting pore space shows a bimodal pore size distribution.

Owing to several advantages compared to particulate packing, monolithic structure provides high effective surface area and high column permeability, which simultaneously offers high separation efficiency and reduces in column pressure drop. Since it was accepted as a large family of chromatographic stationary phases, their numerous applications are focused on high performance liquid chromatography (HPLC) and capillary electrochromatography (CEC), while an application in gas chromatography (GC) is fairly limited in number. In contrast, the utilization of monolithic material in an indirect chromatographic application, for example the use of monolithic materials for sample preparation procedure to concentrate and purify analytes from liquid matrix, in other words solid phase material for solid phase extraction (SPE), pre-concentrator and sorbent trap, are not well-known. These functions were based on physical adsorption between chemistry of supporting material and analyte, while the adsorbent should possess extremely high surface area. Choice of sorbent is therefore a key factor because of its properties, such as selectivity and capacity.

In the analysis, sample preparation method is an important process to clean-up and pre-concentrate an analyte to improve efficiency of method and instrument. Food is one of several samples that frequently require a tedious sample preparation process due to a complex matrix. Fat is the main matrix contained in animal food sample that needs to be eliminated prior to analysis. Even though solid phase extraction (SPE) was a widely used technique in sample preparation method, a few drawbacks of conventional SPE limit a requirement that the separation should be as fast and as cheap as possible. Some drawbacks are time consuming in solid phase packing preparation, SPE optimization and only one time use before disposal that eventually cause high waste volume and cost. Moreover, it often requiring many steps before reaching to a suitable concentrate extract for instrumental analysis, which only a small proportion is actually introduced.

The motivation of this study originated from a tedious process in sample preparation method for the determination of volatile nitrosamines by GC-FID. The analysis of nitrosamine compounds in frankfurter samples extracted by superheated water extraction (SWE) was previously studied (Subprasert, 2007). Matrix interference, such as fat and pigment, typically exists in the sample; a clean up step is then needed prior to the analysis. In the previous work, elimination of matrix interference was performed by a conventional solid phase extraction (SPE). According to some drawbacks of conventional SPE, including a current trend in analytical chemistry to miniaturize an extraction system driven by environmental concerns, the steadily increasing costs of solvent disposal and limited amounts of samples, a capillary monolithic column was proposed to be an alternative. Accordingly, its feasibility in using as an on-line sorbent trap applied to GC-FID, replacing a conventional SPE, will be further investigates.

In this study, three monolithic materials namely polystyrene-co-divinylbenzene (PS-DVB), polydivinylbenzene and silica monolith were of interest to be used as a sorbent trap for a fatty sample. PS-DVB and polydivinylbenzene were chosen on a basis of their high hydrophobicity and thermal stability. While silica monolith was chosen because silica has been successfully used as a fat-retainer and it

also provides high thermal stability. Some information and fabrication processes of three types of monolithic material were described below.

1. Polystyrene-co-divinylbenzene (PS-DVB) monolith

In preparation process, the inner wall of a bare fused-silica capillary tubing was pretreated with the heterobifunctional coupling agent, γ-(trimethoxysilyl)propyl methacrylate (γ-MAPS), in order to provide a large number of free methacrylate groups on the capillary wall. These groups served as anchoring sites during the formation of polymer and provided good connection between the monolith and capillary surface. The fused silica capillary wall became hydrophobic after modification with γ-MAPS as shown in Figure 2.

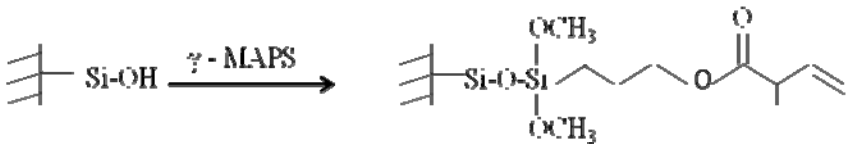


Figure 2 Reaction of silica surface with heterobifunctional coupling agent (γ-MAPS)

In polymerization process, styrene and divinylbenzene were used as a monomer and a cross-linking agent, respectively, to form an interconnected skeleton of monolithic structure as shown in Figure 3. Decanol and tetrahydrofuran were used as a porogenic agent to provide a bimodal pore structure, i.e. through pores and micro pore, of PS-DVB monolith, and dibenzoyl peroxide was used as a thermal polymerization initiator. After polymerization at 70 °C for 24 h, a complete conversion of monomer to polymer was obtained.

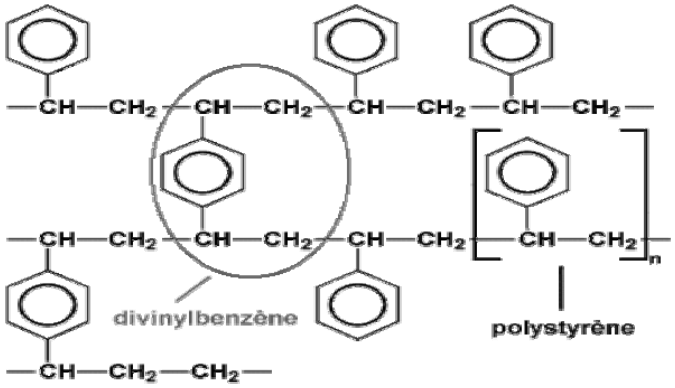


Figure 3 Molecular structure of styrene-co-divinylbenzene monolith.

Hydrophobic properties and thermal stability of PS-DVB were particular attractive features. Owing to their high hydrophobicity, PS-DVB monolith is very well-suited for the extraction of non-polar compounds. In contrast, more polar compounds are less retained during the sorption step. Their interactions with the analytes according to the hydrophobic characteristic of the sorbent are basically the π–π sites of the aromatic rings that form the sorbent structure (Fontanals *et al*., 2005).

2. Polydivinylbenzene monolith

Similar to PS-DVB monolith preparation, the inner wall of fused silica capillary tubing have to pre-treat with heterobifunctional coupling agent, γ-(trimethoxysilyl)propyl methacrylate (γ-MAPS), in order to provide an anchoring site for grafting the polymer monolith to the capillary wall.

In the polymerization process, polymerization mixture consisting of divinylbenzene which was used as a monomer to form an interconnected skeleton of cross-linked polymeric monolithic structure as shown in Figure 4. Either dodecanol or toluene was used as a porogenic agent to provide a bimodal pore structure of monolith, and dibenzoyl peroxide was used as a polymerization initiator (Svec and Kurganov, 2008). After polymerization at 70 °C for 20 h, a complete conversion of monomer to polymer was obtained.

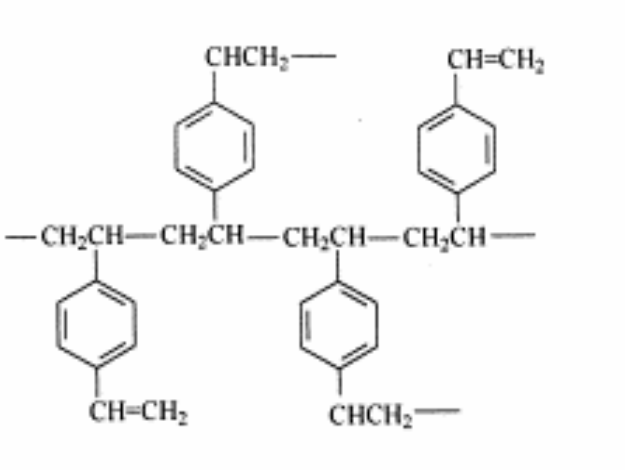


Figure 4 Molecular structure of poly(divinylbenzene) monolith.

An important feature of poly(divinylbenzene) is its very hydrophobic property and thermal stability. Thermal gravimetric analysis (TGA) measurement investigated by Svec and Kurganov (2008) indicated that porous poly(divinylbenzene) monolith did not undergo any significant thermal degradation until a temperature of 380 °C was reached. Due to very hydrophobic surface area of poly(divinylbenzene) matrix, non-polar compounds are retained more strongly than polar compounds. Similar to PS-DVB characteristic, their interactions with the analytes are basically the π– π sites of the aromatic rings that form the sorbent structure.

3. Silica monolith

In contrast to polymer monolith, silica monoliths are prepared through a two-step process via a sol-gel method which involves the sequential hydrolysis and polycondensation of alkoxide silicon precursor. The synthesis mixture consisted of Tetramethoxysilane (TMOS) as a sol-gel precursor. Polyethyleneglycol (PEG) was used as a porogen which act as a through-pore template and a solubilizer of silane reagent. Acetic acid was used as a solvent and acid catalysis hydrolysis. The addition of urea in the starting material was aimed to prepare mesopores, which provided high effective surface area. The first step was the formation of a sol of TMOS precursor via a hydrolysis process. A sol is a colloidal suspension of solid species in a liquid. The sol was converted into a gel through polycondensation of the sol, leading to a wet

structure and finally linking together to form an infinite network as shown in Figure 5. After these steps, a bimodal pore structure was obtained.

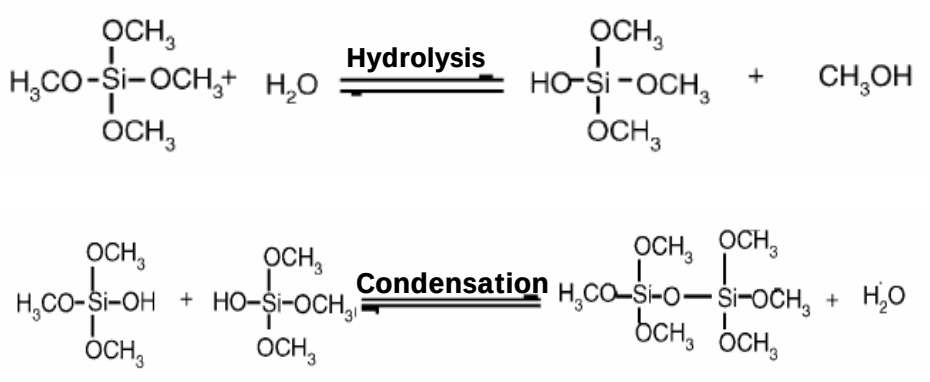


Figure 5 Sol-gel process of silica monolith.

A great advantage of monoliths lies in their relatively simple and cheap preparation in laboratory, making monolith attractive to be used as an inexpensive homemade sorbent trap. Moreover, for an on-line sorbent trap coupling with GC column, it would give benefits in avoiding high organic solvent consumption, and time-consuming sample preparation.

Nitrosamines

Nitrosamine is a class of carcinogenic, mutagenic and teratogenic compounds for animals and humans. Chemical structure of nitrosamine is shown in Figure 6.

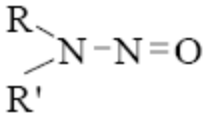


Figure 6 General chemical structure of nitrosamine.

Nitrosamines are generated in food by a reaction between nitrite and, secondary amines. Under acidic conditions the nitrite forms nitrous acid (HNO_2),

which splits into nitrosonium cation $N{=}O^+$ and hydroxide anion OH^-. The nitrosonium cation then reacts with an amine to produce nitrosamine as shown in Figure 7.

$$NO_2^- + 2\ H^+ \rightarrow NO^+ + H_2O$$
$$R_2NH + NO^+ \rightarrow R_2N\text{-}N{=}O + H^+$$

Figure 7 Formation reaction of nitrosamine.

Nitrosamines are found in many foodstuffs, especially in bacon, beer, fish, and also in meat and cheese products. Several methods have been applied for a determination of volatile nitrosamines, including solvent extraction, supercritical CO_2 extraction, steam distillation and vacuum distillation in a mixture of mineral oil and base. The review of the extraction method of nitrosamine was given in the Literature review section. However, these methods are time consuming, high energy and also require the use of toxic organic solvents.

Because US Environmental Protection Agency regulations are designed to reduce the use of solvents that are very harmful to environment and to reduce the cost of solvent disposal, better methods of extraction are needed. Superheated water extraction (SWE) was attractive to be used.

Superheated water is liquid water under pressure at temperatures between the usual boiling point (100°C) and the critical temperature (374°C). It is also known as subcritical water and pressurised hot water. As the temperature of liquid water is raised under pressure, between 100 and 374 °C, the polarity of water decreases markedly and it can be used as an extraction solvent instead of toxic organic solvent for a wide range of analytes. So in this study, SWE was developed for the determination of nitrosamines in frankfurters. SWE can be significantly faster and easier than the conventional extraction methods and does not require the use of toxic and expensive solvents. The co-extraction of lipid during the extraction can cause some serious problems to the analysis. So the use of fat-retaining adsorbent is

necessary. According to the main purpose of this research was to minimize a tedious sample preparation process in the analysis of nitrosamines compounds in frankfurters. Thus, matrix interference elimination was performed by coupling an on-line sorbent trap of monolithic column with GC column applied to GC-FID.

An integration of superheated water extraction (SWE) and on-line sorbent trap monolithic column was expected to provide cleaner, faster and cheaper analysis than the conventional method.

OBJECTIVES

1. To fabricate a reproducible capillary monolithic column and characterize its morphology.

2. To study capability of the fabricated monolithic column as an on-line sorbent trap for non-polar matrix adsorption by coupling with GC for the determination of nitrosamines in frankfurter sample.

3. To investigate sensitivity, reusability and efficiency of the coupling method between SWE-monolithic column sorbent trap-GC.

LITERATURE REVIEW

Monolithic column has emerged since the last two decades. Despite the short time, lots of their applications have been found in a variety of fields. This new category of porous media is very common and certainly well-known to be used as a stationary phase especially in HPLC and CEC, while an application in GC is fairly limited in the number. A production of monolithic columns is often a single-step process involving in situ polymerization that avoids the problems of both frit formation and packing. Due to their unique properties including highly effective surface area, highly porosity that provided a higher column permeability and the ease of preparation, these monolithic materials have recently attracted considerable attention from many different research groups. Some copolymerized monolithic materials, namely polystyrene-divinylbenzene (PS-DVB) and polydivinylbenzene, as well as silica monolithic column synthesis, characterization and their applications were summarized chronologically as follows.

Gusev *et al.* (1999) reported a preparation of porous rigid monolithic capillary columns for capillary electrochromatography (CE) using polymerizing mixtures of chloromethylstyrene, divinylbenzene and azobisizobutyronitryle (AIBN) in presence of various porogenic solvents, namely methanol, ethanol, propanol, toluene and formamide, within the silanized capillary. These columns possess positively charged surface, where acidic and basic peptides could be separated. Unfunctionalized poly(styrene-co-divinylbenzene) monolith was also used for the separation of tripeptides (Gly-Gly-Phe and Phe-Gly-Gly) in a pH 7 buffer.

Gusev *et al.* (1999) prepared a monolithic capillary column in a 75 μm id. silanized fused-silica capillary by an in situ copolymerization of divinylbenzene with styrene or vinylbenzyl chloride in the presence of porogenic solvent. It was applied for a separation of protein and peptides by micro high-performance liquid chromatography (μ-HPLC) compared with capillary electrochromatography (CEC). A silanization solution was prepared from 50% (v/v) of γ-(trimethoxysilyl)propyl

methacrylate in DMF containing 0.01% of 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) and purged with helium for 10 min. Subsequently, the capillary was heated at 120° C for 6 h, and then washed with acetone and dried with a nitrogen stream. For polymer monolith fabrication, a styrene to divinylbenzene ratio was approximately 2:1and the mixture contained 0.1% AIBN initiator. Six parts of five different porogens were mixed with four parts of monomer. The polymerization was performed in a water bath thermostat at 70 °C for 24 h. Efficiency of fabricated monolithic column in CEC was significantly higher than that in μ-HPLC in the same way as observed for capillary columns having conventional particulate packing.

Premstaller *et al.* (2000) prepared a polystyrene-divinylbenzene (PS-DVB) and octadecylated PS-DVB (PS-DVB-C18) monolithic capillary column inside a 200 μm id. silanized fused silica capillary tubing. PS-DVB mixture comprising 50 μL styrene, 5 μL ivinylbenzene, 130 μL decanol, 20 μL tetrahydrofuran and 10 mg ml^{-1} AIBN. The polymerization was performed at 70 °C for 24 h. Afterwards, the capillary was flushed with acetonitrile at a flow rate of 5.0 μL min^{-1} and finally cut into 60 mm long pieces. Octadecylated PS-DVB was synthesized followed Huber, 1993. They demonstrated excellent applications of these polymer-based materials for gradient elution ion pairing reversed-phase (IP-RP-HPLC) analysis of single and double stranded nucleic acids. Monolithic capillary columns were compared with capillary columns packed with micropellicular, octadecylated poly-(styrene/divinylbenzene) particles. An improvement in column performance of approximately 40% was obtained, enabling the analysis of 18-mer oligodeoxynucleotide with a column efficiency of more than 190000 plates per meter.

Svec and Kurganov. (2008) prepared monolithic poly(divinylbenzene) stationary phase and preliminarily studied as an analytical column applied for gas chromatography (GC) to separate 11 model compounds. A fused-silica capillary was firstly treated with 3-(trimethoxysilyl)propyl methacrylate to assure good adhesion of the monolith to the wall. The polymerization mixture consisted of 40% divinylbenzene, 52% 1-dodecanol, 8% toluene and 1% AIBN (% with respect to

divinylbenzene). The polymerization proceeded at a temperature of 70 °C for 20 h to achieve a complete conversion of monomer to polymer. Subsequently, capillaries were washed with methanol and dried in a stream of helium. Thermal gravimetric analysis (TGA) measurement result indicated highly thermal stability of polydivinylbenzene column in which the porous monolith did not undergo any significant thermal degradation until a temperature reach to 380 °C. Eleven model compounds, namely methanol, ethanol, acetonitrile, acetone, 1-propanol, methyl ethyl ketone, 1-butanol, toluene, ethylbenzene, propylbenzene, and butylbenzene, was separated using 50 cm x 100 and 320 mm id. monolithic capillary columns with a temperature gradient of 120–300 °C at a rate of 20 °C min^{-1} and an inlet pressure of 0.55 MPa. The chromatogram results exhibited very low asymmetric resolved peaks.

Coufal *et al.* (2002) prepared a monolithic capillary columns by a radical polymerization of butylmethacrylate (BMA) and ethylenedimethacrylate (EDMA) in a presence of porogenic solvent containing propan-1-ol, butane-1,4-diol and water inside a 320 μm id. silanized capillary tubing. The silanization solution containing 40 μL γ-(trimethoxysilyl)propyl methacrylate (γ-MAPS) in 10 mL 6 M acetic acid and then thermostatted at 60 °C for 20 h. The composition of polymerization mixture was optimized to attain minimum HETP of the order of tens micrometers. Various polar tested compounds were toluene, ethylbenzene, aniline, phenol, benzene, uracil and anthracene. Some chromatographic parameters, namely separation performance and selectivity, were also examined. Separation performance of 320 μm id. monolithic columns in capillary liquid chromatography (CLC) was similar to that observed for 100 and 150 μm id. that was previously reported. Moreover, the 320-μm id. column was easier to operate in CLC and exhibited a higher sample loadability than 100- and 150-μm id. column.

Oberacher *et al.* (2004) prepared a monolithic capillary column by copolymerization of styrene and divinylbenzene inside a 200 μm id. fused silica capillary using a mixture of tetrahydrofuran and decanol as a porogen. An important chromatographic features of the synthesized columns were characterized and critically

compared to the properties of columns packed with micropellicular octadecylated poly(styrene-co-divinylbenzene) (PS-DVB-C_{18}) particles. The permeability of a 60 mm long monolithic column was slightly higher than that of an equally dimensioned column packed with PS-DVB-C_{18} beads and was invariant up to at least 250 bar column inlet pressure, indicating the high-pressure stability of the monolithic columns. The specific surface area of 32-43 m^2/g determined by inverse size exclusion chromatography was obtained. Interestingly, monolithic columns showed a 3.6 times better separation efficiency for oligonucleotides than granular columns. All kind of diffusional band broadening investigated from Van Deemter Plots was reduced two to five times for monolithic columns. Moreover, batch-to-batch reproducibility (0.5-3 %) was proved to be better with granular stationary phases compared to monolithic stationary phase.

Quigley *et al.* (2004) fabricated a polystyrene-based nano-LC monolithic column within 100 and 50 μm id. silanized capillary tubings. Practical advantages of the monolithic columns when compared to a conventional packed column for the separation of protein molecules under the same conditions were compared. Nano-LC polystyrene monolithic columns were more advantageous than conventional packed columns in the analysis of protein molecules with a one-step fabrication process and the analysis time was twice faster due to the higher permeability. With the monolith the flow rate allowance was more than four times of the maximum flow rate allowed by the commercial column. The limit of detection (LOD) of ribonuclease A obtained for monolithic column was about 7.3 fmol lower than that for the commercial column, which was in the order of 41.3 fmol.

Korolev *et al.* (2006) examined influential conditions of preparation of divinylbenzene-based polymer monoliths used as a stationary phase in gas adsorption chromatography. Capillary was firstly treated with 3-trimethoxylylpropy methacrylate in acetone (30 wt %) and left to react at room temperature for 24 h. After that it was washed with acetone and dried in a helium flow. The polymerization mixture was prepared by dissolving AIBN in a monomer mixture of divinylbenzene, dodecanol

and toluene at a monomer : porogen ratio of 4 : 6. Polymerization was conducted at 70–78 °C for 30–240 min. Subsequently, the monolithic column was washed with methanol and dried in a helium stream at 120 °C for 60 min. Polymerization time, temperature and composition of the polymerization mixture affected the chromatographic properties of the columns. The monolithic capillary columns prepared under the optimal conditions had a height equivalent to theoretical plate (HETP) at a level of 20−30 μm, which is an order of magnitude below that of conventional open tubular column.

Guihua *et al.* (2007) prepared high-efficiency poly(styrene-divinylbenzene) (PS-DVB) porous layer open tubular (PLOT) within a 10 μm id. capillary column by in-situ polymerization. The polymer layer was covalently attached to the wall of capillary, thus no column frit was needed. Then the fabricated column with relatively high permeability and loading capacities was applied to analyse human epidermal growth factor receptor (EGFR), a large transmembrane tyrosine kinase receptor with heterogeneous phosphorylation and glycosylation structures, and coupled on-line with a linear ion trap MS (LTQ). Column-to-column retention time reproducibility was 3% RSD, and relative retention time was 2% RSD. Relatively high loading capacities, about 100 fmol for angiotensin I and 50 fmol for insulin, were obtained with a 4.2 m x 10 μm. id. PLOT column. Low detection levels (attomole to sub-attomole) were achieved when the column was coupled on-line with a linear ion trap MS (LTQ). Due to its open porous structure, the PLOT column demonstrated high efficiency for the separation of large peptides as well as peptides with phosphorylated and glycosylated modifications.

Schley *et al.* (2006) prepared a monolithic column based on poly-(styrene-divinylbenzene) (PS-DVB) and utilized it for both pre-concentration (in a 10 mm×0.20 mm id. format) and analytical separation (in a 60 mm×0.20 and 0.10 mm id. format) of peptides and proteins in column switching micro-scale high performance liquid chromatography. Trapping efficiency, especially for small and hydrophilic peptides, was optimized by using 0.10% heptafluorobutyric acid instead of 0.050%

trifluoroacetic acid as a solvent additive during sample loading. Using a 10 mm×0.20 mm id. monolith pre-concentration column, loadabilities between 0.5 and 1.6 ug were obtained by frontal analysis of proteins and bioactive peptides, respectively. An 11–20% decrease in the average peak widths of nine peptides was obtained upon combining a pre-concentration column with an analytical column as compared with a setup using the analytical column only. Peptide recoveries for early eluting hydrophilic small peptides and those for the medium hydrophobic peptides were in a range of 80–110%. Moreover, monolithic columns are very robust and withstand several hundreds of injections without column blocking or loss in loading capacity.

Chang et al. (2006) prepared monolithic silica capillary by sol-gel process and investigated it as an adsorbent for a pre-concentration of airborne trichloroethylene and tetrachloroethylene. The 100-, 200- and 320- μm.id. of fused silica capillaries were pretreated with 1 M NaOH at 40 °C for 3 h. A 2-mL of TMOS was added drop by drop to a solution containing 0.46 g PEG and 0.45 g urea in 5 mL 0.01 M acetic acid. The mixture was stirred at 0 °C for 30 min, and then filled into the capillary with hand-held syringe. It was allowed to react at 40 °C for 24 h., and then the temperature was raised to 120 °C for 3 h, and finally heat at 350 °C for 24 h to decompose of organic moieties. A 10 mL gas sample was flowed through the 50 mm long silica monolithic column at a rate of 1 mL min^{-1}. Any adsorbed trichloroethylene and tetrachloroethylene were then desorbed by applying 50 μL liquid hexane at a flow rate of 10 uL min^{-1} followed by GC-MS analysis. The detection limits were 0.44 μg L^{-1} and 0.34 μg L^{-1} for trichloroethylene and tetrachloroethylene, respectively. The recoveries calculated by applying a standard additions method were ranged from 81% - 117%.

Viktorova *et al.* (2007) studied an effect of synthesis conditions of divinylbenzene-based monolithic capillary column on their chromatographic characteristics. It was demonstrated that the porosity and permeability of the column change significantly even at small deviations from optimum conditions of polymerization of monolith in the column. By contrast, a minimum value of HETP proved to be only slightly sensitive to the conditions of synthesis, ranging between

10–20 μm. Conditions of polymerization of monolith produced more pronounced effect on the slope of the right branch of a van deemter curve (parameter C), with the flattest curve being observed for columns prepared under optimum conditions. A minimum value of HETP for polymer monolithic capillary columns was found to be close to that for silica gel monolithic capillary columns, but the latter characterized by C values was approximately an order of magnitude lower.

An application of monolithic columns in gas chromatography (GC) has been rarely reported, since such porous material has been initially attempted to be used as an analytical column in HPLC. Although, an application of monolith in GC is one of the least common, some interesting studies of physical and chromatographic properties of monolithic material as an analytical column deserve to be summarized as follows.

Korolev *et al.* (2006) studied permeability of monolithic silica gel capillary column with respect to helium carrier gas in gas chromatography. The permeability of monolithic capillary column was compared to that of a hollow capillary column and a column packed with finely dispersed sorbents. The resulting data showed that size of the channels in monolithic column under studied corresponded to a column packed with particles 11–16 μm in size. This was agreed with the results of electron microscopy analysis, which monolith's domains lies within 1–2 μm. Interstitial fraction (or total porosity) of the monolithic columns was found to be very high about 0.95. The results obtained by gas chromatography and liquid chromatography were found to be in good agreement. It was demonstrated that the permeability of the monolithic capillary column studied is almost three orders of magnitude lower than that of hollow capillary column with the same diameter but two orders of magnitude higher than that of columns packed with micro-scale particles.

Korolev *et al.* (2007) studied loading capacities of monolithic capillary columns based on silica gel and divinylbenzene for two carrier gases, CO_2 and N_2. It was demonstrated that efficiency of the column was more dependent on column overloading than retention times of isobutane and n-butane, especially for the CO_2

carrier gas. The loading capacity of a silica gel monolithic column decreased significantly when changing from N_2 to CO_2. For divinylbenzene columns, the loading capacity was virtually the same for both carrier gases. For both monolithic columns, the loading capacity per one meter of column length was found to be 10 times or higher than that for a standard open capillary column.

Korolev *et al.* (2007) applied monolithic capillary columns based on silica gel in high-speed gas-chromatographic separations of a five-component mixture of C1–C4 hydrocarbons. It was found that short-length monolithic columns could be used because of their high specific efficiency which allowed shortening a column dead time and duration of analysis. The column performance of about 1000 theoretical plates per second was reached. The test C1-C4 mixture was completely separated on a 58.5-cm column with an efficiency of about 18700 theoretical plates in a time shorter than 17 s. It was noted that CO_2 and N_2O should be predominantly used as carrier gases.

In our work an application of synthesized monolith was for oil and fat removal from fatty matrix prior to gas chromatographic analysis, therefore similar material, namely conventional porous polymer used as trapping sorbent was surveyed for its similar usage and only one article was reported as given below.

Dodo *et al.* (1999) applied a 100% porous polydivinylbenzene (poly-DVB) packing LC column to remove lipid material from fish extract before an analysis of several semivolatile organic pollutants including polynuclear aromatic hydrocarbons (PAHs) by gas chromatography–mass spectrometry (GC–MS). SEC of the fortified aliquot was performed at room temperature using one 10 cm x 22 mm and two 50 cm x 22 mm, 100 A° Jordi-Gel DVB columns in series The 10 cm x 22 mm column was used as a guard column. The collected fraction was isocratically eluted with methylene chloride as the mobile phase to allow concentration. The packing material was found to be robust because the column could be operated up to 900 psi. resulting in high separation efficiency. Recoveries and relative standard deviations for 18 polynuclear aromatic hydrocarbons fortified into the fish extract and cleaned up by

multiple DVB columns in series were in a range of 86 to 123% and 4 to 11%, respectively.

Other than analytical column, less common application of monolithic materials is their employment as solid-phase support for extraction and pre-concentration. The utilization of both polymer based and silica based porous monolithic materials for an on-line solid phase microextraction (SPME) has been increasingly received attention. Nowadays, the integration was mostly applied to miniaturized HPLC system. A number of the mentioned applications have been given here.

Fan *et al.* (2005) investigated an efficiency of poly(methacrylic acid-ethylene glycol dimethylate) monolithic capillary column as an in-tube solid-phase microextraction (SPME) for an extraction of amphetamine, methamphetamine and their methylenedioxy derivatives in complicated urine samples. The monolithic capillary column showed high extraction efficiency towards target analytes, due to its larger loading amount of extraction phase than conventional open-tubular extraction capillaries and a convective mass transfer procedure provided by its monolithic structure. The extraction mechanism was studied, and the results indicated that the extraction process of target analytes was involved with hydrophobic interaction and ion-exchange interaction. The polymer monolith in-tube SPME-HPLC system with UV detection was successfully applied to the determination of those compounds in urine samples, yielding the detection limits of 1.4–4.0 ng mL^{-1}. Method reproducibility (RSD < 2.9%) was found over a linear range of 0.05–5 μg mL^{-1}, and the time for overall analysis was only about 25 min.

Bones *et al.* (2006) developed an on-line solid phase extraction using a micro-reversed-phase monolithic silica column to simplify pre-concentration method for ultra-trace level of pharmaceuticals in natural waters coupled to LC-UV-ESI-MS. Initially, a use of 10.0 mm x 4.6 mm i.d. C_{18} monolithic silica guard cartridge (Chromolith, Merck) for an on-line rapid pre-concentration of ultra-trace levels of

pharmaceuticals from large volume natural water samples was investigated. Effects of sample loading conditions such as pH, loading rates, precision and reusability of short monoliths pre-concentrator were also investigated. After that, the on-line monolithic pre-concentrator column was connected to an analytical separation section which was performed using two 100.0 mm x 4.6 mm i.d. Chromolith Performance RP-18e silica columns joined in series using a Chromolith zero dead volume column coupler. Acceptable recoveries of more than 70% were obtained for majority of compounds investigated and the detection limits were lied in a low ng/l region. The monolithic column could be washed and conditioned on-line with no sample carryover and used reproducibly for up to eight extractions each. The method allowed very rapid trace enrichment from large volume (500 ml) samples with minimal sample handling.

Wen *et al.* (2006) developed an on-line method for simultaneous determination of four endocrine disruptors (17β-estradiol, estriol, bisphenol A and 17α-ethinylestradiol) in environmental water by coupling in-tube solid-phase microextraction (SPME) to high-performance liquid chromatography (HPLC) with fluorescence detection. A poly(acrylamide-vinylpyridine-*N*,*N*_-methylene bisacrylamide) monolith was synthesized inside a polyether ether ketone (PEEK) tube and it was selected as an extraction medium. Several parameters including flow-rate, extraction time, pH, inorganic salt and organic solvent content in the sample matrix, were investigated to achieve an optimum extraction performance. A water sample was simply prepared by filtering it through a nylon membrane filter and then adjusting the pH to 6.0 with phosphoric acid before a chromatographic injection. Low detection limits (S/N = 3) and quantification limits (S/N = 10) were achieved in a range of 0.006–0.10 ng/mL and 0.02–0.35 ng/mL from spiked lake waters, respectively. The method reproducibility obtained by intra- and inter-day precisions gave the RSDs less than 12 and 9.8%, respectively.

Nitrosamines are well-known carcinogenic compounds contaminated in food. An original analytical methods for nitrosamines determination in water was developed by the Canadian Ministry of Environmental (MOE, 1990), since their appearance has been confirmed in drinking water and groundwater. The procedure was based on EPA

method 607, which describe a liquid-liquid extraction in a separatory funnel at neutral pH, using NDMA-d6 as a surrogate/internal standard, followed by an isotope dilution and detection by single ion monitoring (SIM) - GC-MS.

A number of extraction and clean-up procedures for the determination of N-nitrosamines in food have been described, these included steam distillation, solvent extraction, supercritical fluid extraction and solid-phase extraction. Nowadays, gas chromatography (GC) and high-performance liquid chromatography (HPLC) combined with detection system using either thermal energy analyzer (TEA) or mass spectrometer (MS) were general methods for quantifying nitrosamines after the extraction. A number of analytical methods of nitrosamine determination in food were summarized chronologically as follows.

Maxwell *et al.* (1993) applied supercritical fluid extraction (SFE) to couple with an offline trapping technique for extraction of nitrosamines from frankfurters. The optimum SFE conditions were as follows: extraction temperature at 40 °C, valve temperature at 110 °C, static time of 2 minutes, dynamic time of 20 minutes, CO_2 flow rate of 3.0 L min^{-1} and pressure of 680 bar. In this work, silica gel was used as a solid-phase extraction for a clean-up procedure. A 30% ethyl ether in dichloromethane was used to elute N-nitrosamines from the extraction column, 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%.

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) used as an internal standard. The aqueous filtrate was further extracted with dichloromethane, pre-concentrated, and then cleaned up with basic alumina with dichloromethane solvent. After wards an analysis of eluent was performed by gas chromatography combined with thermal energy analyzer (GC-TEA). The recovery of volatile nitrosamines lied between 60

and 90%, depending on both types of compound and types of food sample. NDMA was detected in several salted and dried fish at levels ranging from trace amounts of 66.5 ug/kg, while NDMA and NPYR were frequently detected in several vegetables at levels ranging between 1-95.1 ug/kg and 0-146 ug/kg, respectively.

Andrade *et al.* (2005) applied headspace sampling coupling with solid phase microextraction and gas chromatography-thermal energy analyzer detection (HS-SPME-GC-TEA) to determine volatile nitrosamines, namely NDMA, NDEA, NPIP and NPYR, in sausages. Two fused silica fibers, polyacrylate (PA)-coated fiber and polydimethylsiloxane-divinylbenzene (PDMS-DVB)-coated fiber were investigated for extraction of nitrosamines in vapor phase. Various factors including influence of equilibrium time, ionic strength, extraction time and extraction temperature were evaluated by employing a factorial fractional design. The results showed that NDMA and NDEA were commonly detected in sausages. 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 °C temperature, 10 min equilibrium time, 25 min extraction time and no ionic strength adjustment. The method was rapid with sufficient linearity, sensitivity and recovery.

Yurchenko *et al.* (2006) determined the level of five nitrosamines namely (N-nitrosodimethylamine, N-nitrosodiethylamine, N-nitrosodibutylamine, N-nitrosopiperidine and N-nitrosopyrrolidine) in 294 various fish samples and in 77 oil samples during 2001-2005. Two-step solid-phase extraction with Extrelut and Florisil sorbents was used for a clean-up method. The sample was initially mixed with NaOH, applied to the Extrelut column and then eluted with hexane/dichloromethane solution. After that, the eluent was applied to the Florisil sorbent and nitrosamine were finally eluted with dichloromethane/methanol 95:5 (v:v) solution. An analysis of eluent was performed with GC-MS with positive-ion chemical ionization (PCI). Ammonia was used as a reagent gas in a selected ion monitoring mode (SIM) with pulsed splitless injection. In this work, limit of detection and of quantification of nitrosamines were approximately 0.10 and 0.35 ug/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 ug/kg, respectively. Their recoveries in smoked fishery products varied from 79 to 88%.

Grebel *et al.* (2006) developed a method to determine N-nitrosamine in water by using solid-phase microextraction (SPME). Four SPME fibers namely Carbowax/divinylbenzene (CW/DVB), Carboxen/polydimethylsiloxane (CAR/PDMS), polyacrylate (PA), and polydimethylsiloxane/divinylbenzene (PDMS/DVB) were investigated for NDMA extraction efficiency. Eight parameters, namely SPME fiber coating, extraction mode, NaCl concentration, pH, sample volume, were optimized, as well as headspace optimization of extraction temperature and time. 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 °C and extraction time of 45 min. The recoveries of nitrosamines extraction were lied between 73-259% and the detection limits of this method for NDMA ranged from 30 to 890 ng L^{-1}.

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 non-polar, moderate-polar and. polar compounds. Its interesting applications are extraction of PAHs, pesticides from environmental samples, essential oils from plant materials, flavors and fragrances from plant material and food. Some superheated water applications in food have been given here.

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 composition. 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.

Fernandez and Luque de Castro (2003) developed a method for cholesterol removal from low- and high cholesterol-content foods based on superheated water extraction. Parameters affecting the extraction process and preconcentration step were optimized by a central composite experimental design. The SWE optimum conditions were 135 °C with 3.0 mL 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 C 18-bonded cartridge was used and the retained cholesterol was eluted with 2 mL of 1:20 methanol-trichloromethane. The method validation was obtained 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 sample was 105%. Precision of the method in term of relative standard deviation was less than 6.5% for all substances.

MATERIALS AND METHODS

Materials

1. Reagents

For polymer-based monolithic column synthesis, γ-(trimethoxysilyl)propyl methacrylate or γ-MAPS (98.0%) obtained from Acros (New Jersey, USA) and glacial acetic acid from BDH (Poole, England) were used as silanization solutions. Styrene (99.0 %) from Fluka (USA) and divinylbenzene (80.0%) from Aldrich (Milwaukee, WI, USA) were used as monomers. Decanol (99.0%) from Acros (New Jersey, USA), Tetrahydrofuran (99.9%) from Fisher (Loughborough, UK) and dodecanol (>99.5%) from Fluka (USA) were used as porogenic agents. Dibenzoyl peroxide (75%) from Acros (New Jersey, USA) was used as an initiator. Fabricated columns were flushed with analytical grade acetonitrile from Ajax Finechem (Auckland, New Zealand) and dried with 99.99% oxygen free nitrogen gas (OFN).

For silica-based monolithic column synthesis, polyethyleneglycol (PEG) (MW = 10,000 g mol^{-1}) (AR grade) from Fluka (Germany), urea (AR grade) from Merck (Darmstadt, Germany), sodium hydroxide and tetramethoxysilane (TMOS) (98.0%) from Merck (Darmstadt, Germany) were used.

Both of polymer-based and silica-based monolithic column were performed inside a 0.32 mm i.d. x 10 m bare fused- silica capillaries tubing with polyimide coating purchased from SGE (SGE Analytical Science, Melbourne, Australia).

Double deionized water having a resistance of 18 MΩ was used for superheated water extraction (SWE). 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 1000 ppm stock standard solution in analytical grade methanol (Merck, Darmstadt, Germany). Standard nitrosamines, namely *N*-

nitrosodiethylamine of NDEA (99.0%) from Fluka (Buchs, Switzerland), *N*-nitrosopiperidine or NPIP (99.0%) from Sigma (Steinheim, Germany) and *N*-nitroso-pyrrolidine or NPYR (99.0%) from Aldrich (Milwaukee, WI, USA) were used in this study. Each 1000 ppm stock nitrosamine standard solution was prepared in methanol.

For an aqueous extraction step, NaCl (99.5%) used 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). Dichloromethane (99.5%) from BDH (Poole, England) was used as an organic trapping solvent.

2. Apparatus

A HPLC pump (LC-10ADVB, Shimadzu, Japan) and an oven (Binder, Germany) were used to fabricate a monolithic column and the synthesized column was characterized by using a scanning electron microscope (JSM-5600LV, JEOL, Japan).

The SWE extraction system was equipment built in our laboratory. It consisted of double deionized water contained in a glass reservoir, a HPLC constrametric 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, Massachusette, 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 that a 0.004 mm. id. x 1/16 inch od. tubing was used. The schematic diagram of superheated water extraction is shown in Figure 8.

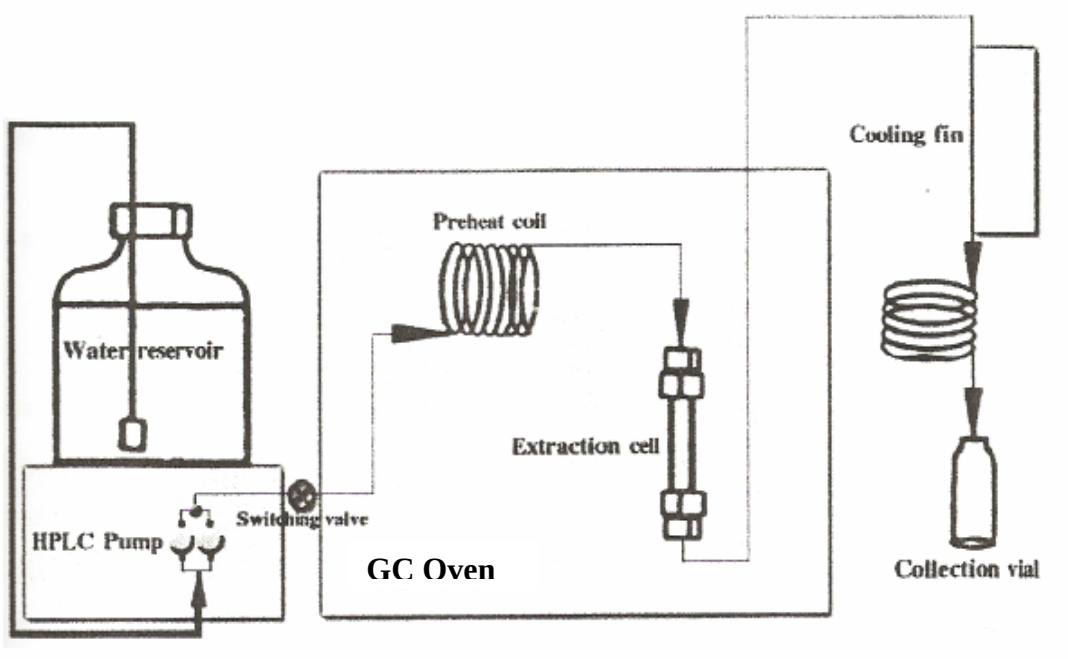


Figure 8 Schematic diagram of superheated water extraction.

After the superheated water extraction the organic layer was dried with anh. Na_2SO_4, centrifuged with an EBA 20 centrifuge (Hettich Zentrifugen, Germany) and filtered through a 13 mm x 0.45 μm nylon filter with glass microfiber (GMF) obtained from vertical chromatography (Bangkok, Thailand).

Chromatographic separation and detection were carried out using a HP5890 series II Gas Chromatograph with flame ionization detector (Hewlette Packard, Polo Alto, CA, USA). A gas chromatograph with mass spectrometric detection (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). From an injection port a fabricated monolithic column was connected with either a 0.32 mm id. x 30 m x 0.50 μm film thickness 5% phenyl 95% dimethylpolysiloxane (ZB-5) column (Phenomenex, USA) or a 0.25 mm i.d. x 25 m x 0.52 μm film thickness 5% phenyl 95% methylsilicone (HP-5) column (Hewlett-Packard, Polo Alto, CA, USA) by using a 4.0 cm glass connector from Alltech (Deerfield, IL, USA) for separation prior to flame ionization or mass detection.

Methods

1. Monolithic column fabrication

1.1 Polystyrene-divinylbenzene (PS-DVB) monolithic column

1.1.1 Silanization of the capillary's inner wall

Silanization solution was prepared from 1.817 g of glacial acetic acid mixed with 20 μL of γ-(trimethoxysilyl)propyl methacrylate into a 5.00 mL volumetric flask and adjusted to the volume by decanol. The formulation of silanization method was followed Coufal *et al*. (2002). Due to very small diameter of a 0.32 mm i.d. x 100 cm bare fused silica capillary tubing, the prepared solution can not be filled into the tubing by using a typical syringe. The solution was then poured into a tightly sealed vial with liner. The liner was penetrated with a typical 5 or 10 mL plastic syringe and a 0.32 mm. i.d. x 100.0 cm bare fused silica tubing of which one end dipped into the solution and the other end into an empty vial as shown in Figure 9. The plastic syringe was used as a hand pump to rinse and fill the solution into the bare fused silica capillary. When completely filled, both ends of the capillary were tightly sealed with septum. The sealed capillary was subsequently incubated in an oven at 60 °C for 20 h. After cooling down to room temperature, it was rinsed with acetone to remove the silanizing solution, dried with a gentle flow of nitrogen gas for 1 h and finally cut into 10.0 cm with a capillary cutter.

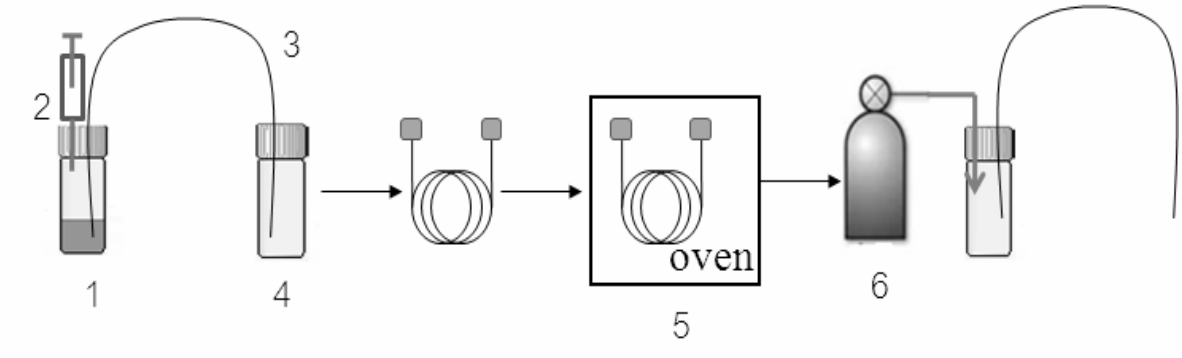


Figure 9 Schematic diagrams of silanization in capillary: 1, silanization solution; 2, plastic syringe; 3, fused silica capillary tubing; 4, waste vial; 5, oven; 6, nitrogen gas.

1.1.2 In-situ polymerization of PS-DVB monolith

Polystyrene-divinylbenzene (PS-DVB) monolith was formed inside the silanized capillary tubing by an in situ polymerization of a styrene-divinylbenzene mixture in the presence of a suitable porogen. Polymerization mixture consisted of 200 μL styrene as a monomer, 200 μL divinylbenzene as a cross-linking agent, 520 μL decanol and 80 μL tetrahydrofuran as a porogenic agent, and 10 mg mL^{-1} dibenzoyl peroxide as an initiator. Preparation of in-situ PS-DVB polymerization was performed by following the method of Premstaller *et al*. (2000). Each 0.32 mm. i.d. x 10.0 cm. pre-treated capillary tubing was subsequently filled with the solution about 5 cm via capillary effect as described in section 1.1.1. Both ends of the capillary were tightly sealed and put in an oven at 70 °C for 24 h to polymerize. Afterwards, the capillary was flushed with acetonitrile at a flow rate of 10 uL min^{-1} for 3 h by using a high pressure pump in order to remove the porogen and monomeric residues. Finally, it was purged with nitrogen gas at ambient until dryness to remove any volatiles.

1.2 Polydivinylbenzene (poly-DVB) monolithic column

1.2.1 Silanization of the capillary's inner wall

The same silanization solution and the same process used in 1.1.1 were also used for the inner wall capillary tubing pretreatment for polydivinylbenzene (poly-DVB).

1.2.2 In-situ polymerization of poly-DVB monolith

Polydivinylbenzene (poly-DVB) monolith was formed inside 0.32 mm. i.d. x 10.0 cm pre-treated capillary tubing. Polymerization mixture contained 400 μL divinylbenzene as a monomer, 520 μL dodecanol and 80 μL toluene as a porogenic agent, and 10 mg mL^{-1} dibenzoyl peroxide as an initiator. In-situ poly-DVB polymerization was achieved by following the method of Svec and Kurganov (2008). Each 0.32 mm. i.d. x 10.0 cm. pre-treated capillary tubing was subsequently filled with the solution about 5 cm via either capillary effect or a plastic syringe used as a hand pump, similar to one previously described in section 1.1.1. Both ends of the capillary were tightly sealed and put in an oven to polymerize at 70 °C for 20 h. After polymerization, the column was flushed with methanol at a flow rate of 10 μL min^{-1} for 3 h with a high pressure pump in order to remove the porogen and monomeric residues and then it was purged with nitrogen at room temperature until dryness to remove any volatile.

1.3 Silica monolithic column

A fabrication method for silica monolithic column was similar to one previously reported (Tanaka, 2002). In brief, a bare fused silica capillary tubing (0.32 mm. i.d. x 100 cm) was firstly pretreated with 1 M NaOH. Both ends were tightly sealed with septum and heated in an oven at 40 °C for 6 h. After that the tubing was washed with deionized water and dry by blowing with nitrogen gas. A sol-gel solution for silica monolith synthesis was prepared by mixing 400 μL of tetramethoxysilane

(TMOS) with 0.088 g polyethyleneglycol (PEG) and 0.09 g of urea in 0.01 M acetic acid. The mixture was stirred in an ice bath for 45 min and then filled into the pretreated capillary tubing. Subsequently, it was placed in an oven at 40 °C for 24 h, after that the oven temperature was raised to 120 °C for 6 h. After cooling down to room temperature, it was finally washed with methanol to remove any residue, and then dried at room temperature with nitrogen gas.

2. Monolithic column surface characterization by scanning electron micrograph (SEM)

Well-fabricated monolithic PS-DVB, poly-DVB and silica columns were cross-sectional cut into 0.50 cm long pieces with a capillary cutter. The specimens were stuck on an aluminum stud cell by using a double-sided carbon tape and then coated with gold nanoparticle for 5 min by using an SPI Sputter (IB-2, Eiko engineering, Japan). After that their morphologies were characterized by using a scanning electron microscope (JSM-5600 LV, JEOL, Japan). The scanning electron micrograph was operated at 20 kV.

3. Preliminary test of monolithic short column as a sorbent trap

3.1 Gas Chromatographic set up

Chromatographic and quantitative analysis was performed by using GC-FID. Both injector and detector were kept constant at 250°C and 99.99% oxygen free nitrogen was used as carrier gas. *N*-nitrosodipropylamine was used as an internal standard for quantification. The oven temperature program was held at 60 °C, then ramped from 60 °C to 100 °C at 7 °C min^{-1} and held for 8 min, then raised to 120 °C at 7 °C min^{-1} and held for 5 min. The connection of fabricated monolithic column was shown in Figure 10. In brief, a 0.32 mm id. x 5.0 cm bare fused silica tubing was firstly connected with an injection port of the GC at one end and the other end connected with a fabricated monolithic column where a glass connector was leaded to

a capillary GC column (ZB-5) or a 0.32 mm i.d. x 10 m bare fused silica tubing. This setup provided an ease of replacement of fabricated monolithic column.

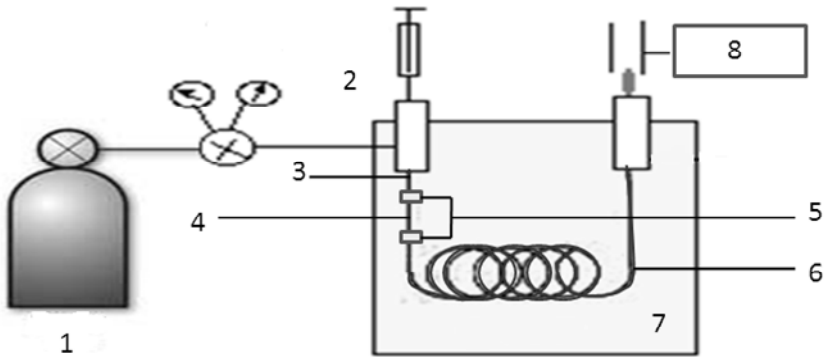


Figure 10 Schematic diagrams of an on-line coupling between monolithic sorbent trap and gas chromatographic column: 1, carrier gas (N_2); 2, injection port; 3, 0.32 mm i.d. x 5.0 cm bare fused silica capillary; 4, fabricated monolithic column; 5, glass connector; 6, a 0.32 mm i.d. x 10 m fused silica capillary tubing or an analytical capillary column; 7, GC oven; 8, flame ionization detector.

3.2 Separation of oil added nitrosamines sample on an analytical column with and without monolithic sorbent trap

The fabricated monolithic columns were preliminarily studied as a sorbent trap for an analysis of nitrosamines in a fat fortified sample applied to GC-FID. A fat fortified sample was prepared from 250 uL of 1000 ppm nitrosamines in methanol mixed with 250 uL of refined rice bran oil (Chim brand, Amornchai, Ayuddhaya, Thailand), and the volume was finally adjusted to 1.00 ml with dichloromethane.

In order to avoid a ZB-5 capillary column to be contaminated from non-polar matrix compounds, the experiment was performed in three sequential steps. Firstly, one microliter of the test fortified sample was repeatedly injected with splitless mode into a 0.32 mm id. x 10 m bare fused-silica tubing to get a chromatogram with all eluted peaks of fat and nitrosamines. Secondly, the same

sample was applied to a 0.32 mm i.d. x 4.0 mm fabricated monolith connected with the 0.32 mm id. x 10 m fused silica tubing. Finally, the sample was introduced to the same monolith connected with a ZB-5 analytical column. Schematic diagram of the three steps is illustrated in Appendix Figure 3.

3.3 Separation of nitrosamines on an analytical column coupled with a monolithic sorbent trap

Effect of fabricated monolithic column, namely PS-DVB, poly-DVB and silica, used as an on-line sorbent trap for a simultaneous separation of nitrosamines compounds were primarily studied. The experiment was performed by connecting the fabricated monolithic capillary prior to a ZB-5 column and the column flow rate was adjusted to 1.4 mL min^{-1}. The mixture was prepared by mixing each 200 µL of stock NDEA, NPYR, NPIP standard solution with a 100 µL of NDPA and the total volume was adjusted to 1000 µL with dichloromethane. One microliter of the solution was injected with splitless mode. Each type of fabricated columns were examined five times (n = 5) under the same GC-FID condition.

4. Superheated water extraction (SWE)

4.1 Standard solution preparation

Nitrosamine standard stock solutions were prepared by dissolving 0.0300 g of each standard with 30.00 mL of methanol. The internal standard was prepared by dissolving 0.0200 g NDPA in 20.00 mL of methanol and the final concentration of all nitrosamines were 1000 ppm.

4.2 Preparation of frankfurter samples

Refrigerated frankfurter samples were purchased from local supermarket in Bangkok. After cooling down to room temperature, the sample was chopped into small pieces and homogenized by hand mixing. After that the chopped frankfurter

was weighted into 1.0 g, spiked with 20 uL each of nitrosamine stock solution, and left for solvent evaporation at room temperature for 10 min

4.3 Superheated water extraction (SWE) procedure

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 Meric 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 sample. The cell was inserted with glass wool at both ends to prevent the frit being plugged and then closed with 2-um stainless steel frits and screw caps at the either end. Both preheating coil and extraction cell were placed in an oven that was maintained constant at 140 °C for 5 min. Afterwards, the water was flowed through the sample cell at a flow rate of 1.0 mL min^{-1} for 10 min. 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 to a cooling system consisting of a stainless steel cooling coil wrapped with aluminium fins where the high temperature of the extractant was reduced in order to avoid loss of some volatiles. The aqueous extract was collected in a vial and closely sealed for further cleaning up step.

4.4 Cleaning up sample

After SWE, a 10 mL extract was added with 2 g sodium chloride as a salting out reagent and further extracted with 2.0 mL dichloromethane as a trapping solvent. A cloudy emulsion occurred in the vial and transferred to centrifuge at 1,100 rpm for 7 min. After that the dichloromethane layer was taken to filter through a 0.45 µm Nylon 6,6 membrane with glass microfiber. A clear solution was obtained and then dried with anhydrous sodium sulfate. In order to pre-concentrate, the organic phase was purged with nitrogen gas at room temperature and adjusted to 100 µL with dichloromethane.

5. Monolithic sorbent trap after SWE

5.1 Sensitivity of nitrosamines in the presence of silica monolith

To study if the sorbent trap reduces the nitrosamine sensitivities, a comparison between the sensitivity of NDEA, NPYR, NPIP and NDPA on flame ionization detector in the presence and absence of silica monolith sorbent trap was performed. Standard solution of each nitrosamine was prepared in the mass ranged of 2 μg – 400 μg. One microliter of each standard solution was injected to the GC with and without a connection of a short silica monolithic column prior to an analytical column (ZB-5) under the same chromatographic optimal condition. The concentration range close to the detection limit was repeatedly injected to find a lowest concentration which provided a peak height of 2-3 times to baseline noise.

A graph was plotted between peak heights and mass injected. Linear regressions, slope, intercept, correlation coefficient, standard deviation at very low concentration and detection limit were calculated and a comparison of each parameter obtained from the results for the absence and presence of silica monolithic column was performed.

5.2 Capability and reusable of a short silica monolithic column

A 1.0 g of unfortified frankfurter sample was subjected to SWE under optimal conditions. After SWE, a 10 mL extract was cleaned up as previously described. The organic solution was purged with nitrogen gas at the room temperature to pre-concentrate and the volume was finally adjusted to 100 μL with dichloromethane. In order to avoid an analytical column (ZB-5) to be highly contaminated from the non-polar matrix such as fat and oil from the frankfurter extracted, a 0.32 mm. i.d. x 10 m bare fused silica capillary tubing coated with polyimide was replaced the ZB-5 analytical column.

The frankfurter sample matrix was firstly investigated by injection of the SW extract of the unfortified frankfurter extract into bare fused silica tubing. The GC temperature program was started at 60 °C, then ramped from 60 °C to 220 °C at 15 °C min^{-1} and held at 220 °C for 5 min to ensure that high molecular weight matrix were completely eluted. After that, 1 μL of unfortified frankfurter extract were repeatedly injected onto 0.32 mm i.d. x 3.0 cm fabricated silica monolithic column connected with the bare fused silica tubing with a glass connector. Under chromatographic optimal conditions a number of injections before the fat leaching were examined. Triplicate analysis (n=3) was studied for monolithic column, then the capability and a number of recycling of silica monolithic sorbent were investigated.

5.3 Simultaneously separation and detection of nitrosamines

A 1.0 g of frankfurter sample was fortified with each 20 μL of 1000 ppm standard stock NDEA, NPYR, and NPIP solution and the fortified sample was subjected to SWE under optimal condition. After SWE, the clean up method was liquid-liquid extraction with dichloromethane as previously described in section 4.4. Finally, 10 μL of 1000 ppm standard stock NDPA solution was used as internal standard for quantification and the volume was adjusted to 100 μL with dichloromethane.

Initially, the mixture of standard solution was applied to the ZB-5 analytical column in the absence of silica monolithic column. A following injection of the same solution was applied to the ZB-5 in the presence of short silica monolithic column. The final introduction was the injection of fortified frankfurter extract into the ZB-5 in the presence of short silica monolithic column. These studies were performed under the same GC-FID optimal conditions.

5.4 Injection-to-injection repeatability and column reproducibility

As a silica monolithic sorbent trap column may be reusable for the analysis of nitrosamine compounds in a frankfurter extract, column repeatability and reproducibility for quantitative analysis were consequently investigated.

To study an injection-to-injection repeatability of monolithic column, fortified frankfurter extract was injected onto the same monolithic sorbent trap column connected with a ZB-5 under GC-FID optimal condition. The sorbent trap column were repeatedly injected (n=5) five times. Standard deviation (SD) and relative standard deviation (%RSD) for injection-to-injection repeatability were calculated.

To study a column-to-column reproducibility of monolithic column, the same spiked frankfurter extracted sample was repeatedly injected onto a short silica monolithic sorbent trap connected with the ZB-5 column. Four monolithic columns (n=4) were studied. Standard deviation (SD), relative standard deviation (%RSD) for each monolithic column were calculated and compared in terms of reproducibility.

Identification of extracted nitrosamines was confirmed by GC-MS analyses. Spectra for identification of the compounds were obtained by a direct splitless injection of 1 µL of each extract solution into a 0.25 mm i.d. x 25 m x 0.52 µm film thickness 5% phenyl 95% methylsilicone (HP-5) column with the same temperature program applied for the GC-FID. The injector and detector temperature were kept at 250 °C and 280 °C, respectively. The mass detector was performed in an electron ionization (EI) mode with electron energy of 70 eV. Helium gas was used as a carrier gas. Temperatures of the ion source and of the quadrupole mass analyser were kept at 200 °C and 100 °C, respectively. To confirm NDEA, NPYR, and NPIP in frankfurter a selected ion monitoring (SIM) technique was applied by choosing molecular ions at m/z 102, 100 and 114 respectively, while the m/z of 70 was selected for NDPA internal standard molecular ion.

6. Efficiency of the method

6.1 Detection limits by GC-MS

Each calibration curve of NDEA and NPIP was prepared from an injection of low mass range between 30 to 70 ng. The mass range between 50 to 90 ng was injected for NPYR. The graph was plotted between peak area ratio of analyte and internal standard concentration. Linear regressions, slope, intercept, correlation coefficient, and standard deviation were subsequently calculated.

6.2 Recoveries

Recoveries of fortified frankfurter extract under the optimal condition were also determined. A 1.00 g of sample was fortified with 60 µg each of NDEA, NPIP and NPYR and then extracted by SWE under optimal conditions. The SW extract was added with 2 g NaCl for salting out, cleaned up and pre-concentrated by liquid-liquid extraction with 2 mL dichloromethane. The organic layer was separated, centrifuged and filtered through a 0.45 µm nylon 6,6 filter with glass microfiber. A 60 µL of 1000 ppm *N*-nitrosodipropylamine was added 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.

RESULTS AND DISCUSSION

As monolithic material fabricated in a capillary was of interest to be utilized to trap fat and oil for nitrosamine determination, a number of fabrication methods were followed several research reports as mentioned in the previous section. However, three monolithic capillaries, namely PS-DVB, poly-DVB and silica, were synthesized, and in our work their characterization was performed for monolithic characteristic verification prior to examination as a sorbent trap.

1. Polystyrene-co-divinylbenzene (PS-DVB)

Since PS-DVB is generally used in chromatography as a non-polar packing material, it was therefore postulated the feasibility of PS-DVB monolith to be used as a sorbent trap for non-polar fat and oil.

1. 1 Polystyrene-co-divinylbenzene (PS-DVB) monolithic column synthesis

Initially, the inner wall of a 0.32 mm i.d. bare fused silica capillary tubing was treated with a silanization solution containing γ-(trimethoxysilyl)propyl methacrylate or γ-MAPS. γ-MAPS is a heterobifunctional coupling agent to provide anchoring sites for grafting of silanol groups on silica surface with the styrenic monomer.

After the silanization process, the homogeneous polymerization mixture containing styrene, divinylbenzene, decanol, toluene and dibenzoyl peroxide was carefully filled into pre-treated capillary tubing and tightly sealed with septum in order to avoid air bubble or trace oxygen from air, which is an effective inhibitor of free radical polymerization. Polymerization mixture composition and condition was similar to one previously report (Premstaller *et al*., 2000), except that azobisisobutyronitrile (AIBN) was replaced with dibenzoyl peroxide to be used as a thermal initiator, since AIBN is chemically explosive, then not allowed to import.

With a reaction at 70 °C for 24 h the PS-DVB monolith was formed inside the pre-treated 0.32 mm i.d. x 10 cm capillary tubing.

The newly fabricated column was characterized by using a scanning electron microscope, giving SEM micrographs presented in Figure 11.

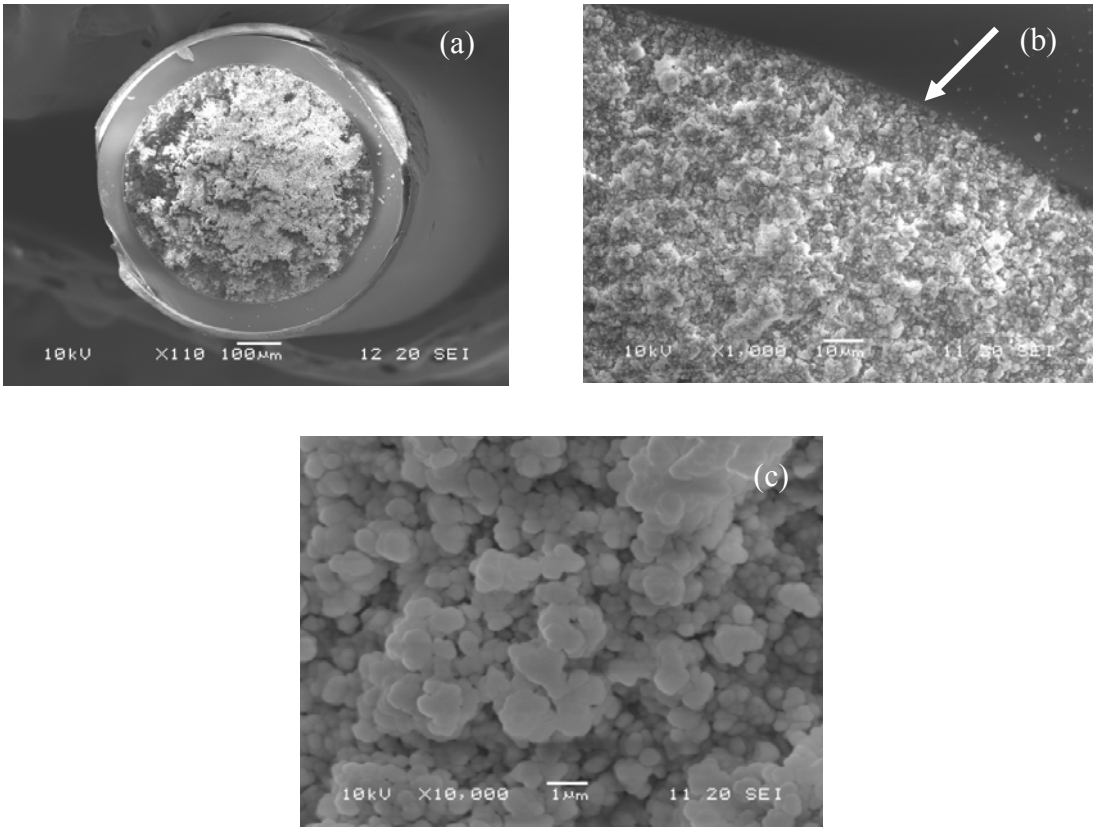


Figure 11 Scanning electron micrographs of (a) PS-DVB monolithic packing in a 0.32 mm i.d. fused silica capillary of which (b) the inner wall pretreated with γ-(trimethoxysilyl)propyl methacrylate and (c) a cauliflower structure of typical polymer monolith.

In polymerization process styrene and divinylbenzene were used as a monomer and a cross-linking agent, respectively, to form an interconnected skeleton of monolithic structure. Decanol and tetrahydrofuran were used as a porogenic agent to provide a bimodal pore structure of PS-DVB monlith and dibenzoyl peroxide was used as a polymerization initiator (Premstaller *et al*., 2000). A highly cross-linked

porous monolith, which was bonded to the silanized wall of capillary, was formed inside 0.32 mm i.d. capillary tubing as illustrated in Figure 11 (a). The cleft between the monolith and the inner wall of the capillary was not observed as shown in Figure 11 (b). Such evidence confirmed that silanization process facilitated a strong attachment between the silica surface and the styrenic monolith. Figure 11 (c) shows the cauliflower structure of fabricated PS-DVB monolith, which is a typical structure of typical polymer monolith. It was found that the PS-DVB monolithic structure consists of small globuli about 0.5 um that were slightly fused together into a single piece to form a rigid yet highly permeable support and provided high effective surface area. These results confirmed that dibenzoyl peroxide can be used instead of AIBN. The effective surface area about 32-43 m^2 g^{-1} and the total porosity by inverse size exclusion chromatography (ISEC) about 0.70-0.71 cm^3 g^{-1} (70-71%) was obtained (Oberacher *et al*., 2004).

According to a single step preparation by an in situ polymerization, hydrophobic properties and thermal stability of PS-DVB monolithic column, the feasibility of fabricated PS-DVB monolith as a non-polar sorbent trap were further investigated.

1.2 Preliminary study of fabricated PS-DVB monolith as a sorbent trap

High molecular weight matrix components such as lipids are frequently present in fatty food extract and need to be eliminated to allow a more definitive identification of lower limit residues and to minimize adverse effects on the detection instruments and compounds of interest. A cleaning-up of fatty samples is very tedious and time consuming, and sometimes more than one step is required to remove lipids. The commonly post-clean-up procedures were used, for example adsorption columns or solid-phase extraction (SPE). However, some drawbacks of conventional off-line SPE were slow separation, high organic solvent consumption and tedious work. In order to improve the clean-up step of fatty extract prior to analysis, this study was focused on a development of fabricated monolithic column as an on-line sorbent trap applied to GC-FID.

Nowadays, monolithic materials were widely used in many applications including an on-line SPE, to HPLC. Separation of interested analyte from matrix compounds was based on a mechanism of interaction between sorbent and analyte similar to that of off-line SPE. In a typical setup for an on-line SPE-HPLC, both extraction and separation columns are connected to a six-port injection valve. The analyte of interest can be separated from matrix compounds by the utilization of six-port switching valve (Svec F, 2006).

In contrast to HPLC, six-port column switching valve was not available for a typical GC instrument. In this study, the set up of monolithic column as an on-line sorbent trap applied to GC-FID was performed as described in section 3.1. An extraction of analyte from matrix compounds was controlled by the chemistry of sorbent materials and temperature programming. First, extracted sample in an organic solvent was injected and then evaporated at the injection port. Low temperature at the beginning of the temperature program was used to condense vapor sample components, allowing their adsorption on the monolithic sorbent. Subsequently, raising temperature released the analytes out of the monolithic sorbent and passed to an analytical column. The feasibility of an in situ trapping of lipids from fatty samples by using fat retaining monolithic sorbents coupling with temperature program in the analysis of nitrosamines was investigated.

1.2.1 Matrix investigation of rice bran oil in dichloromethane

The experiment was aimed to examine an eluting temperature range of oil matrix. In this study rice bran oil was imitated the fatty matrix of nitrosamine extract from food. As it could not be directly applied to the ZB-5 column because the column would be contaminated and deteriorated, it was then initially investigated by an injection of one microliter of refined rice bran oil dissolved in dichloromethane into a 10 m x 0.32 mm.id bare fused silica tubing, which was used as a connecting tool between injection port and detector. Temperature program was optimized, giving a chromatogram shown in Figure 12.

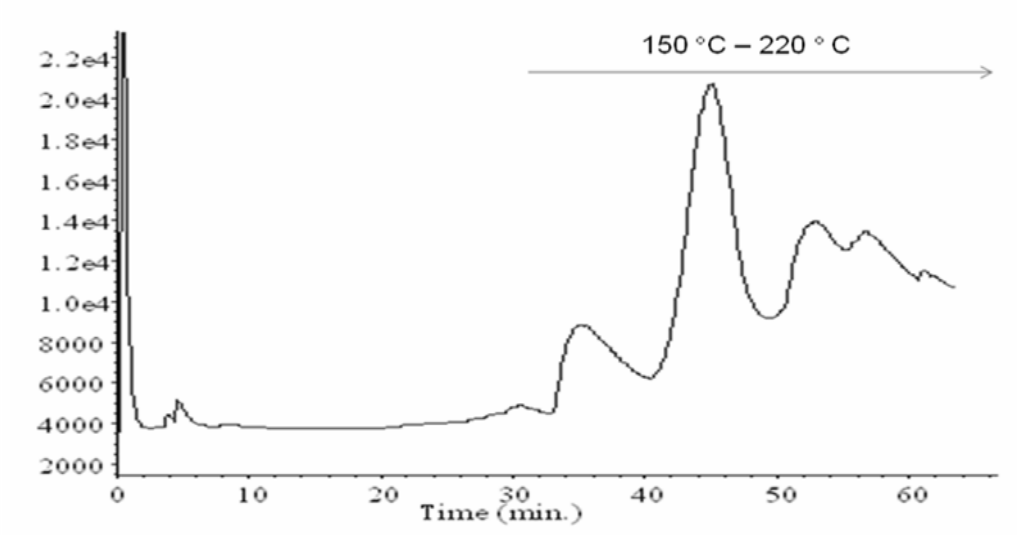


Figure 12 GC Chromatogram of refined rice bran oil in dichloromethane injected into a bare fused silica tubing with a temperature program of 40 °C, ramping to 90 °C at 5 °C min^{-1} and held for 10 min, then rising to 150 °C at 5 °C min^{-1} and held for 10 min, then further rising to 220 °C with 5 °C min^{-1} and held for 10 min.

The GC chromatogram demonstrated that mostly oil matrix peaks were eluted in a range of temperature of higher than 150 °C while a small matrix peaks were eluted at approximately 80 °C as shown in Figure 12. High molecular weight matrix components were eluted at high temperature as expected. From the chromatogram it can be postulated that the suitable temperature program used in this study should be lower than 150 °C, since nitrosamine compounds possessed boiling temperature of lower than 150 °C. In addition, a further raising to higher temperature than 150 °C was not recommended, because the heat could remove the oil matrix out of the monolithic stationary phase to enter the analytical column.

1.2.2 Separation of rice bran oil mixed with NDPA

The experiment was aimed to test the possibility of a separation between NDPA as a nitrosamine representative and the oil matrix, together with and without using PS-DVB monolith trapping. Therefore, after investigating the matrix, a

new test sample was prepared from 250 μL of 1000 ppm *N*-nitrosodipropylamine (NDPA) in methanol mixed with 250 μL refined rice bran oil and then adjusted the solution to 1.00 mL with dichloromethane. One microliter of the test sample was injected in a splitless mode into a 0.32 mm i.d. x 10 m bare fused-silica tubing. The following injection of the same sample was applied to a 0.32 mm i.d. x 4.0 mm fabricated PS-DVB monolith connected with the same fused silica tubing, and the final injection was applied to the PS-DVB monolith connected with the analytical column (ZB-5). The chromatograms from three cases connection were compared in Figure 13.

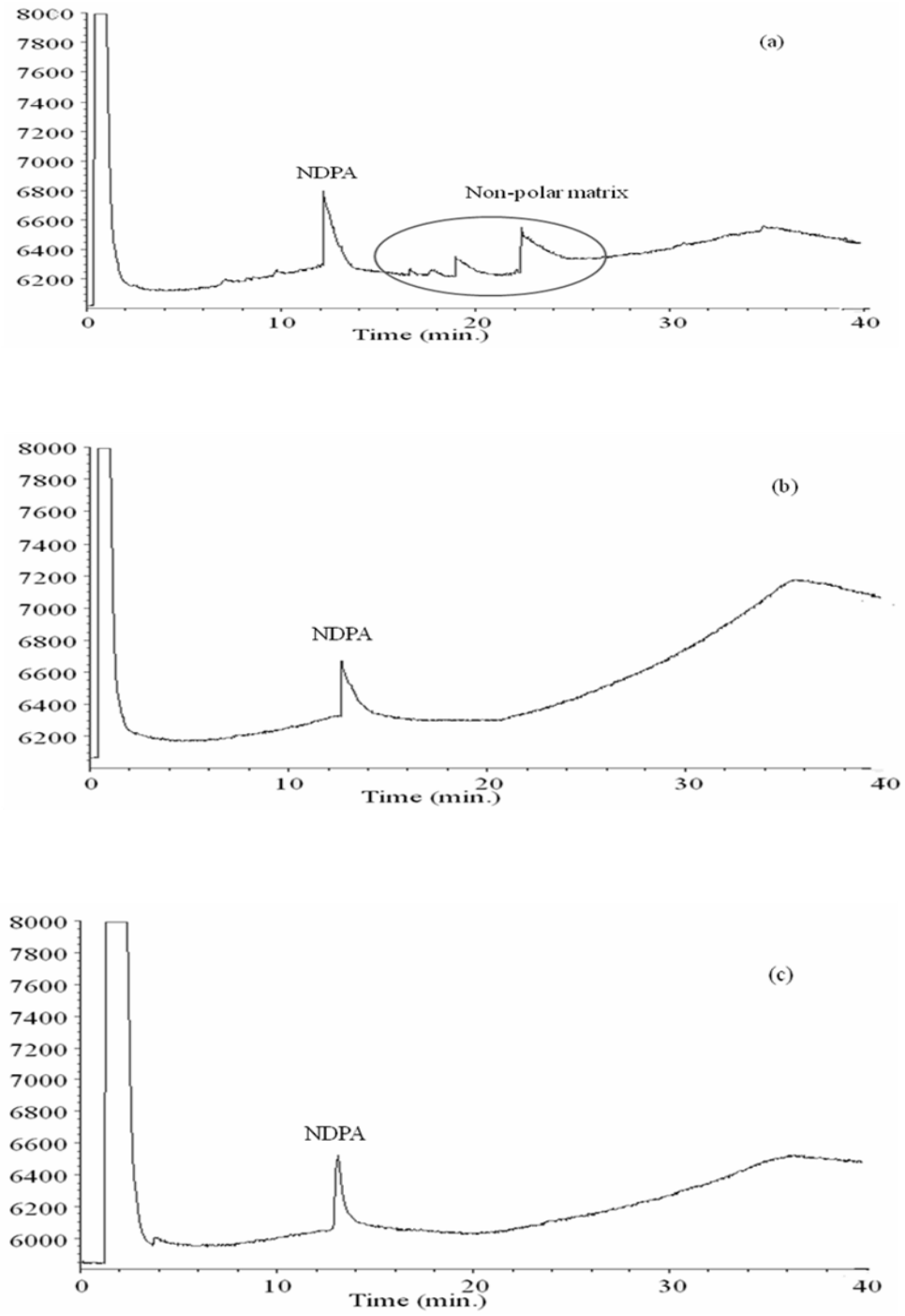


Figure 13 GC Chromatogram of rice bran oil mixed with NDPA obtained from (a) fused silica tubing, (b) PS-DVB monolith connected with fused silica tubing and (c) PS-DVB monolith connected with ZB-5. (Temperature program: 60 °C for 2 min, then ramped from 60 °C to 90 °C at 3 °C min^{-1} and held for 8 min, then rising to 120 °C at 2 °C min^{-1} and held for 5 min).

Figure 13 (a) and 13 (b) show two chromatograms obtained from rice bran oil mixed with NDPA injected into the fused silica tubing compared between an absence and presence of short PS-DVB monolithic column, respectively. As shown in Figure 13 (a), NDPA peak and non-polar matrix peaks were well separated. The non-polar matrix was speculated to be high molecular weight fat and oil presented in the sample, since the oil sample without NDPA was also applied, resulting only the matrix peaks in a similar retention. In a subsequent experiment, it is clearly shown in Figure 13 (b) that NDPA peak was eluted while non-polar matrix peaks were disappeared in the presence of PS-DVB monolith. When coupling with PS-DVB monolith, vapor of oil matrix that occurred at injection port was condensed and adsorbed on PS-DVB monolithic column. Their interaction mechanisms are mainly based on hydrophobic Van der Waals interactions between the oil matrix component and the PS-DVB sorbent.

In order to improve the NDPA peak shape, which was rather broad and tailing because of an interaction with silanol groups on fused silica tubing, the newly fabricated PS-DVB monolithic column was connected prior to the analytical column. A peak possessing better shape was obtained as shown in Fig 13 (c).

All of the above results were clearly shown that only oil matrix compounds were trapped, while NDPA was released from the sorbent. These proved the ability of PS-DVB monolith as a high efficiency non-polar sorbent.

From the satisfactory results in the previous study, rice bran oil mixed with standard NDPA was then repeatedly injected into the same fabricated PS-DVB monolithic column connected with ZB-5 in order to study an injection-to-injection precision. The precision calculated in term of %RSD is given in Table 1.

Table 1 Injection-to-injection precision based on PS-DVB monolith as a sorbent trap connected with ZB-5.

Monolith	Column length (mm)	Flow rate (ml min^{-1})	Injection-to-injection[1] precision[1] (%RSD)
PS-DVB	4.0	2.20	2.26

[1] n=5

Table 1 shows injection-to-injection precision of rice bran oil mixed with NDPA on styrene-divinylbenzene copolymer as a sorbent trap. The column length of 4.0 mm was used. Although, in this study, a column length of PS-DVB monolith was limited to 4.0 mm by high resistance to carrier gas flow, it was compensated with high effective surface area of the monolithic structure, since the result showed that no interference peaks were found despite the fifth time of injection and the injection-to-injection precision was 2.26.

To study a column-to-column precision, rice bran oil mixed with NDPA was injected onto a 4.0 mm PS-DVB monolithic column connected with ZB-5. Five monolithic columns were studied under the same chromatographic condition. The average peak area and average retention time (t_R) of NDEA for each PS-DVB monolithic sorbent trap are compared and given in Table 2.

Table 2 Comparison of average peak area and average retention time (t_R) of NDPA (IS) for each PS-DVB monolithic sorbent trap connected with ZB-5.

PS-DVB monolithic column no.	Average peak area[1]	Average retention time (t_R) (min)
1	19861	12.72
2	20951	12.71
3	19639	12.70
4	21076	12.71
5	18961	12.72
Average	20098	12.71
%RSD	4.48	0.066

[1](n = 5)

From Table 2, column-to-column precisions of average peak area and average retention time (t_R) of NDEA are 4.48 and 0.066, respectively. The good precision indicated the reproducibility of the fabrication of PS-DVB monolith.

From this preliminary study, the results demonstrated the promising of PS-DVB monolith as a reusable sorbent trap to remove non-polar matrix from rice bran oil mixed with NDPA. Then, utilization of PS-DVB monolith sorbent trap for selected volatile nitrosamines, namely NDEA, NPYR, NPIP and NDPA, determination was further investigated.

1.2.3 Separation of standard nitrosamines on an analytical column coupled with monolithic sorbent

A feasibility of short PS-DVB monolith as an on-line sorbent trap was previously investigated, resulting that the oil matrix peaks were trapped on PS-

DVB monolith, while NDPA peak was eluted. However, as a group of volatile nitrosamines, i.e. NDEA, NPYR, NPIP and NDPA, they are usually simultaneously analysed. Without mixing with rice bran oil, a mixture of four standard nitrosamines was prepared. The solution was injected to a well fabricated PS-DVB monolithic column with a length of between 3.0 – 10.0 mm connected to the ZB-5 analytical column with the same chromatographic conditions.

Limitation of connecting between a typical PS-DVB sorbent column and an analytical column is length of monolithic sorbent. As a rule of thumb, an increase in a chromatographic column length enhances effective surface area, but drops column permeability. Although monolithic structure provides higher column permeability than normal particle packed column with the same length, the rule was remained.

The effect of the length of PS-DVB monolithic sorbent column coupling with ZB-5 on pressure was shown in Table 3. In this work, the column flow after coupling with the column length of 3.0-10.0 mm, was adjusted to 1.4 ml min^{-1} and the pressure for each column length obtained from GC instrument was observed from the column head pressure gauge in the front of the GC instrument panel. At high carrier gas flow through column, high resistance occurred, therefore higher pressure was needed to apply to pass the coupling between the sorbent and analytical column to obtain a required column flow rate. Only maximum pressure of 40 psi was allowed due to a limitation of GC instrument.

Table 3 Effect of PS-DVB[1] monolith column length on column head pressure.

Column length (mm)	Average pressure (psi)
10.0	No flow
8.0	No flow
6.0	33.0
5.0	24.0
4.0	17.5
3.0	12.5

($n^1 = 5$) at constant flow rate = 1.4 ml min^{-1} (maximum pressure = 40 psi)

From Table 3, it is observed that shorter monolithic column length provided higher column permeability and lower pressure was required to adjust to a column flow of 1.4 mL min^{-1}. No column flow was observed for a column longer than 6.0 mm. When the column length was gradually cut off, the required pressure was 33.0, 24.0, 17.5 and 12.5 psi for 6.0, 5.0, 4.0 and 3.0 mm, respectively. This evidence indicated that even the higher effective surface area was obtained with the longer column, lower column permeability which leaded to higher resistance to carrier gas occurred.

The effect of the length of PS-DVB monolithic sorbent column on chromatographic observation of four standard nitrosamines separation was investigated as shown in Figure 14. A flow column with different lengths between 3.0-6.0 mm was connected with the ZB-5 column.

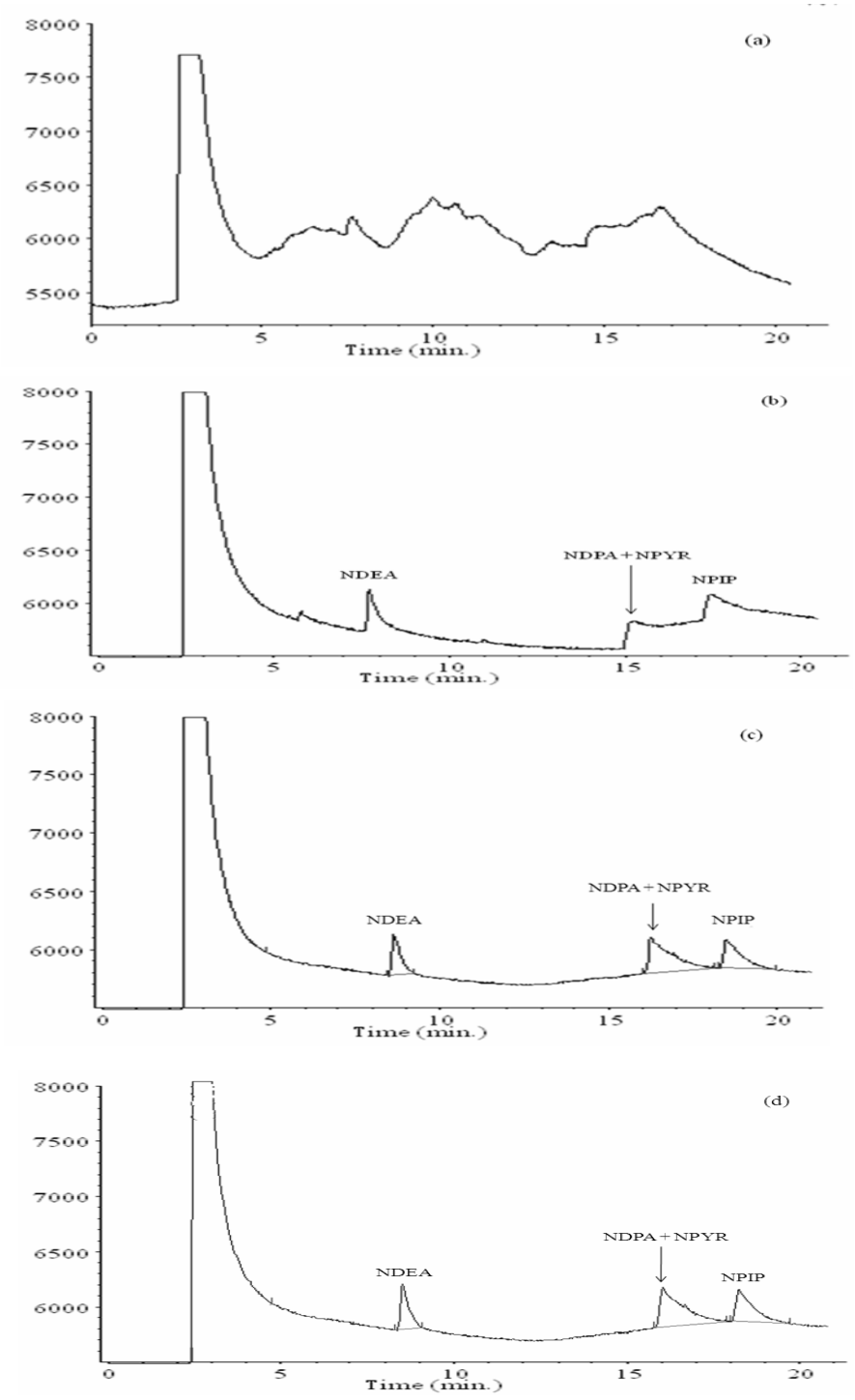


Figure 14 Separation of standard nitrosamines on ZB-5 column coupled with on-line PS-DVB monolith sorbent of (a) 6.0 mm, (b) 5.0 mm, (c) 4.0 mm and (d) 3.0 mm. Temperature program: 60 °C, then ramped from 60 °C to 100 °C at 7 °C min^{-1} and held for 8 min, then raised to 120 °C at 7 °C min^{-1} and held for 5 min.

Apart from the pressure, the length of PS-DVB monolithic sorbent column also affects chromatographic separation of standard nitrosamines as shown in Figure 14. Chromatogram with very noisy baseline was obtained and nitrosamine peaks were not found (Figure 14 (a)). When the column length was reduced to 5.0 mm, nitrosamine peaks could be observed with baseline drift. Broad tailing peak of NDEA and NPIP was obtained while NDPA and NPYR peaks were completely overlapped as shown in Figure 14 (b). The broad tailing peak was postulated to occur from strong interaction of nitrosamines with polymeric sorbent and with silanol groups on fused silica. At a column length of 4.0 mm and 3.0 mm, better peak shape of nitrosamines were obtained, however, NDPA and NPYR peaks still completely overlapped as shown in Figure 14 (c) and (d), respectively. The PS-DVB monolithic column was no longer reduced due to too low effective surface area that was not useful to be sorbent.

Although the fabricated PS-DVB monolith was successfully utilized as a non-polar sorbent trap of rice bran oil mixed with NDPA, a coupling of PS-DVB monolith with the ZB-5 column was not suitable to quantitatively analyse NDEA, NPYR, NPIP and NDPA, because NDPA and NPYR peaks did not resolve.

2. Polydivinylbenzene (poly-DVB)

From the previous study of polystyrene-co-divinylbenzene (PS-DVB) monolithic column as an on-line sorbent trap for fatty sample, the result was clearly shown that PS-DVB monolith was promising for trapping fat and oil. However, several problems were observed. Firstly, too low column permeability of PS-DVB monolith resistance to the flow of gas stream led to a limitation of length of trapping column, only maximum of 4.0 mm was allowed. Secondly, fabricated PS-DVB monolith affected a simultaneous separation of the selected volatile nitrosamines. Broad and tailing peaks of nitrosamines were obtained, resulting in unresolved peaks of NPYR and NDPA. Therefore, poly(divinylbenzene) or poly-DVB were fabricated and preliminarily studied.

Since macroporous divinylbenzene is widely spread used as a disposal polymeric sorbent cartridge to remove lipid from food or physiological sample due to a very high selectivity for hydrophobic substance, it was, therefore, postulated the feasibility of polydivinylbenzene monolith to be used as a sorbent trap for non-polar fat and oil.

2. 1 Polydivinylbenzene (poly-DVB) monolithic column synthesis

A P-DVB monolithic column was fabricated as previously described in Section 1.2. It was then characterized by using a scanning electron microscope, giving SEM micrographs presented in Figure 15.

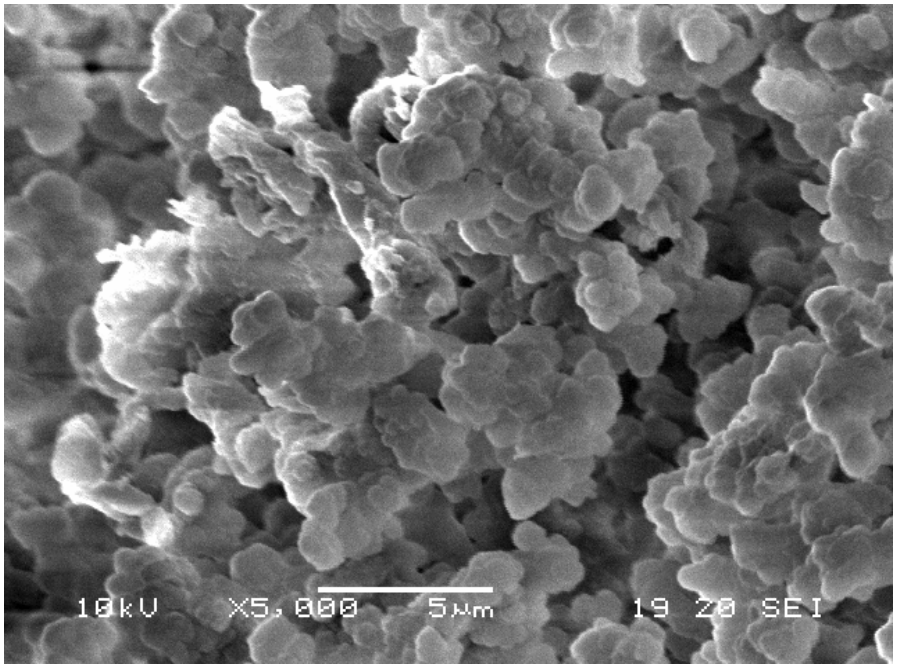


Figure 15 Scanning electron micrograph of polydivinylbenzene monolithic packing in a 0.32 mm i.d. fused silica capillary.

In the silanization process, γ-MAPS provided a large number of free methacrylate groups on the capillary wall to serve as anchoring sites during the formation of polymer and provided good connection between the monolith and

capillary surface. The fused silica capillary wall became hydrophobic after modification with γ-MAPS.

In polymerization process, divinylbenzene was used as a monomer to form an interconnected skeleton of cross-linked polymeric monolithic structure. Either dodecanol or toluene was used as a porogenic agent to provide a bimodal pore structure of monolith and dibenzoyl peroxide was used as a polymerization initiator (Svec and Kurganov, 2008). A highly cross-linked porous monolith, which was bonded to the silanized wall of capillary, was formed. A conversion of divinylbenzene to crosslinked polymer occurs when the structure consists of enough solid materials to make it stable. From SEM micrograph, the cauliflower structure is observed and it clearly shows that polydivinylbenzene monolith provided larger through pores compared with PS-DVB (Figure 11), because larger porogen, 1-dodecanol and toluene, were used as porogenic agents which provided larger through pores, leading to higher column permeability, in addition with the sufficient number of small pores within microglobules which afforded large effective surface area. The large effective surface area about 460 m^2/g determined from the adsorption and desorption isotherms of nitrogen using monolith was obtained. Large surface area indicates that the microglobules are permeated by a large number of small mesopores and micropores (Svec and Kurganov, 2008).

According to thermal stability and hydrophobic properties including higher column permeability and higher effective surface area of poly(divinylbenzene) monolithic column, the feasibility of fabricated poly(divinylbenzene) monolith as a non-polar sorbent trap was investigated.

2. 2 Preliminary study of fabricated polydivinylbenzene monolith as a sorbent trap

From the previous study of PS-DVB monolithic column, two problems needed to be solved was their low column permeability and unresolved peaks of nitrosamines. As the column permeability was limited by a column length, the length

of fabricated poly(divinylbenzene) monolith was, therefore, firstly investigated. Secondly, the poly-DVB monolithic sorbent coupled with a ZB-5 analytical column would be examined for a feasibility to separate a mixture of standard nitrosamines. Provided that results of satisfying separation of the mixture were given, the ability in trapping fat and oil matrix of fabricated poly(divinylbenzene) monolith would be further investigated.

2.2.1 Separation of standard nitrosamines on an analytical column coupled with monolithic sorbent

A mixture of each standard nitrosamine namely NDEA, NPYR and NPIP was prepared by using NDPA as an internal standard in dichloromethane and it was injected into a well fabricated polydivinylbenzene monolithic column with a length of between 5.0-30.0 mm connected to the ZB-5 analytical column operated under the same chromatographic condition. The effect of the length of polydivinylbenzene monolithic sorbent column coupling with ZB-5 on pressure reading from the column head pressure gauge in the front of GC instrument panel and chromatographic observation were investigated.

Effect of the length of polydivinylbenzene monolithic sorbent column coupling with ZB-5 on pressure is shown in Table 4. In this study, the column flow after coupling with a column length of 5.0-30.0 mm of polydivinylbenzene monolith was adjusted to 1.4 mL min^{-1}.

Table 4 Effect of poly(divinylbenzene)[1] monolith column length on pressure.

Column length (mm)	Average pressure (psi)
30.0	No flow
25.0	No flow
20.0	35.0
15.0	24.0
10.0	19.0
5.0	13.5

n^1 = 5 at constant flow rate = 1.4 ml min^{-1} (maximum pressure = 40 psi)

Table 4 shows the effect of poly(divinylbenzene) monolithic column length on pressure. Since the flow of monolithic sorbent capillary of 1.4 mL min^{-1} was needed. From observation, the shorter the monolithic column, the lower the pressure required to adjust to 1.4 mL min^{-1}. No column flow was observed when a poly(divinylbenzene) monolith column longer than 20.0 mm was used. When the column length was gradually reduced to 20.0, 15.0, 10.0 and 5.0 mm, the pressure decreased to 35.0, 24.0, 19.0 and 13.5 psi, respectively. As expected, poly(divinylbenzene) monolithic column provided higher column permeability compared with PS-DVB monolithic column. A comparison of pressure resulted from different lengths of PS-DVB and poly-DVB column is illustrated in Figure 16. To achieve the same flow of 1.4 mL min^{-1} poly-DVB column of three times longer than PS-DVB could be used. For example, the same flow was obtained for 20.0 mm poly-DVB column, compared to 6.0 mm of PS-DVB. Lengthening column resulted in higher effective surface area. The higher permeability of poly-DVB than PS-DVB was due to the larger porogenic agent in poly-DVB fabrication (1-dodecanol) than that used in PS-DVB synthesis (decanol). Subsequently, the formation of larger through pores and higher column permeability of poly-DVB was obtained.

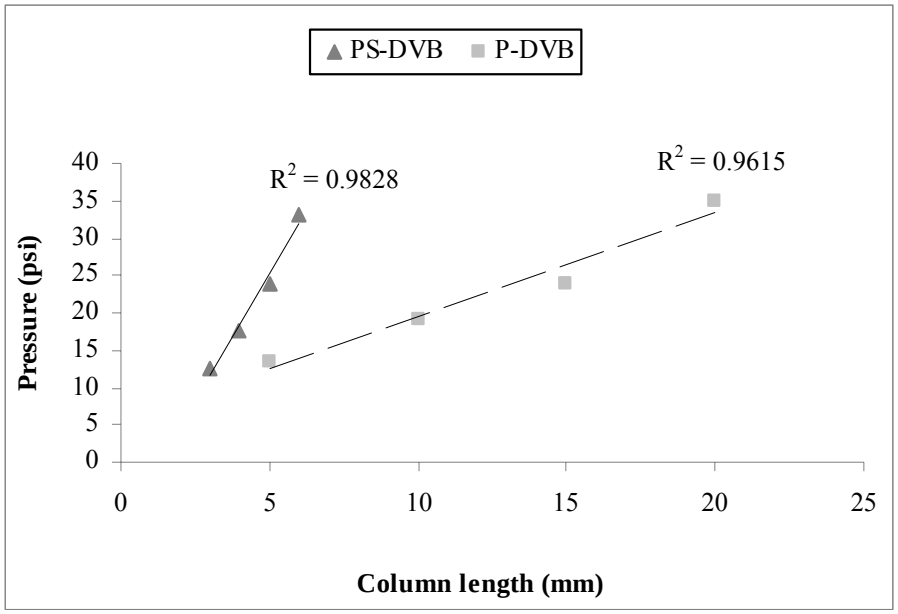


Figure 16 Comparison of column head pressure at different column length of PS-DVB and poly-DVB monolith.

Effect of length of poly-DVB monolithic sorbent column on chromatographic observation of standard nitrosamines separation was investigated as shown in Figure 17 (a) flowed column with different lengths between 5.0-20.0 mm was connected with the ZB-5 column.

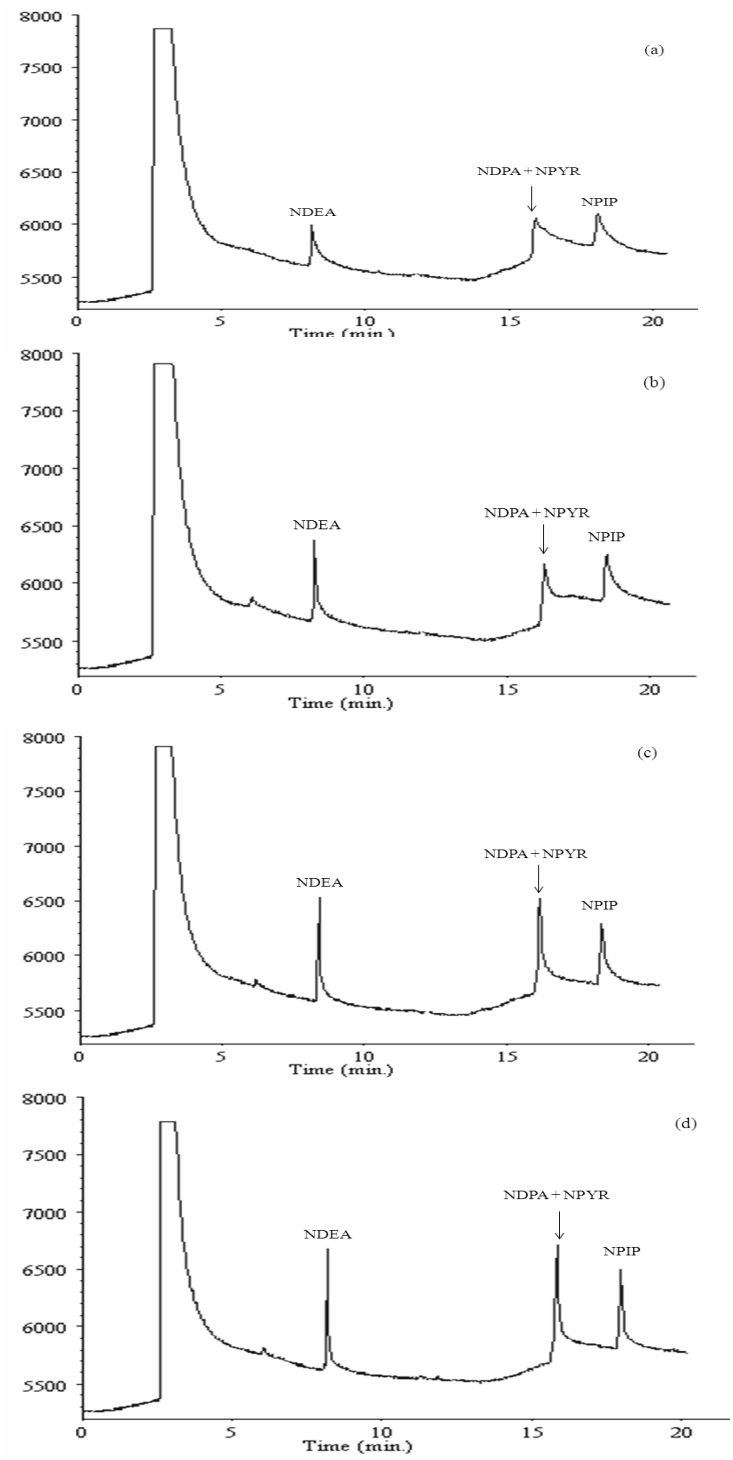


Figure 17 Separation of nitrosamines on ZB-5 column coupled with on-line sorbent poly-DVB monolith sorbent of (a) 20.0 mm, (b) 15.0 mm, (c) 10.0 mm and (d) 5.0 mm. Temperature program: 60 °C, then ramped from 60 °C to 100 °C at 7 °C min^{-1} and held for 8 min, then raised to 120 °C at 7 °C min^{-1} and held for 5 min.

In Figure 17 (a) a chromatogram with all broad tailing peaks and unresolved peaks of NPYR and NDPA was obtained. To balance between column permeability and chromatographic separation efficiency, the column length was gradually reduced. When the column was cut into 15.0 mm and standard nitrosamines were injected, better peak shape of nitrosamines especially NDEA was observed. However, the separation of NPYR and NDPA was still not possible as shown in Figure 17 (b). Reduced column length to 10.0 mm and 5.0 mm, peak shape of nitrosamines was improved, as well as higher peak height, but NDPA and NPYR peaks remained completely overlapping, as shown in Figure 17 (c) and (d), respectively. Despite of higher permeability and better peak shape for all peaks, but the two compounds were not separated, the column length was finally not shortened.

From these results, it was shown that fabricated poly(divinylbenzene) column provided higher column permeability compared with PS-DVB monolithic column resulting in longer column length to be able to used. However, a set up of on-line poly-DVB monolith sorbent trap connected with the ZB-5 analytical column could not receive well-separate peaks of NPYR and NDPA. Therefore, separation of oil added nitrosamine sample by coupling the poly-DVB monolithic sorbent trap with the ZB-5 analytical column were not further investigated.

3. Silica

Since silica gel porous sorbent has successfully been used as a lipid-retainer with complex fatty materials (Subprasert, 2007), the feasibility of silica monolith to be used as a sorbent trap for non-polar fat and oil was therefore postulated.

3. 1 Silica monolithic column synthesis

A solution for silica monolith synthesis was prepared from tetramethoxysilane (TMOS), polyethyleneglycol (PEG), urea and acetic acid. The mixture was filled into 0.32 mm i.d. NaOH pretreated fused silica tubing and subsequently tightly sealed with septum. By heating at 40 °C for 24 h, and further

raising temperature to 120 °C for 6 h, the sol-gel reaction occurred and the monolithic structure was formed.

The newly fabricated column was characterized by using a scanning electron microscope, giving SEM micrographs presented in Figure 18.

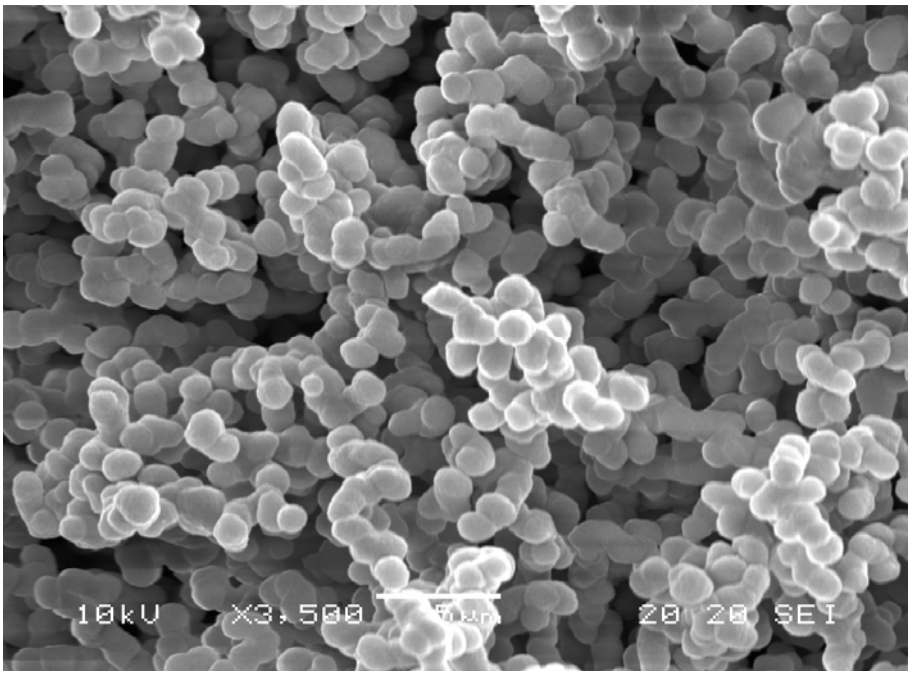


Figure 18 Scanning electron micrographs of silica monolithic packing in a 0.32 mm i.d. fused silica capillary.

Figure 18 shows a scanning electron micrograph of silica monolithic packing in a 0.32 mm i.d. fused silica capillary. Silica skeleton about 1 µm and large through pores or macropores was observed. In contrast to polymer monolith, silica monoliths are prepared through a two-step process via a sol-gel method which involves sequential hydrolysis and polycondensation of alkoxide silicon precursor. In the polymerization mixture, TMOS was a sol-gel precursor. Polyethyleneglycol (PEG) was used as a porogen which act as a through-pore template and solubilizer of silane reagent. Acetic acid was functioned as both solvent and acid catalyst for hydrolysis. The addition of urea in the starting material was to prepare mesopores which provide high effective surface area (Siouffi *et al*., 2003). From this solution,

total porosity exceeds to 90% of the column volume (Tanaka *et al*., 2002) and high specific surface area about 300 m^2 g^{-1} investigated by inverse gas chromatography (IGC) was obtained (Sasook, 2008).

According to their high column permeability, high effective surface area and also high thermal stability, the feasibility of fabricated silica monolith as a sorbent trap for fatty sample was further investigated.

3.2 Preliminary study of monolithic short column as a sorbent trap

Since monolithic column was interested to be used as a sorbent trap for fatty sample, fabricated silica monolithic columns were preliminarily studied as follows. Firstly, capability for trapping fat and oil of silica monolithic material was studied by using standard nitrosamines mixed rice bran oil as a sample. Secondly, optimum column length of silica monolith as an on-line sorbent was then investigated by using a mixture of standard nitrosamines sample.

3.2.1 Separation of oil added nitrosamines sample

In this study, rice bran oil mixed with standard nitrosamines was prepared and initially injected into 0.32 mm i.d. x 10 m bare fused silica tubing. The following injection of the same sample was performed on silica monolith connected with fused silica tubing and the final injection was performed on silica monolith connected with ZB-5. In this study, bare fused silica tubing was primarily tested in order to avoid the 0.32 mm i.d. x 30 m ZB-5 analytical capillary column to be contaminated by fat. These three types connection were investigated under the same GC optimal condition, resulting GC chromatograms given in Figure 19.

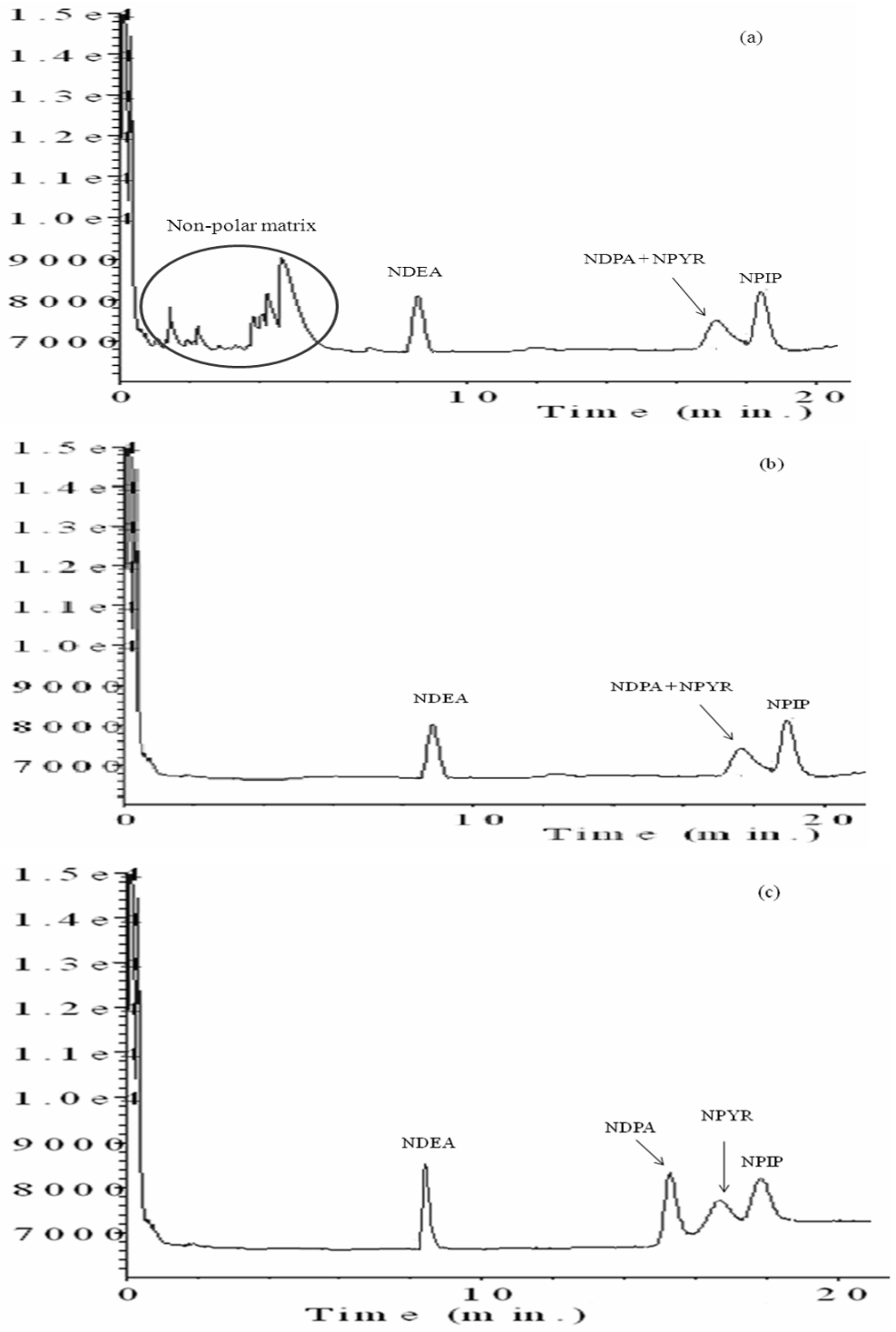


Figure 19 Chromatogram of nitrosamines mixed rice bran oil test mixture compounds obtained (a) fused silica tubing, (b) silica monolith connected with fused silica tubing and (c) silica monolith connected with ZB-5. Temperature program: 60 °C, then ramped from 60 °C to 100 °C at 7 °C min^{-1} and held for 8 min, then raise to 120 °C at 7 °C min^{-1} and held for 5 min.

Figure 19(a) and 19(b) show two chromatograms obtained from nitrosamines mixed rice bran oil injected into the fused silica tubing in the absence and presence of silica monolithic column, respectively. As shown in Figure 19(a), solvent peak of methanol and dichloromethane, nitrosamine sample peaks and non-polar matrix peaks were eluted and separated by using temperature programming. The non-polar matrix was speculated to be high molecular weight fat and oil presented in the sample, since the oily sample without nitrosamines was also applied, resulting only the matrix peaks in a similar retention. In a subsequent experiment, it was clearly shown that only methanol, dichloromethane and nitrosamine peaks were eluted, while non-polar matrix peaks were disappeared in the presence of 0.32 mm i.d. x 5.0 cm silica monolith as shown in Figure 19(b). Coupling with silica monolith, oil matrix component that was evaporated at the injection port was condensed and adsorbed on silica monolithic column.

Subsequently, in order to obtain better separation of NPYR and NDPA and to improve peak shape of all nitrosamines, which was rather broad due to an interaction with silanol groups on fused silica tubing, the newly fabricated silica monolithic column was connected prior to the analytical column. As shown in Figure 19(c), solvent peaks of methanol and dichloromethane, and all nitrosamine sample peaks were resolved, while non-polar matrix peaks were not observed. With silica monolith sorbent coupled with the ZB-5, it was found that better shape was obtained for all peaks and a separation of NPYR and NDPA peaks was succeeded.

These results show that silica monolith was promisingly a high efficiency non-polar sorbent, which provided high effective surface area. It was also proven that only oil matrix components were retained, while nitrosamines could be released from the sorbent.

3.2.2 Separation of standard nitrosamines on an analytical column coupled with monolithic sorbent

The effect of the length of silica monolithic sorbent column coupling with a ZB-5 analytical column on pressure was shown in Table 5. The pressure was measured from the column head pressure gauge in the front panel of GC instrument. The maximum scale of the column head pressure gauge is 40 psi. In this study, after coupling the ZB-5 with a silica monolith column with a length between 70.0-30.0 mm, the column flow was adjusted to 1.4 mL min^{-1} and the column head pressure was observed.

Table 5 Effect of length of silica monolithic column[1] on column head pressure.

Column length (mm)	Average pressure (psi)
70.0	10.0
60.0	9.5
50.0	8.9
40.0	8.3
30.0	8.0

[1] n = 5

The results in Table 5 show that shorter monolithic column length providing higher column permeability and lower pressure was required to adjust the column flow to 1.4 mL min^{-1}. The pressure required to maintain the flow was 10.0, 9.5, 8.9, 8.3 and 8.0 psi for 70.0, 60.0, 50.0, 40.0 and 30.0 mm, respectively. A comparison of average pressure resulted from different lengths of PS-DVB, poly-DVB and silica column was illustrated in Figure 20. Permeability of silica monolith was not affected by the column length, since the flow was almost constant when increasing column length, compared to that for PS-DVB and poly-DVB. This led to the feasibility in using a longer column. For example, a silica monolith of 70 mm long

used in this study was approximately 10 times and 3.5 times of allowable PS-DVB and poly-DVB lengths, respectively.

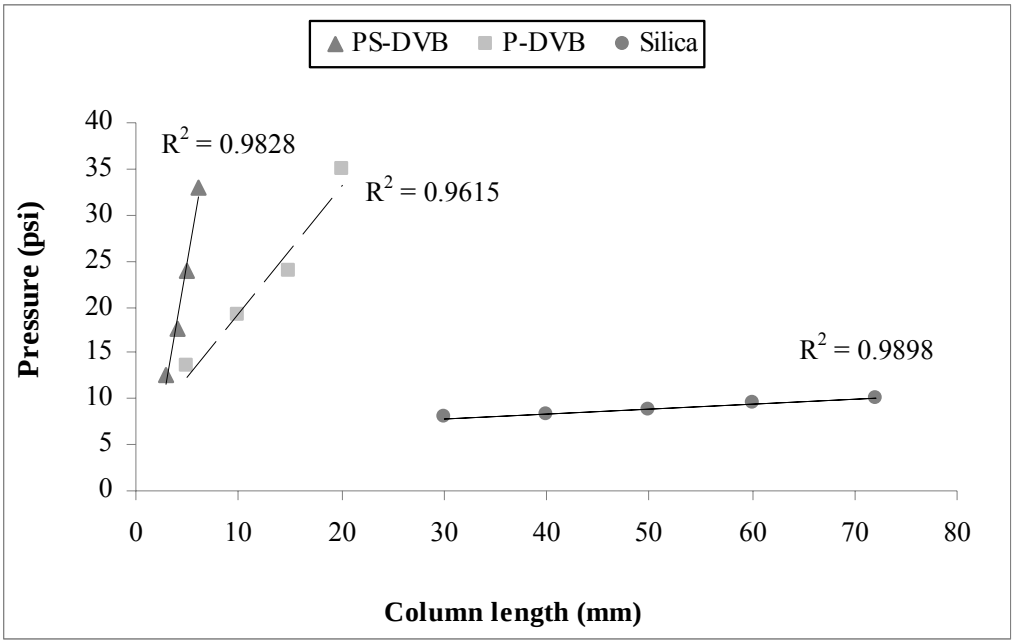


Figure 20 Comparison of column head pressure at different column length of PS-DVB, poly-DVB and silica monolith.

In contrast to polymer monolithic structure, silica monolith provided several times higher column permeability as a result of the largest through pore among those. Besides, its total porosity exceeds to 90% of the column volume (Tanaka *et al*., 2002). The result was confirmed by a comparison of scanning electron micrographs of PS-DVB, poly-DVB and silica monolith shown in Figure 21.

The effect of the length of silica monolithic sorbent column on chromatographic observation of standard nitrosamine separation was investigated. Each column listed in Table 5 was coupled with the ZB-5, and mixture of standard nitrosamine was injected, resulting chromatograms shown in Figure 22.

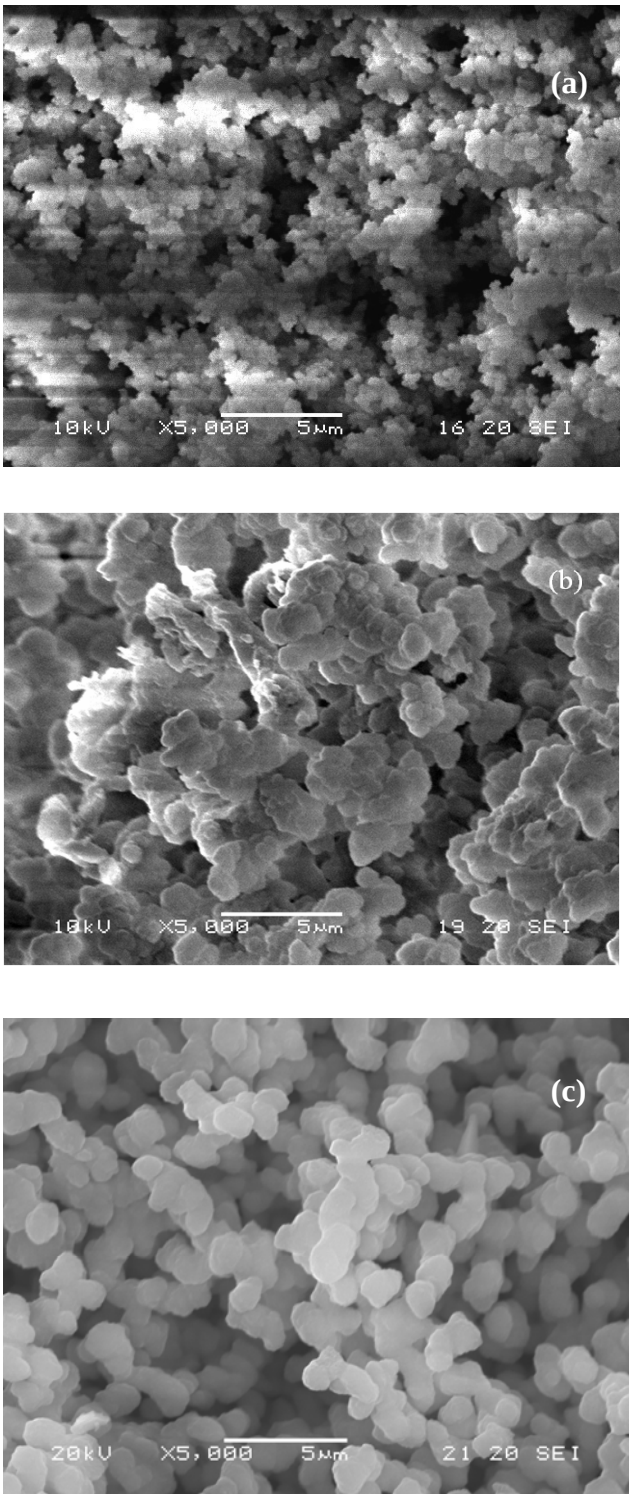


Figure 21 Scanning electron micrographs of (a) polystyrene-co-divinylbenzene monolithic column, (b) polydivinylbenzene monolithic column and (c) silica monolithic column.

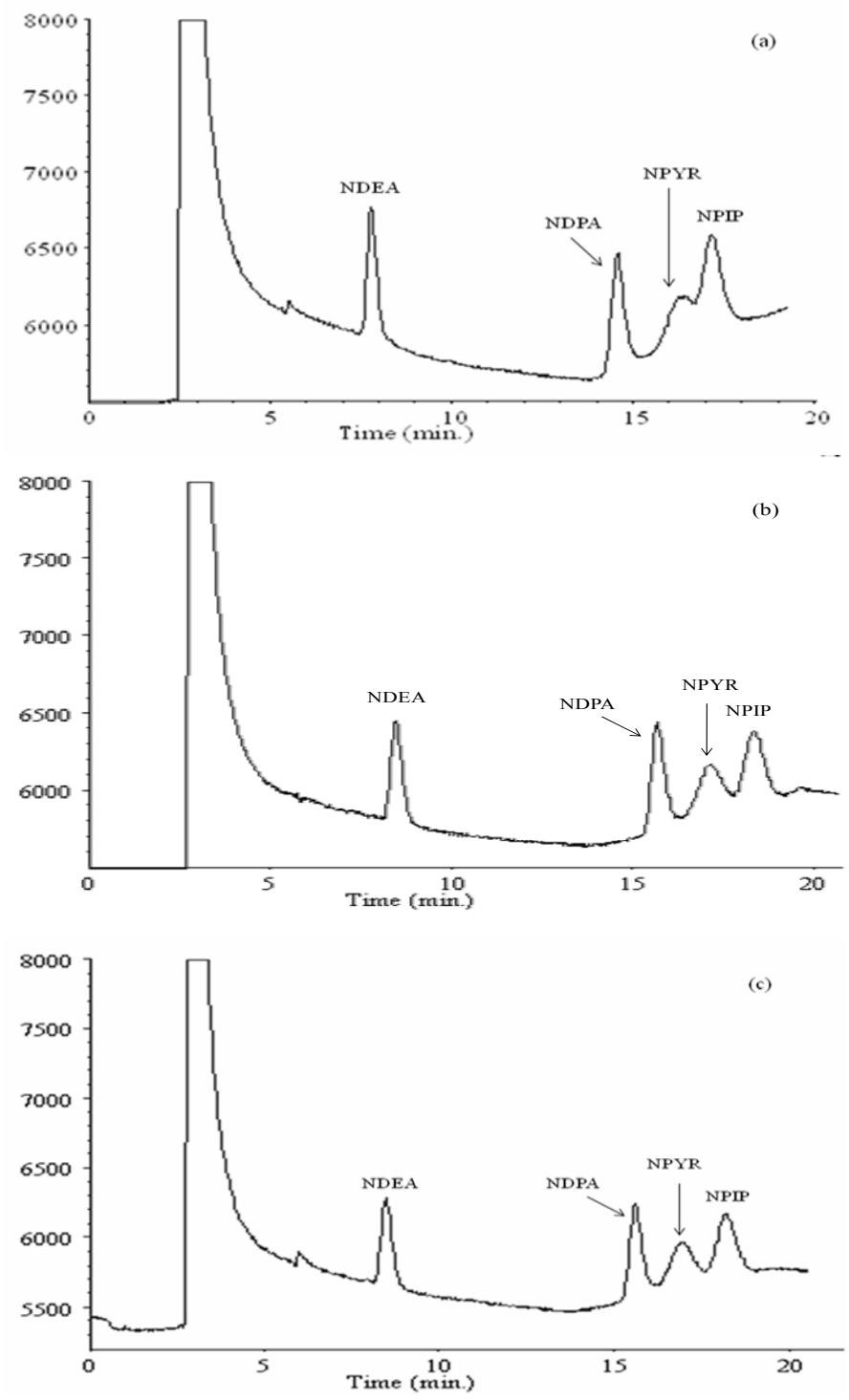


Figure 22 Separation of standard nitrosamines on ZB-5 analytical column coupled with an on-line silica monolith sorbent of (a) 70.0 mm, (b) 60.0 mm, (c) 50.0 mm, (d) 40.0 mm and 30.0 mm. Temperature program: 60 °C, then ramped to 100 °C at 7 °C min^{-1} and held for 8 min, then raised to 120 °C at 7 °C min^{-1} and held for 5 min.

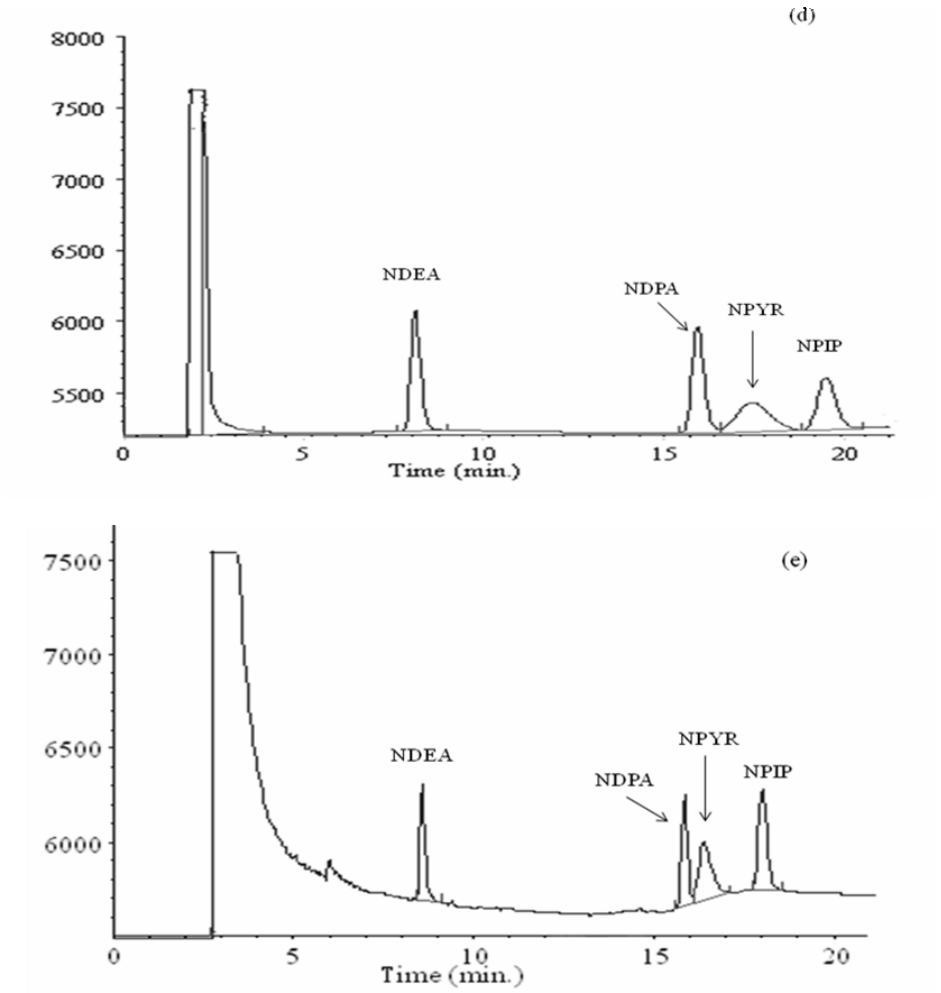


Figure 22 (Continued).

The chromatograms shown in Figure 22 demonstrate an interaction of interested nitrosamine compounds with silica monolithic column of different length. Better separation of nitrosamines was obtained when a short silica monolith was used owing to lower interaction of nitrosamines with silica sorbent. At 70.0 mm (Figure 22 (a)) NDEA and NDPA peaks were well-separated, but NPYR and NPIP peaks were overlapped. Better separation of NPYR and NPIP was obtained when the column length was reduced to 60.0 mm and 50.0 mm as shown in Figure 22 (b) and 22 (c), respectively. At a column length of 40.0 mm (Figure 22 (d)), though all nitrosamines peaks were well-separated, the peak of NPYR was broad, as a consequence of strong interactions between its polar functional group and polar silica sorbent surface. These include dipole-dipole interactions, and dipole-induced dipole interactions. At 30.0 mm, best separation and peak shape of all nitrosamines was

resulted as shown in Figure 22 (e). Further reducing sorbent column length might cause unfavorable separation and poor non-polar matrix trapping. Besides, the longest sorbent column with the least effect on separation efficiency was required, the silica monolithic column was then no longer cut.

Monolithic column with silica material demonstrated the best performance in trapping non-polar matrix and the length of 30 mm gave the best separation of nitrosamines. It was therefore utilized in the following experiment in which a real sample was applied.

3.3 Sensitivity of standard nitrosamines in an absence and presence of silica monolith sorbent on flame ionization detector

The experiment was aimed to compare sensitivity of nitrosamines by flame ionization detector in the absence and presence of silica monolith capillary as an on-line sorbent trap. Peak height of each nitrosamine was obtained at a very low concentration and the detection limits for both cases were then calculated.

Table 6 shows the lowest mass injected which provided a peak height of 2-3 times to baseline noise of each nitrosamine compared between the absence and presence of 30.0 mm silica monolithic column. The results exhibit the same trend for both cases. It was found that the smallest mass injected of each nitrosamine on ZB-5 was only slightly lower than silica monolith connected with ZB-5. However, the lowest mass injected between the presence and absence of silica monolith was in the same order of magnitude and range.

Table 6 Lowest mass injected of nitrosamines detected by FID on a ZB-5 column compared with a silica monolith trap connected with the ZB-5.

Compound	Lowest mass injected (ng injected)	
	ZB - 5	Silica monolith + ZB -5
NDEA	1.8	3.8
NPYR	4.8	19.8
NPIP	3.8	11.8
NDPA (IS)	1.8	6.8

3.3.1 Sensitivity of standard nitrosamines on ZB-5 by flame ionization detector

Four series of very low concentration close to the lowest mass of four nitrosamines in Table 7 were injected and the concentrations were then converted to injected mass of each component. The average peak height of each compound was plotted against the analyte mass, showing a good linearity. The slope, intercept (y_B) and least square of correlation coefficient (r^2) obtained from calibration curves of all nitrosamines are summarized in Table 7.

Table 7 Statistical values from each regression line of analyte on ZB-5.

Compound	Mass range (ng injected)	Intercept a, y_B	Slope, b	r^2
NDEA	2-8	-15.481	20.013	0.9970
NPYR	5-40	-15.142	6.6011	0.9970
NPIP	4-20	-19.853	10.100	0.9990
NDPA (IS)	2-20	-10.440	14.240	0.9970

Subsequently, the average peak height, standard deviation (SD) and three times of standard deviation (3SD) of NDEA, NPYR, NPIP and NDPA (IS) were determined by 20 injections for NDEA and 15 injections of NPYR, NPIP and NDPA of the concentration given in Table 6. The results were given and calculated for standard deviation (SD) as shown in Table 8.

Table 8 Peak heights of each analyte on ZB-5 for examining the standard deviation (SD).

Number	Peak height			
	NDEA	NPYR	NPIP	NDPA (IS)
1	22.7	22.7	16.0	22.7
2	26.3	21.0	17.3	22.7
3	24.0	25.3	17.3	25.3
4	21.3	26.7	18.7	21.3
5	22.7	24.0	21.3	20.0
6	24.0	24.0	17.3	24.0
7	22.7	26.7	18.7	24.0

Table 8 (Continued)

Number	Peak height			
	NDEA	NPYR	NPIP	NDPA (IS)
8	25.3	25.3	17.3	25.3
9	24.0	22.7	20.0	23.2
10	21.3	21.3	18.7	23.3
11	22.7	24.0	17.0	22.7
12	26.3	25.3	18.3	23.2
13	26.3	25.3	17.0	23.3
14	26.7	26.7	18.3	24.0
15	24.0	24.0	17.0	22.7
16	22.7	-	-	-
17	24.0	-	-	-
18	26.7	-	-	-
19	21.3	-	-	-
20	24.0	-	-	-
SD	1.81	1.82	1.33	1.36

The 3SD was recalculated to examine the true detection limit, by substitution in the equation, $y - y_B = 3SD$, while y is the true detection limit and y_B is the concentration of the blank (or intercept in this case). When y was replaced in the equation of slope 'mx', then x was calculated as the true detection limit which was equal to 0.50, 1.49, 1.57, and 0.45 ng for NDEA, NPYR, NPIP and NDPA (IS), respectively as shown in Table 9.

Table 9 Detection limit of standard nitrosamines on the ZB-5 column.

Compound	Detection limit (ng injected)
NDEA	0.50
NPYR	1.49
NPIP	1.57
NDPA	0.45

3.3.2 Sensitivity of standard nitrosamines on a silica monolith trap connected with ZB-5 by flame ionization detector

Four series of very low concentration close to the lowest mass of four nitrosamines in Table 6 were injected and the concentrations were then converted to injected mass of each component. The average peak height of each compound was plotted against the analyte mass, showing a good linearity. Slope, intercept (y_B) and least square of correlation coefficient (r^2) obtained from calibration curves of all nitrosamines were summarized in Table 10.

Table 10 Statistical values from each regression line of analyte on silica monolith connected with ZB-5.

Compound	Mass range (ng injected)	Intercept a, yB	Slope, b	r^2
NDEA	4-8	0.3453	4.9693	0.9930
NPYR	20-80	0.0017	1.0014	0.9980
NPIP	12-40	-0.7232	2.9140	0.9980
NDPA (IS)	7-40	1.2460	4.3930	0.9990

Subsequently, the average peak height and standard deviation (SD) of NDEA, NPYR, NPIP and NDPA (IS) were determined by 20 injections for NDEA and 15 injections of NPYR, NPIP and NDPA of the concentration above (Table 6). The results were given in Table 11.

Table 11 Peak height of each analyte on silica monolith connected with ZB-5 for examining the standard deviation (SD).

Number	Peak height			
	NDEA	NPYR	NPIP	NDPA (IS)
1	19.0	20.0	33.7	32.0
2	20.0	22.0	32.0	29.3
3	21.3	18.0	33.1	30.7
4	18.7	18.7	34.3	30.7
5	17.3	19.3	32.0	32.0
6	20.3	21.0	33.7	28.7
7	21.0	19.8	34.3	32.0
8	18.7	20.7	35.0	28.0
9	21.3	21.0	31.7	29.3
10	18.7	21.3	32.0	29.3
11	21.3	18.3	33.7	28.7
12	18.7	17.0	32.7	30.7
13	20.0	21.0	31.0	31.0
14	17.3	19.7	34.8	32.0
15	20.3	21.0	32.3	30.7
16	21.0	-	-	-
17	18.7	-	-	-
18	18.7	-	-	-

Table 11 (Continued)

Number	Peak height			
	NDEA	NPYR	NPIP	NDPA (IS)
19	20.0	-	-	-
20	21.0	-	-	-
SD	1.30	1.42	1.22	1.36

The 3SD was recalculated to examine the true detection limit, by substitution in the equation, $y - y_B = 3SD$, while y is the true detection limit and y_B is the concentration of the blank (or intercept in this case). When y was replaced in the equation of slope 'mx', then x was calculated as the true detection limit which was equal to 0.86, 4.26, 1.01, and 1.21 ng for NDEA, NPYR, NPIP and NDPA (IS), respectively, as shown in Table 12.

Table 12 Detection limits of standard nitrosamines on a silica monolith trap connected with ZB-5.

Compound	Detection limit (ng injected)
NDEA	0.86
NPYR	4.26
NPIP	1.01
NDPA	1.21

A comparison of detection limits of nitrosamines obtained from ZB-5 coupled with and without 30.0 mm silica monolith show in Table 13 and Figure 23. It was found that both detection limit values for each NDEA, NPIP and NDPA (IS) were very close to each other, except that a ZB-5 coupling with silica monolith gave significantly higher detection limit of NPYR than the ZB-5. Nevertheless, they were

in the same order of magnitude. Because NPYR is more polar than the other nitrosamines, an interaction of NPYR with silica monolith surface was stronger, resulting broad and tailing peak that led to a reduction in the sensitivity.

Table 13 Compared detection limit of nitrosamines in the absence and presence of silica monolith connected with ZB-5.

Compound	Detection limit (ng injected)	
	ZB - 5	Silica monolith + ZB -5
NDEA	0.50	0.86
NPYR	1.49	4.26
NPIP	1.57	1.01
NDPA (IS)	0.45	1.21

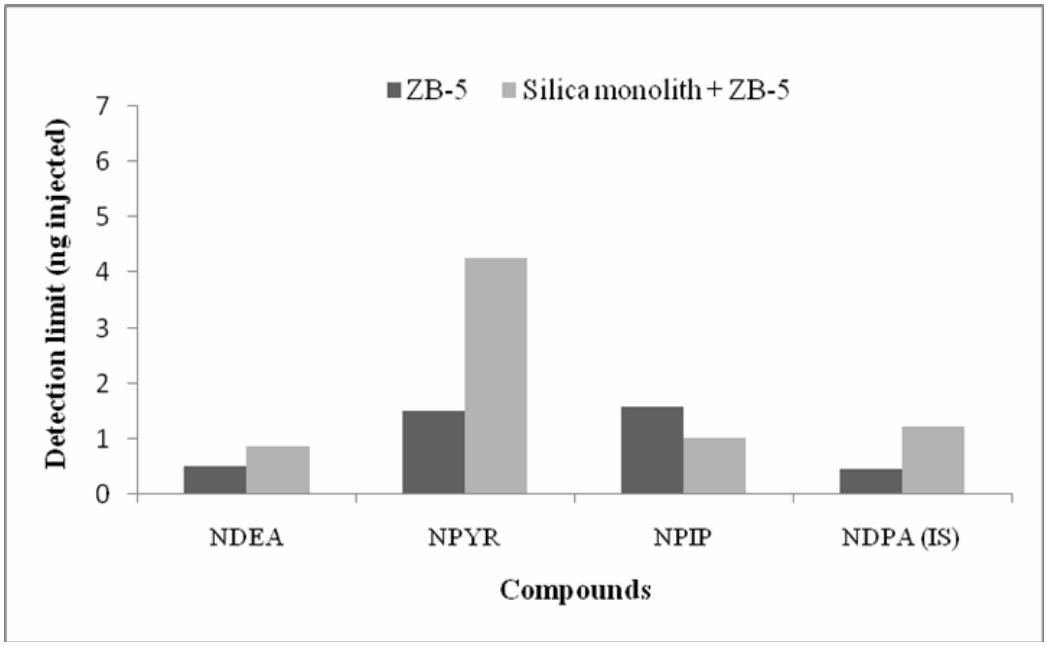


Figure 23 Comparison between detection limits of nitrosamines on a ZB-5 and a 30.0 mm silica sorbent trap connected with the ZB-5.

3.4 Capability and reusability of silica monolithic column as a non-polar sorbent trap

From the preliminary study in section 3.2.1, satisfying result was obtained from using silica monolith as a non-polar sorbent trap for rice bran oil mixed with standard nitrosamines. Since the capability of monolithic column had been expect for trapping fat and oil in a determination of nitrosamine in frankfurter sample. Therefore, this experiment was set up to investigate the monolithic sorbent trap for its fat retaining capability by testing with an extract of blank (or unfortified) frankfurter extract. Preparation of the extract was previously mentioned in Section 5.2. Reusability of the silica monolithic trap was also determined.

In order to avoid a ZB-5 capillary column to be contaminated from non-polar matrix compounds, one microliter of the unfortified frankfurter extract was repeatedly injected with splitless mode into a 30.0 mm silica monolithic column connected with a 0.32 mm id. x 10 m bare fused-silica tubing to examine a number of injection before the fat was leached. The results are present as chromatograms given in Figure 24.

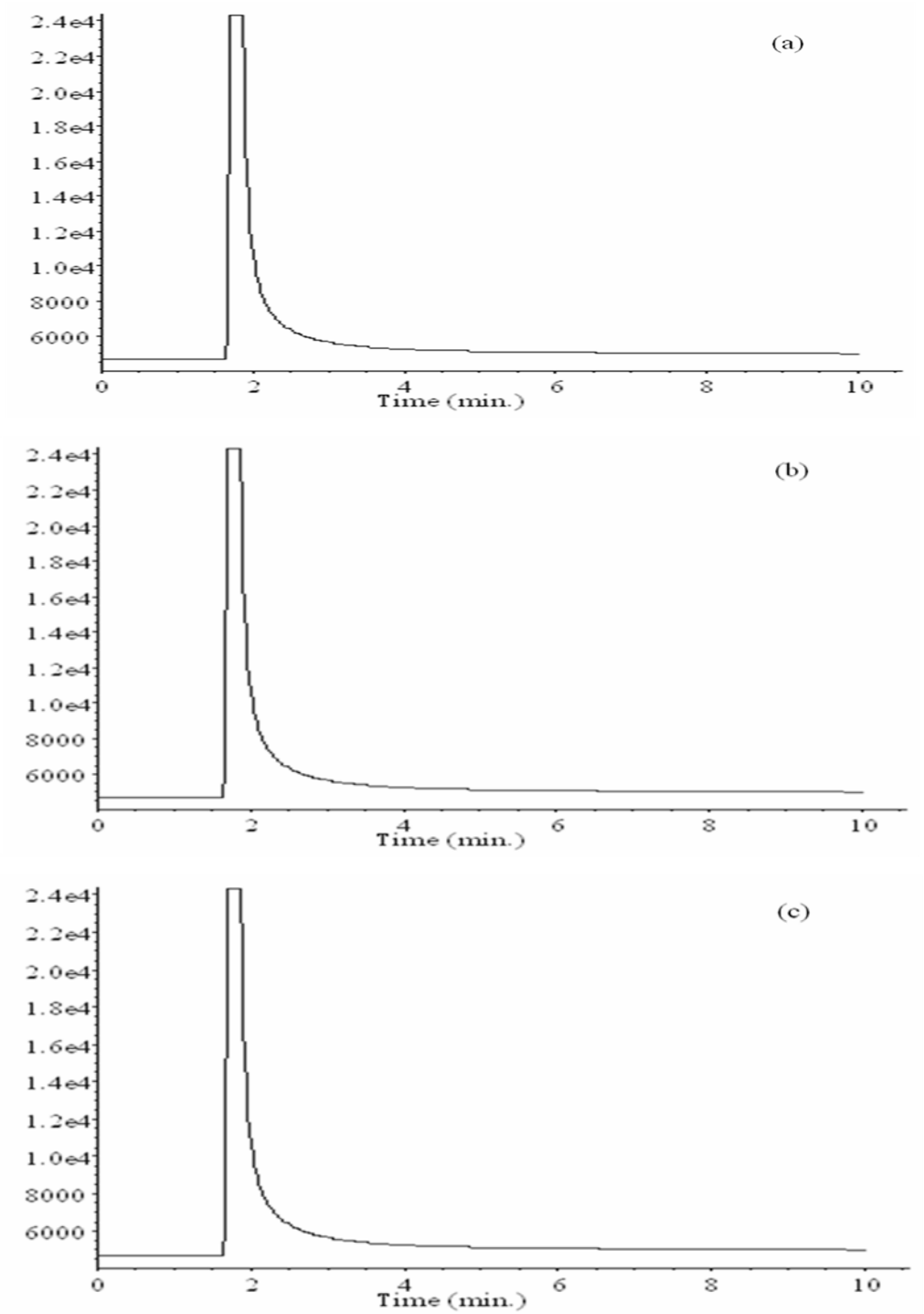


Figure 24 GC chromatograms obtained from repeatedly injection of unfortified frankfurter extract on 30.0 mm silica monolith connected with ZB-5 (a) 10 injection (b) 20 injection, (c) 27 injection, (d) 28 injection, (e) 29 injection and (f) 30 injection.

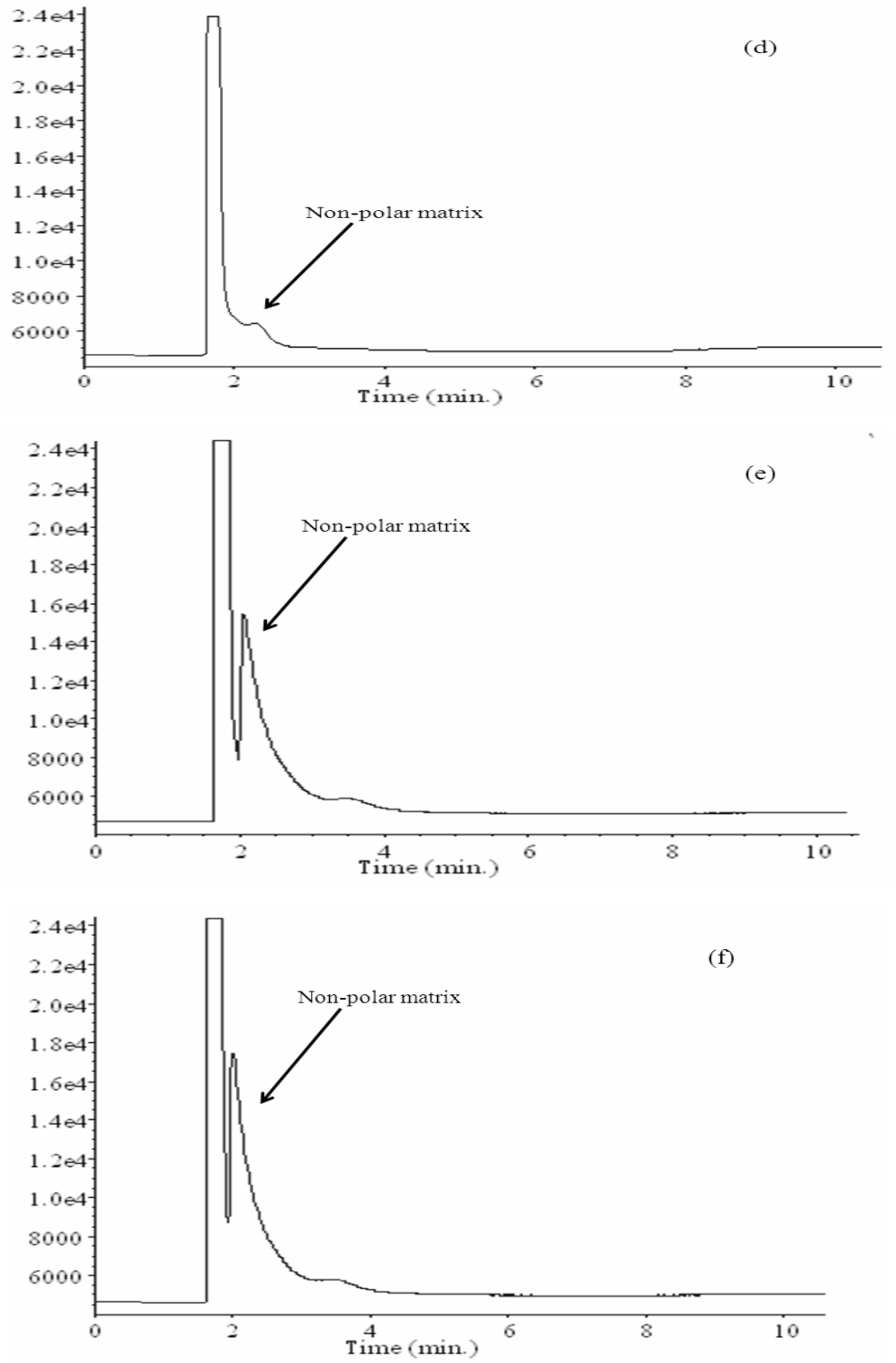


Figure 24 (Continued).

In Figure 24 (a), (b) and (c) the chromatograms demonstrate that only solvent peak of dichloromethane was observed and no fat was leached for 10, 20 and 27 injections. A small peak adjacent to the solvent peak that was speculated to be fat was observed in the chromatogram shown in Figure 24 (d) for 28 injections. The peak was dramatically increased in the following chromatograms obtained from 29 and 30 injections as shown in Figure 24 (e) and (f), respectively.

The peak area of non-polar matrix compound that was speculated to be fat in unfortified frankfurter extract were also plotted against the number of repeatedly injection as shown in Figure 25. It was found that 30.0 mm long silica monolithic column can be reused 27 times before the fat was leached. The number of repeated injection time was also dependent on contaminated fat amount. Again, it is recognized that frankfurter is highly fat food of which fat content is approximately 30% by weight.

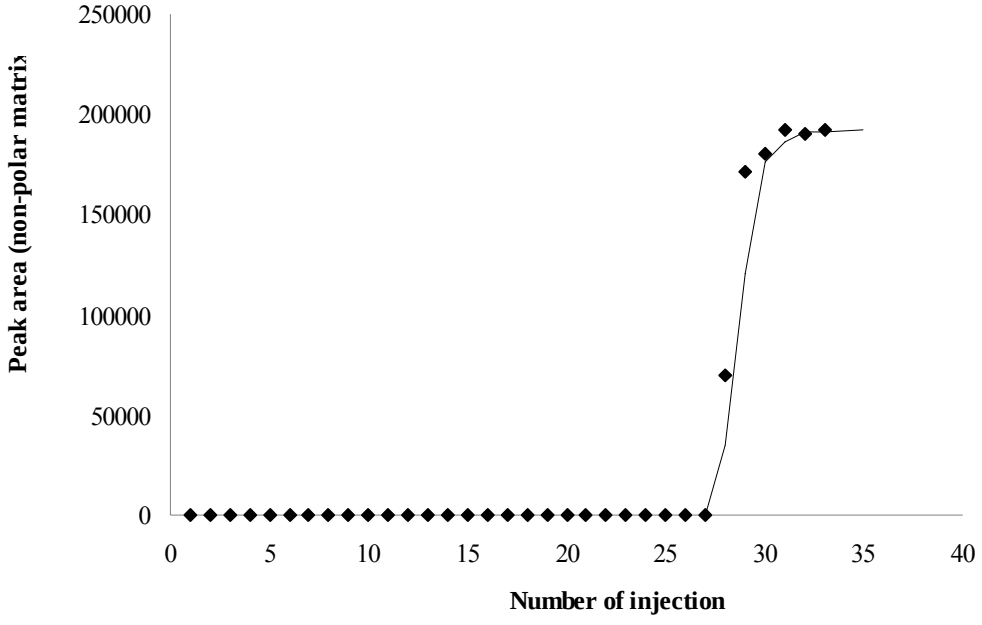


Figure 25 Number of repeated injection of a unfortified frankfurter extract on 30.0 mm silica monolith connected with the ZB-5.

In order to confirm capability of silica monolith as a fat retaining sorbent, fabricated silica monolithic column before and after trapping the fat from extracted frankfurter was investigated via morphology by using scanning electron microscope of which pictures shown in Figure 26.

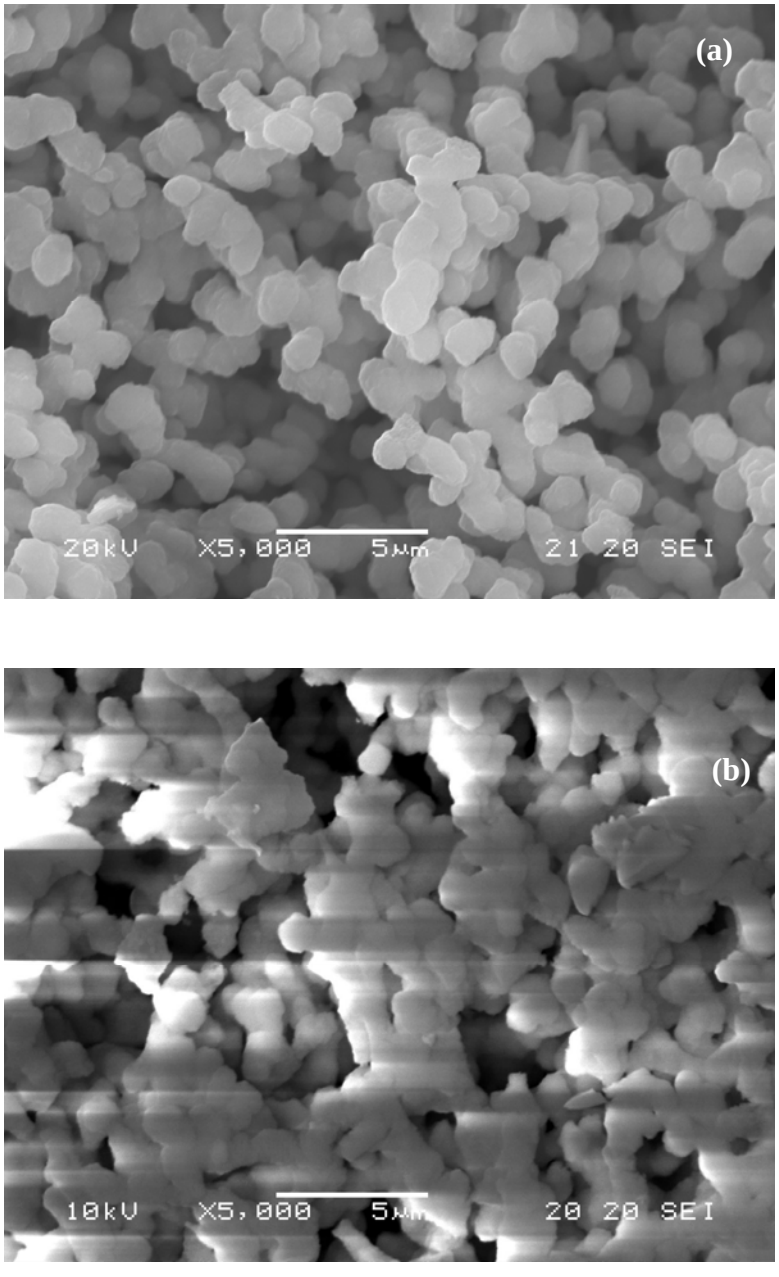


Figure 26 Scanning electron micrograph of 30.0 mm silica monolithic column (a) before, and (b) after use as a sorbent trap for frankfurter extract.

Before trapping matrix from frankfurter extract, the fabricated silica monolithic column showed its interconnected skeleton with small globuli about 1 μm and its large through pore was clearly observed as seen in Figure 26 (a). After 30 time injection of the extract, the thickening on the surface of silica skeleton was observed (Figure 26 (b)), and it was postulated that the fat and other non-polar matrix covered overall monolithic skeleton. The deposition might occur from both condensation and adsorption of the volatile fat and other non-polar components onto silica surface, since the operating temperature was lower than the boiling point of lard (182-205 °C). Such phenomena might also occur when using PS-DVB and poly-DVB sorbent trap. However, since the separations of nitrosamine on those sorbents were not successful, they were not interesting in this work.

From this experiment, it was then concluded that the reusability of 30.0 mm fabricated silica monolithic column as a sorbent trap for fat matrix was up to 27 times. In practice, for analytical column safety, a number of re-use should be reduced a quarter to about 20.

4. Application to real sample

4.1 Chromatographic separation of an extract of nitrosamine fortified frankfurter

The experiment was studied in three cases and aimed to investigate the effect of silica monolith on fortified frankfurter extract. Firstly, standard nitrosamines were injected into ZB-5 column. Secondly, standard nitrosamines were injected into silica monolith connected with the ZB-5. Finally, fortified frankfurter extract was injected into silica monolith connected with the ZB-5. These three cases were performed under the same GC optimal conditions and the results are presented as chromatograms given in Figure 27-29.

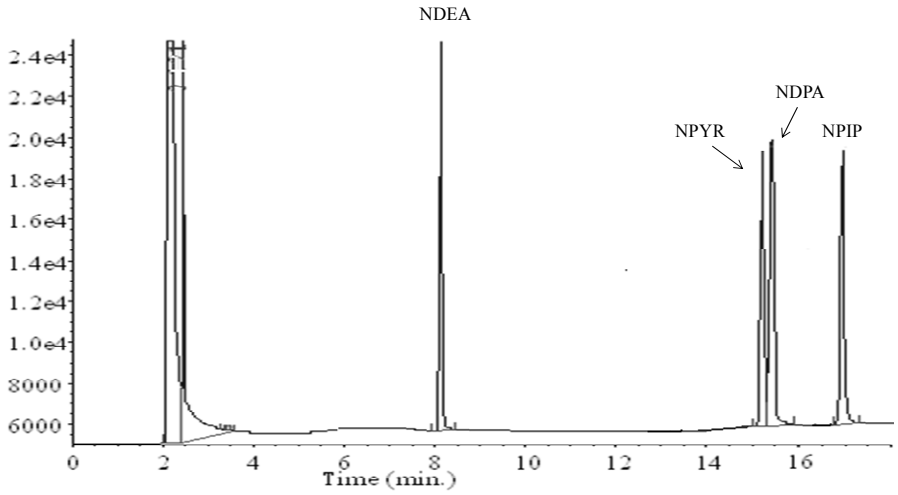


Figure 27 GC chromatogram obtained from standard nitrosamines on ZB-5.

Figure 27 shows GC chromatogram obtained from an injection of standard nitrosamine on ZB-5. The retention time of NDEA, NPYR, NDPA, and NPIP were 8.273, 15.227, 15.496 and 17.138 min, respectively. Although the peak shape was quite symmetry and sharp, it can be seen the overlapping peaks of NPYR and NDPA on the chromatogram.

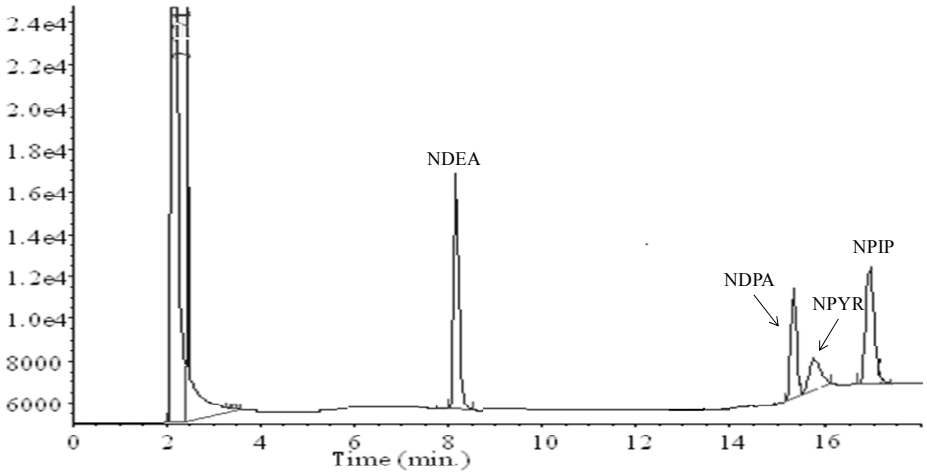


Figure 28 GC chromatogram obtained from separation of standard nitrosamines on a 30.0 mm silica monolithic sorbent column connected with the ZB-5 analytical column.

In general, when a sorbent trap is connected to an analytical column, similar chromatogram to that without the sorbent is expect. To our surprise, the

chromatogram in Figure 28 shows retention times of NDEA, NDPA, NPYR and NPIP were 8.375, 15.350, 15.820 and 16.977, respectively. An elution order of NPYR and NPDA was obviously swapped, when comparing with the chromatogram in Figure 27. Although the NPYR peak was well-separated from the NDPA one, its shape was quite broad, which might be a result of an interaction of the highest polar NPYR to the silica monolith, compared with those of the other analytes. The interaction of nitrosamines with silica monolith was assumed to be dipole-dipole and/or dipole-induced dipole. However, the interaction that caused broad peak was compensated with better separation between NPYR and NDPA peak. The coupling then gave benefits to the nitrosamine separation.

Subsequently an average peak area for each nitrosamine obtained from an injection of standard nitrosamines with absence and presence of silica monolith connected with ZB-5 are summarized in Table 14. The results were subjected to a significance test by using two-tailed t-test to investigate any difference occurring from a connection with silica monolith.

Table 14 Average peak area of each standard nitrosamine on ZB-5 coupled with and without silica monolith.

Compound	Average peak area[1]	
	ZB – 5	Silica monolith + ZB -5
NDEA	54854	56825
NPYR	57405	55696
NPIP	76628	75658
NDPA (IS)	74929	76490

[1] n= 3

By using two-tailed t-test, the |t| values obtained were 0.1532, 0.5815, 0.6317, and 0.2820 for NDEA, NPYR, NPIP and NDPA, respectively. All the |t| value

was lower than t-critical value ($t_{crit,0.05,2}$ = 4.30) at 95% confidence limit and 2 degrees of freedom. This meant that no significant difference between data obtained from the absence and presence of silica monolith. The results also revealed that no significant effect on the yield of analyte when coupling silica monolith to an analytical column.

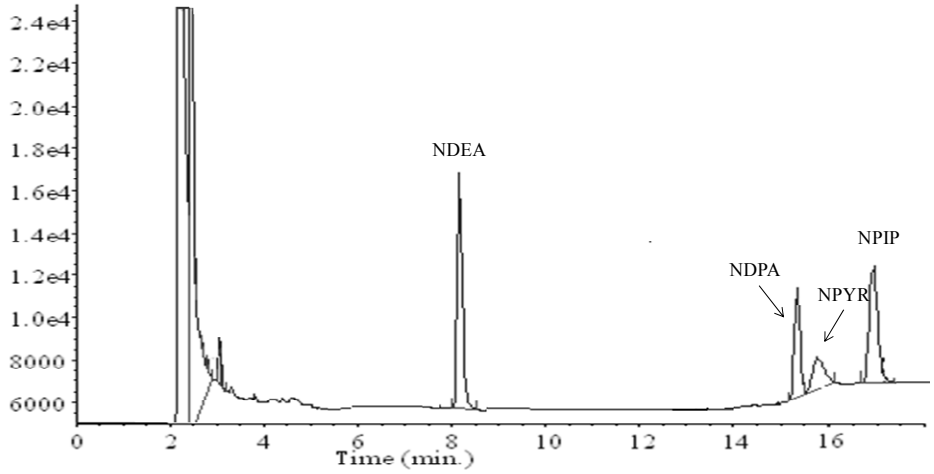


Figure 29 GC chromatogram obtained from an extract of fortified frankfurter separated on 30.0 mm silica monolithic sorbent column coupled with ZB-5.

From figure 29 the retention time of NDEA, NDPA, NPYR and NPIP are 8.297, 15.370, 15.870 and 17.137, respectively. Similar to the chromatogram of pure standard nitrosamines in Figure 28, no extra peak was observed in this chromatogram. It indicates that no fat was co-eluted and any effect to nitrosamine separation subjected to fat retention on the sorbent was also not observed. These results have proved the ability of silica monolith as a fat retaining sorbent for nitrosamine determination.

4.1.1 Recovery determination by GC-FID

To study recovery of the method, 1.0 g frankfurter was fortified with 20 µL of each nitrosamine and used for extraction. After SWE, extracted sample was subjected to dichloromethane extraction, pre-concentration and finally injection onto a 30.0 mm silica monolithic column connected with ZB-5 under GC

optimal conditions with flame ionization detection. Quantitative analysis of nitrosamine was performed and the results in term of % recovery of each nitrosamine are summarized and compared to those obtained from SWE coupled with a conventional SPE in Table 15.

Table 15 Comparison of % recovery of nitrosamines obtained from a LLE-SPE (Subprasert, 2007) and LLE-silica monolith after SWE.

Compound	t_R (min)	%Recovery[1] ± SD (%RSD)	
		SWE-SPE	SWE-silica monolith
NDEA	8.355	93.53±3.9 (4.2)	84.96 ± 0.035 (5.65)
NPYR	16.227	83.15±1.6 (1.9)	72.88 ± 0.020 (6.05)
NPIP	17.847	96.40±2.7 (2.8)	85.45 ± 0.029 (3.65)

[1] n = 3

Table 15 shows the recovery of nitrosamines in frankfurter subjected to the extraction by superheated water, liquid-liquid extraction and extraction using silica monolithic sorbent trap. The recoveries ranged from 73-85%, which was in an acceptable range of 70% to 130%, required by the California Department of Health Services (Grebel *et al*, 2006). Loss of analytes in the procedure may be caused by human error during extraction procedure, solvent evaporation and other systematic errors. From the results, precision of the coupling method was satisfactory, varying between 3.65-6.05%, which confirmed the ability of silica monolithic column as an on-line sorbent trap.

The main purposed of the research was to reduce a tedious sample preparation process of nitrosamine determination after superheated water extraction by using a fabricated monolithic sorbent column. Comparison of recoveries compared between SWE-conventional solid phase extraction (SPE) (Subprasert, 2007) and

SWE-silica monolith were shown in Table 15 and Figure 30. The results exhibited the same trend for both cases. The recovery subjected to SWE-conventional SPE were about 10% higher than those for SWE-silica monolith adsorption, However, to our surprise, the standard deviations obtained from the former technique were about 100 times higher than the latter. This indicated a very high precision of the method, despite a slightly lower accuracy, compared with the former. It must also be taken into account that time consuming, solvent consumption and cost of operation were highly reduced when silica monolith was used instead of SPE. The cleaning up and pre-concentration process by a conventional SPE took about one hour, and the time was reduced to approximately 10 min when the novel adsorbent was used.

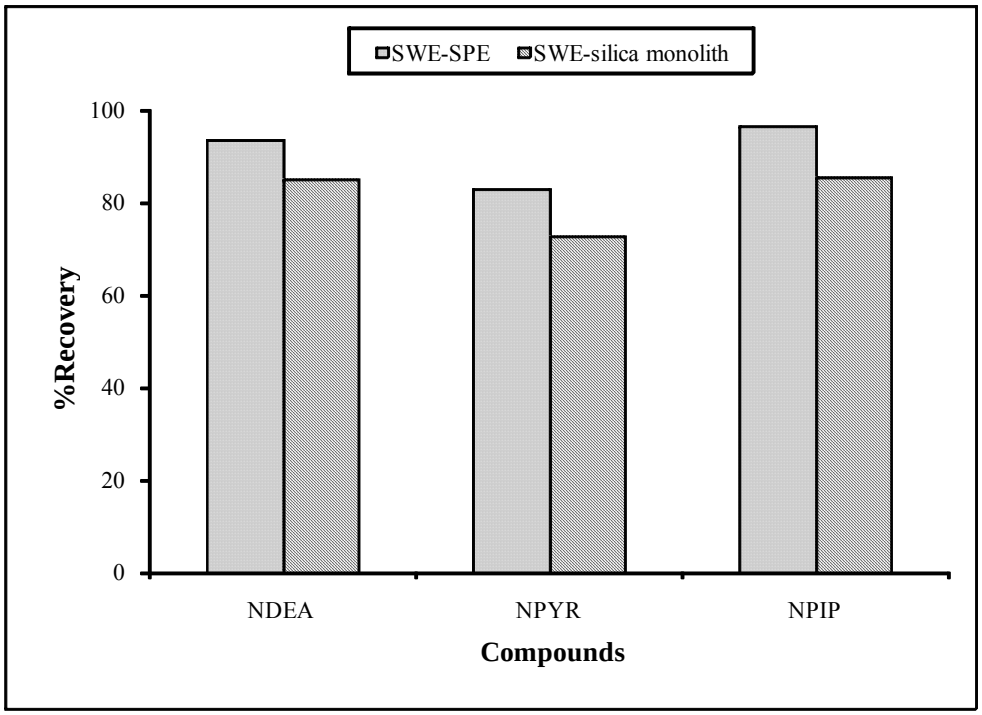


Figure 30 Comparison of % recovery of fortified frankfurter extract subjected to SWE-conventional SPE and SWE-silica monolithic sorbent examined by GC-FID.

4.1.2 Injection-to-injection repeatability and column reproducibility

To be a sorbent trap an investigation of injection-to-injection repeatability and column-to-column reproducibility of silica monolithic column must be performed. In this experiment the fortified frankfurter extract was repeatedly injected on the same silica monolithic sorbent column connected with the ZB-5 analytical column. An average area ratio of each analyte to its internal standard, average retention time (t_R) and relative standard deviation (%RSD) are summarized in Table 16. A good reliable injection-to-injection precision of average area ratio and average retention time were obtained, varying between 0.7908-1.4586% and 0.0943-0.1643, respectively.

Table 16 Injection-to-injection repeatability of average area ratio and retention time (t_R) for each component separated on silica monolithic column connected with ZB-5.

Compound	Average area ratio	%RSD	Average retention time (t_R)	%RSD
NDEA	0.5786	0.7908	8.457	0.0943
NPYR	0.4012	0.9132	15.836	0.1643
NPIP	0.8192	1.4586	17.149	0.1258

To study column-to-column reproducibility, the fortified frankfurter extract was repeatedly injected into four silica monolithic columns successively connected with the ZB-5. Average area ratios of each analyte to its internal standard, average retention time (t_R), and their relative standard deviations (%RSD) are summarized in Table 17. The results demonstrate a good column-to-column precision of average area ratio and average retention time, varying between 2.3981-5.9794% and 0.1623-0.2235, respectively. These results show that the fat adsorption on the silica monolith did not significantly affect both quantification and retention of nitrosamines.

Table 17 Column-to-column precision comparison of the average area ratio and average retention time (t_R) for each component for each of four silica monolithic column connected with ZB-5.

Compound	Average area ratio	%RSD	Average retention time (t_R)	%RSD
NDEA	0.5976 ± 0.0203	3.4031	8.460 ± 0.015	0.1831
NPYR	0.3914 ± 0.0234	5.9794	15.841 ± 0.0354	0.2235
NPIP	0.8774 ± 0.0210	2.3981	17.123 ± 0.0318	0.1856

4.2 Efficiency of the method examined by GC-MS detection.

As GC-MS detection provides much smaller detection quantities of analytes than several classical methods and it was used in our previous study in nitrosamine extraction and quantification, the GC-MS was then re-applied to determine the compounds in order to compare efficiency obtained from the new technique and the conventional method. To demonstrate the efficiency of overall nitrosamine determination process, efficiency of a combination of extraction, clean-up procedure, pre-concentration and chromatographic separation and detection must be investigated. Experiments of detection limit and recovery determination were conducted and the results were compared to those obtained from SWE coupled with a conventional SPE.

4.2.1 Detection limit

This experiment was aimed to investigate detection limits of all analytes, which were simultaneously extracted by superheated water under optimal condition, cleaned up by LLE and finally injected into a 30.0 mm long silica monolithic column connected with HP-5 (similar to the ZB-5 column) under optimal GC condition. Three calibration curves of nitrosamines were prepared from extraction of each nitrosamine fortified frankfurter, and the extract was injected to the GC-MS at a mass range of NDEA and NPIP between 30-70 ng injected, and NPYR between 50-90 ng injected. NDPA was used as the internal standard. Ten extractions were performed. Peak area ratio of each analyte to its internal standard for each concentration were calculated and averaged as the values given in Table 18.

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).

$$y - y_B = 3\, s_B \qquad (1)$$

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).

To estimate the limit of detection, peak height is typically obtained from a chromatogram. In this work the peak area was taken because all nitrosamine peaks were slightly tailing, thus resulting 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 each nitrosamine to internal standard.

Compounds	Peak area compound / internal standard[1]						
	Mass (ng injected)						
	30	40	50	60	70	80	90
NDEA	0.2517	0.3041	0.3841	0.4392	0.4823	-	-
NPIP	0.1549	0.2187	0.2944	0.3679	0.4578	-	-
NPYR	-	-	0.1831	0.2246	0.2547	0.3034	0.3544

[1]n = 3

Average peak area ratio of each compound was plotted against the analyte mass, giving a good linearity with a slope, intercept, and least square of correlation coefficient (r^2) shown in Table 19.

Table 19 Statistical parameters from each linear calibration curve of analyte.

Compounds	Mass range (ng injected)	Intercept a, y_B	Slope, b	r^2
NDEA	30-70	0.0740	0.0060	0.9900
NPYR	50-90	-0.0300	0.0040	0.9910
NPIP	30-70	-0.0780	0.0070	0.9960

To estimate a standard deviation of the blank or unfortified frankfurter extract (s_B), 25 µL NDEA, 25 µL NPIP and 45 µL 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 were calculated and given in Table 20.

Table 20 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
1	0.2389	0.2142	0.1516
2	0.2336	0.2104	0.1502
3	0.2321	0.2266	0.1431
4	0.2304	0.2253	0.1534
5	0.2437	0.2162	0.1507
6	0.2363	0.2193	0.1405
7	0.2455	0.2176	0.1420
8	0.2399	0.2196	0.1527
9	0.2476	0.2028	0.1497
10	0.2357	0.2104	0.1422
S_B	0.0061	0.0072	0.0049

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 given in Table 21.

Table 21 Detection limit of each nitrosamine in fortified frankfurter extract by a SWE-silica monolith adsorption.

Compound	Calculated detection limits (ng injected / 1 g sample)
NDEA	3.05
NPYR	5.40
NPIP	2.10

From Table 21, the method limit of detection of nitrosamine compounds in the fortified frankfurter was found in a range of 2.10-5.40 ng injected per 1 g sample. The detection limits of all analytes obtained were not very low as expected, probably due to an insufficiently high sensitivity of the HP5989 mass spectrometric detector used in the work. It was then postulated that the detection limits could be reduced by using more sensitive and selective detectors, namely tandem mass spectrometric detector (tandem-MS), and thermal energy analyzer (TEA). The TEA is recommended for nitrosamine detection, however it is costly and not available in most laboratories.

4.2.2 Recovery

To study recovery of the method, 1.0 g frankfurter was fortified with 60 µL of each nitrosamine and used for extraction. After SWE, extracted sample was subjected to LLE, adjusted to 1 mL with dichloromethane and finally injected into a 30.0 mm silica monolithic column connected with HP-5. Five determination of nitrosamine were performed by GC-MS and the results in term of % recovery of each nitrosamine were summarized and compared to those obtained from SWE coupled with a conventional SPE shown in Table 22.

Table 22 Comparison of % recoveries of nitrosamines obtained from a LLE-SPE (Subprasert, 2007) and LLE-silica monolith after SWE.

Compound	t_R (min)	%Recovery[1] ± SD (%RSD)	
		SWE-SPE	SWE-silica monolith
NDEA	8.379	95.05 ± 5.9 (6.2)	85.76 ± 0.011 (2.09)
NPYR	16.204	81.82 ± 4.9 (6.0)	74.68 ± 0.014 (2.69)
NPIP	17.613	106.93 ± 7.7 (7.2)	88.45 ± 0.029 (3.85)

[1] n = 5

From Table 22, recoveries of nitrosamines range between 74.68-88.45 % which lies within the acceptable range, 70% to 130%. The precision of the coupling method was satisfactory, varying between 2.09-3.85%. Recoveries of nitrosamines obtained from SWE-SPE (Subpresert, 2007) and SWE-silica monolith examined by GC-MS was compared as shown in Figure 31. The results showed the same trend for both methods. The recovery obtained from SWE-SPE was slightly higher than those from SWE-silica monolith about 7% for NPYR, 10% for NDEA and 18% for NPIP. However, similar to the recovery from FID detection, the precision expressed by %RSD of the former method was about 300-500 times higher than the later one. Silica monolith was obviously proven that it was beneficial in reducing analysis time, solvent consumption and also cost of operation.

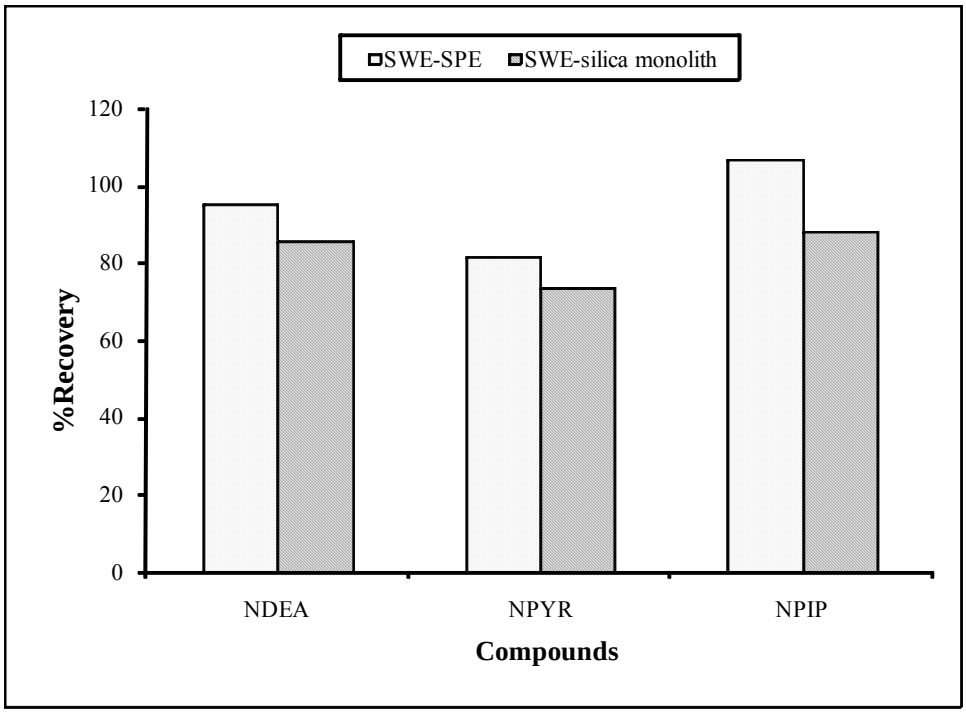
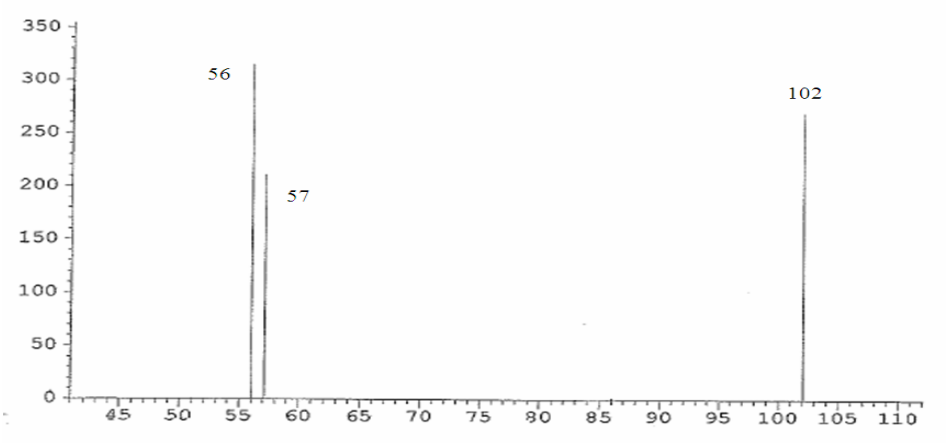
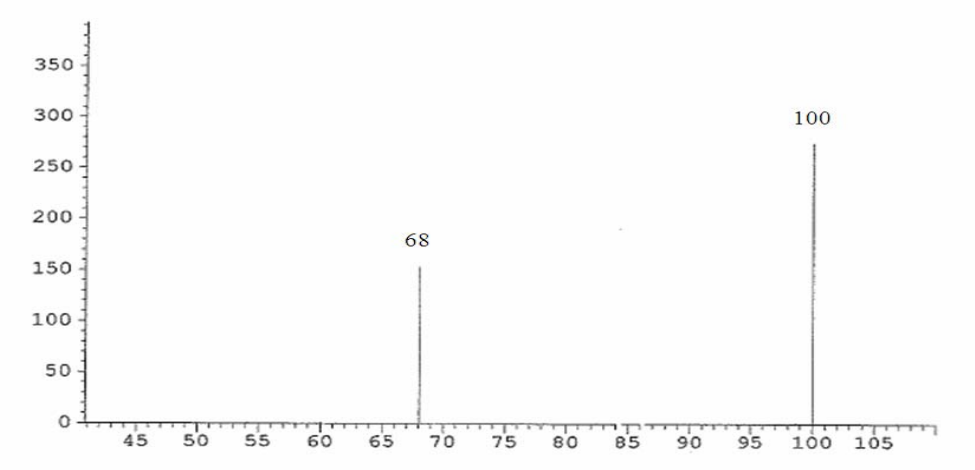


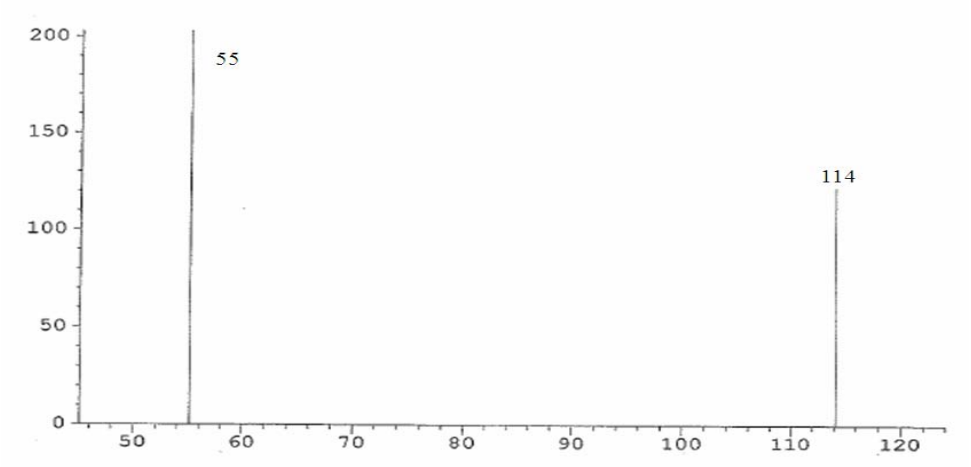
Figure 31 Comparison of % recovery of nitrosamines in fortified frankfurter extract by using SWE-SPE and SWE-silica monolithic sorbent and subjected to GC-MS analysis.



(A)



(B)



(C)

Figure 32 Mass spectrums of extracted nitrosamines by using superheated water coupled silica monolithic column connected with HP-5.

Figure 32 shows the mass spectrum acquired from an injection of 80 μg mL^{-1} concentration of NDEA (Figure 32-A), 100 μg mL^{-1} concentration of NPYR (Figure 32-B) and 80 μg mL^{-1} concentration of NPIP (Figure 32-C). All spectrums obtained from a 1 μL injection of fortified frankfurter extract. The NDEA spectrum showed m/z 102, 57 and 56, while those NPYR and NPIP spectrum showed m/z at 100 and 68; 114 and 55, respectively. No additional ions of significant relative abundance were detected in those spectrums performed in the presence of silica monolithic column coupling with HP-5, indicating that the method was very clean with no interference with similar retention and fragmented mass.

It was confirmed from these results that silica monolithic column coupled with GC column was a remarkable alternative for very cheap and fast determination of nitrosamines in food sample. Besides, the sorbent column also gained benefit in high throughput clean-up method prior to chromatographic analysis.

5. Comparison with other methods

Since the nitrosamines are mutagenic compounds that can be found in meat products and its allowable concentration in meat is very low, the development of analytical method including sample preparation is then necessary. In this work, nitrosamine was extracted with superheated water and then cleaned up by using liquid-liquid extraction (LLE) coupled with a home-made silica monolithic column as an on-line sorbent trap before an application to GC. The overall method was compared with several other previously reported techniques, for example supercritical fluid extraction , conventional SPE, and steam distillation, in term of sample preparation, analytical method, sample mass, % recovery and detection limit, as shown in Table 23.

Although % recovery of the proposed method was quite close to those reference methods, it provided more advantages. For example, only 1 g of food sample was required for SWE, the amount was much reduced for distillation. The extraction time of SWE was much shorter, e.g. the SWE time was less than 20 min

per sample, while it take approximately one hour to finish a steam distillation. For a clean-up step in this work, LLE was coupled with an on-line monolithic column sorbent trap. The volume of dichloromethane for the LLE step was less than 3 mL that was significantly reduced, leading to a fast pre-concentration step. The on-line home-made monolithic sorbent trap was replaced a conventional SPE, providing simpler procedure and significantly reducing cost and time for the clean-up.

In the reference method 1, 2, 3, 4, 5 and 6, many steps of sample preparation, time consuming and large organic solvent volume are required. Particularly, in the AOAC reference method 1, vacuum distillation takes about 75 min per sample, after that the distillate is transferred and extracted with up to 100 mL dichloromethane. The pre-concentration step of about 2 h is totally needed, whereas it was reduced to a few minutes when monolithic adsorbent was used. Moreover, one piece of monolithic sorbent could be reused more than 20 times (or 20 samples). In the reference method 2, the distillate is collected and extracted with 60 and 3 x 180 mL of dichloromethane, or totally 600 mL dichloromethane is consumed for each extraction. Whereas, in the reference method 3, 5 and 6, the required of organic solvent for SPE step are 52, 72 and 30 mL, respectively. In the reference method 5, the cleaning up and pre-concentration process by a conventional SPE takes about one hour, while the time was less than 10 min when monolithic adsorbent was used. The nitrosamine extraction is not obtained with high efficiencies by using the reference method 4.

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 MS detector used in this work. However, the LOD quantification can be improved by using a more sensitive and specific detector.

Table 23 Comparison between various methods for determination of nitrosamines.

Reference	1 AOAC (982.22) (1995)	2 Byun *et. al.* (2004)	3 Yurchenko *et. al.* (2006)	4 Filho *et. al.* (2007)	5 Yurchenko *et. al.* (2007)	6 Subprasert. P. (2007)	Proposed method
Sample matrix	Fried Bacon	Fermented sausage	Fish products	Sausages	Meats	Frankfurter	Frankfurter
Preparation method	Mineral oil vacuum distillation and extraction with DCM	Steam distillation on a steam generator and extraction with DCM	Two-step SPE with Extrelut and Florisil	Supercritical fluid CO_2 coupled with SPE (florisil)	Two-step SPE with Extrelut and Florisil	SWE, LLE couple with SPE (florisil)	SWE, LLE couple with on-line silica monolith sorbent trap
Analytical method	GC-TEA	GC-TEA	GC-MSD	MEKC	GC-MS	GC-MS	GC-MS
Sample mass	25 g	20 g	6.0 g	0.2 g	6.0 g	1.0 g	1.0 g
% recovery	-	87-93 %	79-88 %	21-82 %	79-88 %	86-106 %	75-88 %
Detection limit	-	0.1 ug kg^{-1}	0.10 μg kg^{-1}	-	0.09 μg g^{-1}	0.47-1.48 ng	2.10-5.40 ng

CONCLUSION

Feasibility of home-made monolithic column as a non-polar matrix retainer was investigated. Three monolithic columns, namely polystyrene-co-divinylbenzene (PS-DVB), poly-divinylbenzene (poly-DVB) and silica, were fabricated and morphological characterization by SEM. The results showed satisfactory monolithic structure of fabricated columns. These three types monolithic columns were preliminarily studied as an on-line sorbent trap applied to GC.

Firstly, polystyrene-co-divinylbenzene (PS-DVB) monolith was studied and it shows capability in trapping fat and oil, investigated by an injection of rice bran oil mixed with NDPA. Because of the high resistance to carrier gas flow when PS-DVB monolith was coupling with a ZB-5 analytical column, only a limit length of 3.0-6.0 mm PS-DVB could be used. However, the peaks of NPYR and NDPA were overlapped, then no further experiment of PS-DVB was conducted.

Subsequently, polydivinylbenzene (poly-DVB) was studied. The larger through pores of poly-DVB monolithic structure was revealed by SEM, leading to higher column permeability, thus the column could be lengthened to about 0.5-20.0 mm. However, a coupling of poly-DVB with ZB-5 also gave merged peaks of NPYR and NDPA.

Finally, silica monolith was studied and satisfactory result was obtained. It demonstrated a capability in trapping fat and oil, investigated by an injection of nitrosamines mixed rice bran oil. Only non-polar matrix was trapped, while the nitrosamines were released from the silica sorbent. An optimum column length of silica monolith to provide the resolved peak of NPYR and NPDA was 30.0 mm. Sensitivity of nitrosamines by flame ionization detector compared between the absence and presence of silica monolith was also investigated and there was no significant difference for both cases.

Coupling of silica monolith as an on-line sorbent trap with GC-column was subsequently performed with the real sample. Fortified frankfurter sample was subjected to superheated water extraction (SWE) under optimum conditions. The extract was clean-up and pre-concentrated by liquid-liquid extraction. Separation and identification of components were carried out using a silica monolith sorbent trap coupled with either GC-FID or GC-MS. A 30.0 mm silica monolith provided reusability up to 27 times injection before fat leaching was observed. For chromatographic separation, a good injection-to-injection repeatability and column-to-column reproducibility were also obtained.

When coupling the SWE and silica monolithic sorbent trap, the recoveries of nitrosamine extracted from frankfurter were in a range of 75-88 %. Calibration graphs by GC-MS obtained for calculating limit of detection gave excellent linear correlation coefficients with a range of detection limit between 2.10-5.40 ng injected of analytes extracted from frankfurter sample.

The proposed method was more beneficial than other published methods and SWE-conventional SPE for nitrosamine determination in the following ways: firstly, the sample preparation steps are much more simple and efficient when SPE step was replaced by the on-line monolithic sorbent trap. Secondly, lower cost of operation was obtained, while using only small amount of organic solvents and home-made monolithic column. Thirdly, the overall extraction and determination time necessary for SWE, clean-up and pre-concentration was only 25 min.

It was then concluded that the coupling of superheated water extraction and on-line silica monolithic sorbent trap was an interesting alternative technique, providing several advantages, i.e. simplicity, low cost, reduced analysis time, low organic solvent consumption, thus generating low amount of waste. This method was then supported for a routine analysis of nitrosamines in meat and meat products.

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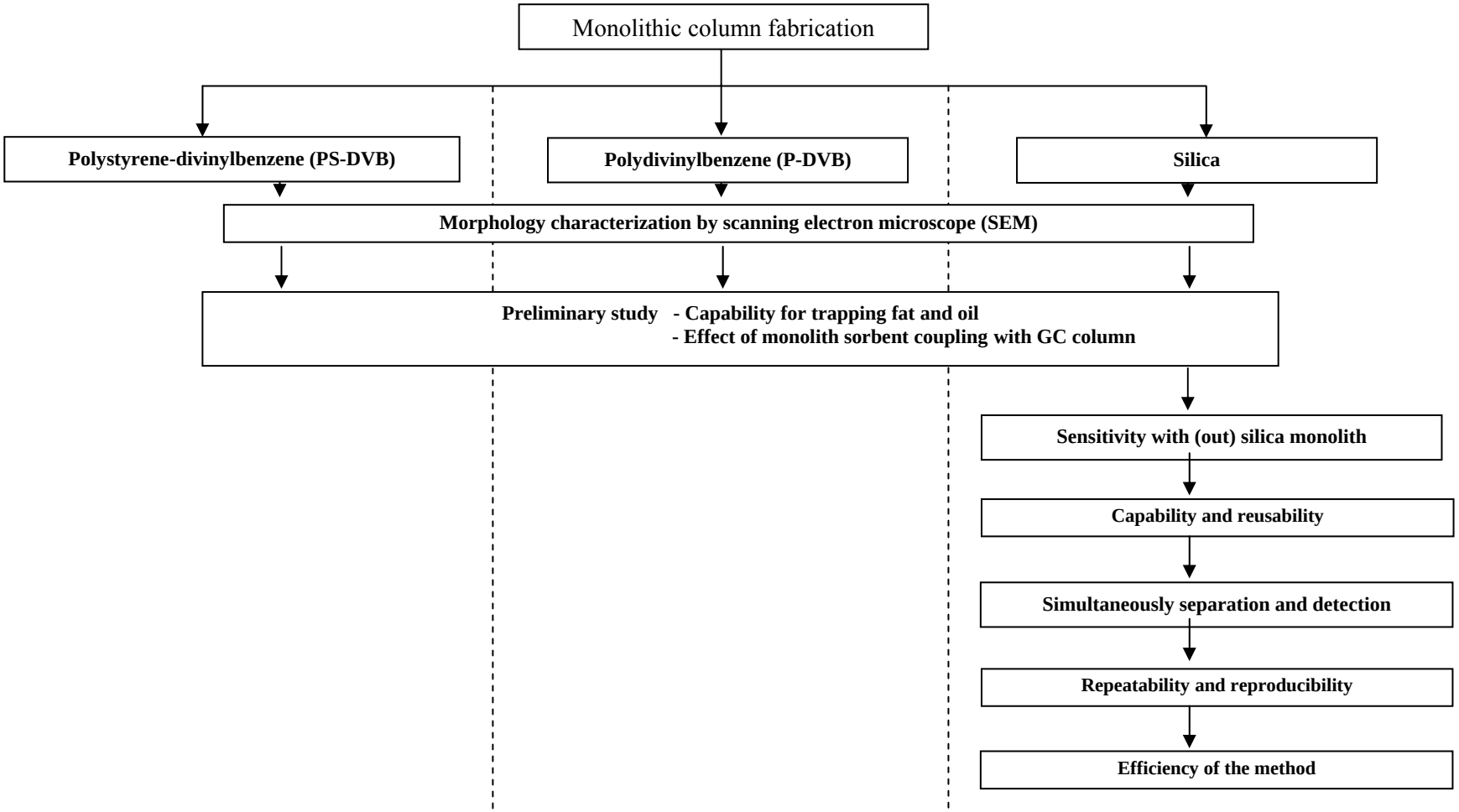
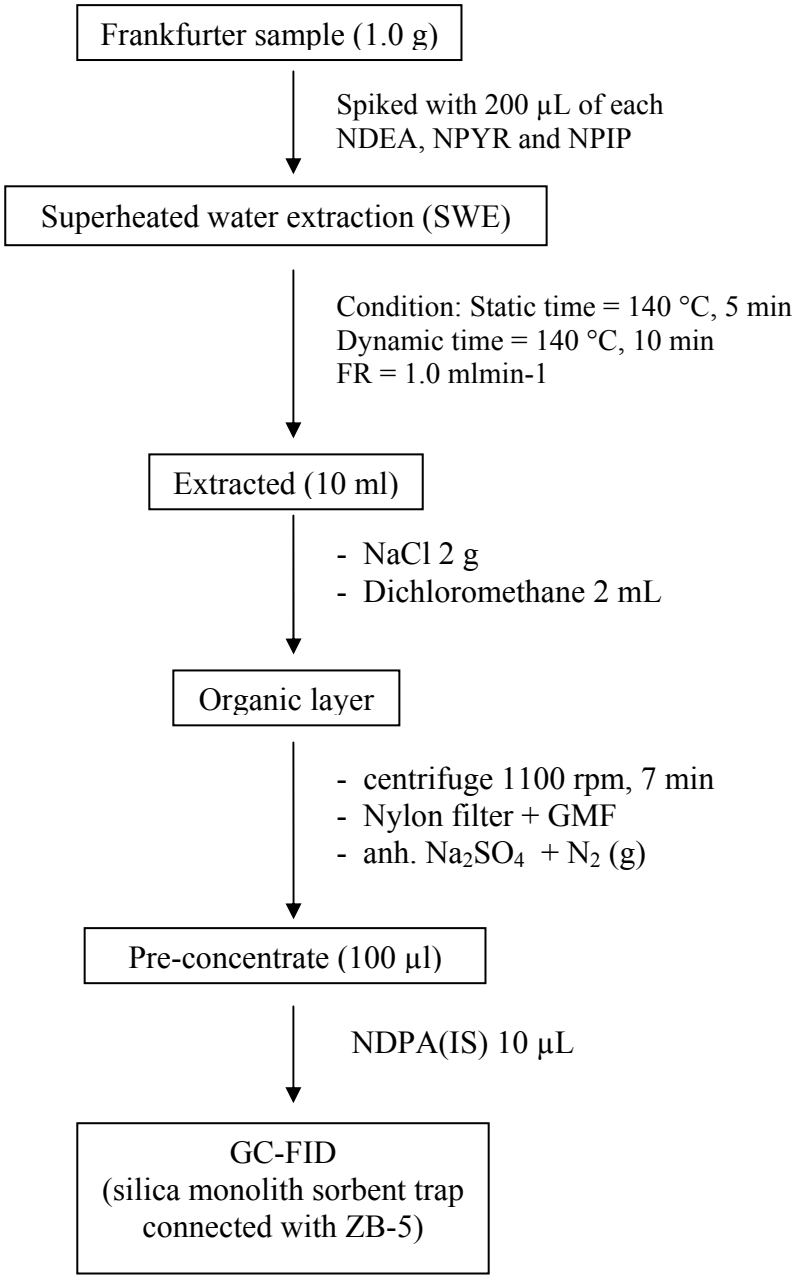
APPENDIX

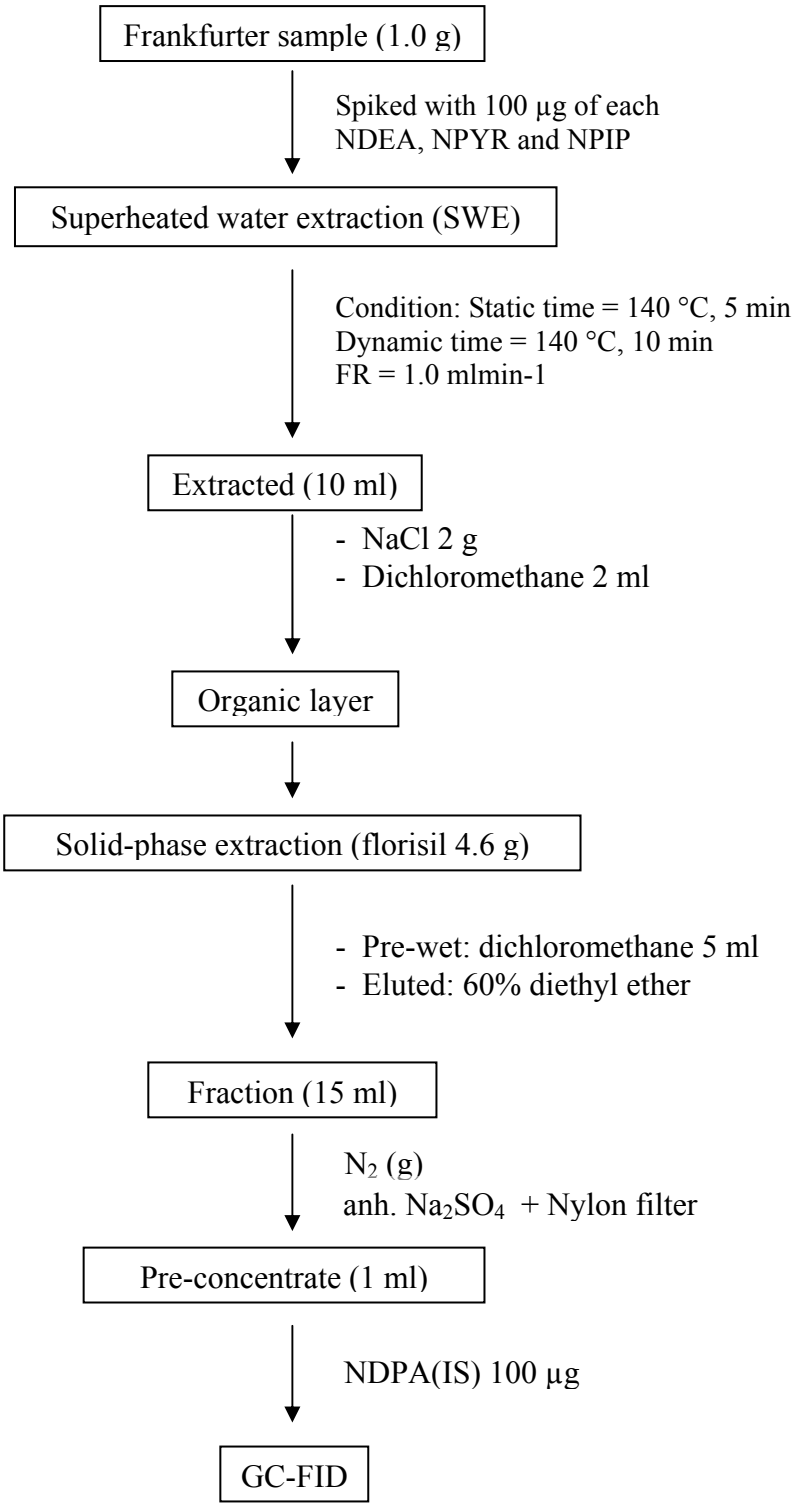
Appendix 1 Overall experiments of the present research.



Appendix 2 Flow chart of SWE coupled with silica monolith sorbent.

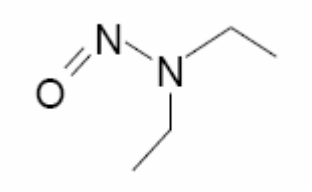


Appendix 3 Flow chart of SWE coupled with SPE (Subprasert, 2007).



Appendix 4 Chemical information of analyte.

N-nitrosodiethylamine (NDEA)



$(C_2H_5)_2NNO$
CAS no. 55-18-5

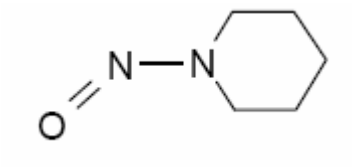
Synonyms: diaethylnitrosamin (German); N,N-diethylnitrosamine; diethylnitrosamine; diethylnitrosoamine; ethylamine, N-nitrosodi-; N-ethyl-N-nitrosoethanamine; nitrosodiethylamine; DANA; DEN; DENA; NDEA.

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 effected organs were liver, esophagus, nasal cavities, kidney, forestomach, lung and larynx.

Appendix 4 (continued)

N-nitrosopiperidine (NPIP)



$C_5H_{10}N_2O$
CAS no. 100-75-4

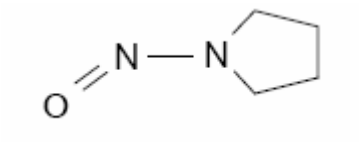
Synonyms: N-nitroso-piperidin (German); 1-nitrospiperidine; NO-Pip; NPIP.

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 4 (continued)

N-nitrosopyrrolidine (NPYR)



$C_4H_8N_2O$
CAS no. 930-55-2

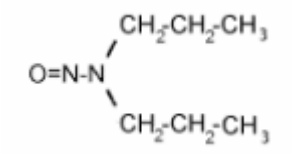
Synonyms: N-nitrosopyrrolidin (German); pyrrolidine, 1-nitroso-; NO-Pyr; NPYR.

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 dose (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 4 (continued)

N-nitroso-di-n-propylamine (NDPA)



$C_6H_{14}N_{20}$
CAS no. 621-64-7

Synonyms: NDPA; Di-n-propylnitrosamine; N-Nitrosodi-n-propylamine; DPNA; DPN; N-Nitroso-N-propyl-1-propanamine; propylamine, N-Nitroso-N-di; Dipropylnitrosamine; N-Propyl-N-nitrosopropylamine

Use information: It is used as a research chemical.

Toxicology: The LD50 for NDPA, administered orally to the rat, is 480 mg/kg. In a chronic experiment, the agent was added to the daily food of the rats. Four dose groups were involved - 30 mg/kg led to cancer of the liver, 15 mg/kg produced liver cancer in all the dosed rats and in four cases metastases into the lung was observed. When 8 mg/kg NDPA was administered, the result was cancer in 15 of 16 animals. The lowest dose, 4 mg/kg, resulted in cancer of the liver in 12 of 14 animals. The mean carcinogenic doses (D50) were 3.2, 1.86, 1.52 and 1.15 g/kg. The mean induction periods (t50) were 120, 155, 202 and 300 days.

Appendix 5 Method of calculation of the nitrosamines recovery from chromatographic data.

Suppose that a standard solution for calculating the response factor was a mixture of 60 ug of the nitrosamine analyte and 60 ug 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 60 ug nitrosamine compounds and extracted. 60 ug internal standard was used and then the solution was applied to GC, resulting the peak area as following example data.

Compound	Peak area	
	Analyte	Internal standard
Standard	2809765	4316629
Frankfurter sample	2001768	3706183

$$\text{Response factor} = \frac{60 \text{ ug} \times 4316629}{60 \text{ ug} \times 2809765} = 1.54$$

$$\% \text{ Recovery} = \frac{2001768 \times 60 \text{ ug} \times 1.54 \times 100}{3706183 \times 60 \text{ ug}}$$

$$= 83.17 \%$$

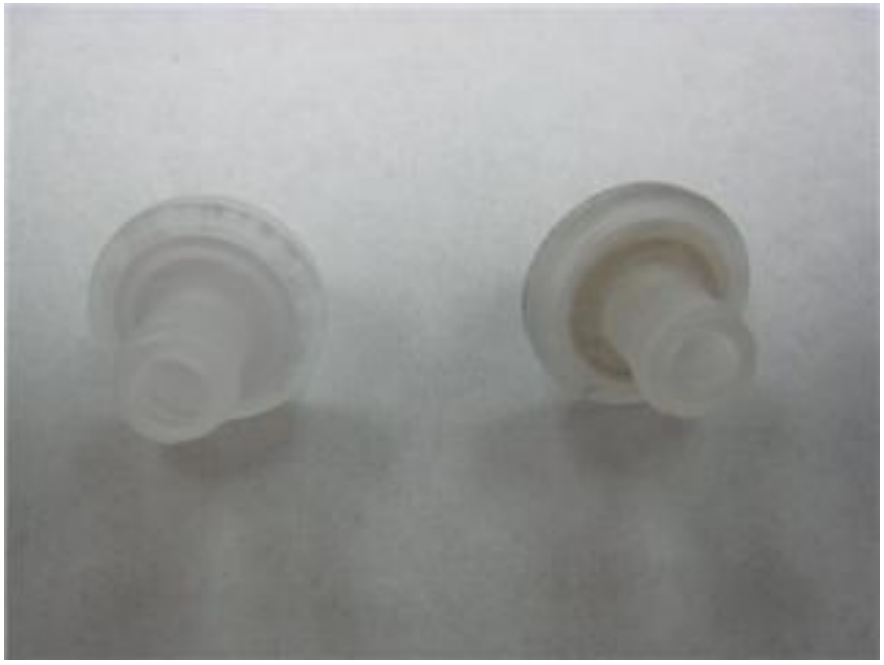
Appendix Table 1 Physical properties of selected nitrosamines.

Analyte	NDEA	NPIP	NPYR
Formula	$(C_2H_5)_2N_2)$	$C_5H_{10}N_2O$	$C_4H_8N_2O$
Molecular weight	102.14	114.15	100.2
Boiling point (C)	177	217	214
Density (g/mL)	0.94	1.06	1.09
Refractive index	1.4386	1.4933	-
Physical appearance	Yellow Liquid	Yellow Liquid	Yellow Liquid
Water solubility (mg/L)	106,000	76,480	1,000,000
Polarity (log Koctanol-water)	0.48	0.36	-0.19
Vapour peressure (mm Hg)	0.86	0.14	0.06
UV absorption (λ max, nm)	231	366	230

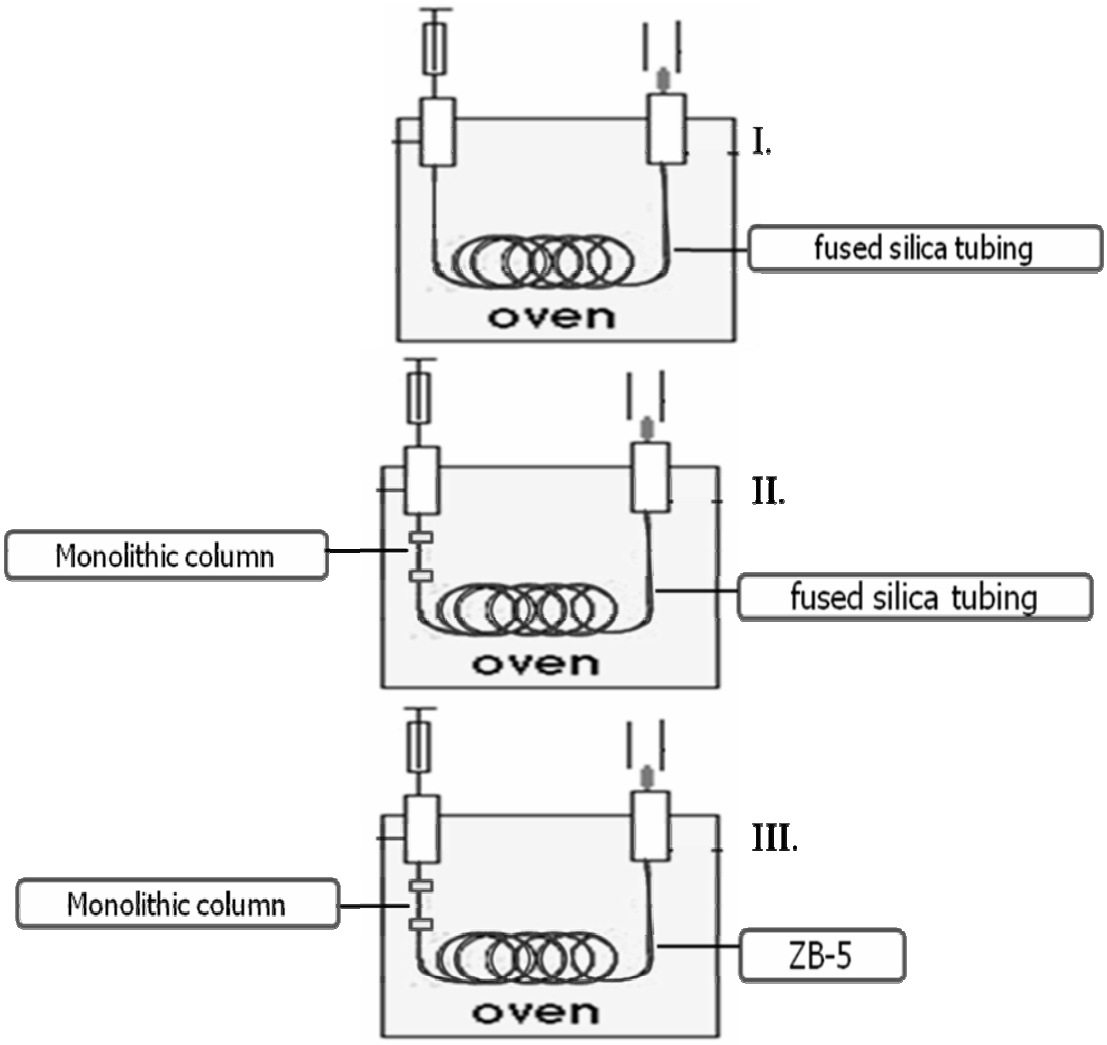
Appendix Table 2 Chemical list.

Chemicals	Formula	Molecular weight	Company
Acetone	C_3H_6O	58.08	Ajax Finechem
Acetonitrile	CH_3CN	41.05	Ajax Finechem
Decanol	$C_{10}H_{22}O$	158.28	Acros
Dibenzoyl peroxide	$C_{14}H_{10}O_4$	242.23	Acros
Dichloromethane	CH_2Cl_2	84.93	BDH
Divinylbenzene	$C_{10}H_{10}$	130.0	Aldrich
Dodecanol	$C_{12}H_{26}O$	186.34	Fluka
Methanol	CH_3OH	32.04	Merck
N-nitrosodiethylamine	$C_4H_{10}N_2O$	102.14	Fluka
N-nitrosopiperidine	$C_5H_{10}N_2O$	114.5	Sigma
N-nitrosodipropylamine	$C_6H_{14}N_2O$	130.19	Supelco
N-nitrosopyrrolidine	$C_4H_8N_2O$	100.12	Aldrich
Sodium chloride	NaCl	58.44	BDH
Sodium hydroxide	NaOH	39.99	Merck
Sodium sulphate anhydrous	Na_2SO_4	142.02	Ajax Finechem
Styrene	C_8H_8	104.15	Fluka
Tetrahydrofuran	C_4H_8O	72.11	Fisher
Tetramethoxysilane	$SiC_4H_{12}O_4$	152.25	Merck
Urea	$(NH_2)_2CO$	60.07	Merck

Appendix Figure 1 Superheated water apparatus



Appendix Figure 2 Nylon filter with glass microfiber (GMF) before and after filter fortified frankfurter extract.



Appendix Figure 3 Gas chromatographic set up for separation of oil added nitrosamines.