THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Chemistry)

DEGREE

Chemistry	Chemistry
FIELD	**DEPARTMENT**

TITLE: Monolithic Materials for Microfluidic System

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THESIS

MONOLITHIC MATERIALS FOR MICROFLUIDIC SYSTEM

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Chemistry)
Graduate School, Kasetsart University
2011

Visakha Chunhakorn 2011:Monolithic Materials for Microfluidic System. Master of Science (Chemistry), Major Field: Chemistry, Department of Chemistry. Thesis Advisor: Associate Professor Orapin Chienthavorn, Ph.D. 102 pages.

A C_8-silica monolith was synthesized in a microchannel of PDMS chip, with channels length of 10 and 20 cm to be used as an analytical column for reversed phase liquid chromatography. Separation of mixture amines, namely methylamine, ethylamine, and trimethylamine, was studied with pre-derivatisation technique using 4-(2-aminoethylamino)-7-(N,N-dimethylsulfamoyl) benzofurazan fluorescencing agent, which gave an emission wavelength of 518 nm. Because of high backpressure occurred in the microfluidic chip; the flow rate was limited to 0.3-0.5 mL min^{-1}. Three amines gave well resolved peaks with retention time of 2.5084. 3.0315, and 3.7355 min when using methanol as a mobile phase. The C_8-silica monolith in the channel was also studied for a separation of riboflavin and of chlorpheniramine in a channel length of 20 cm. Calibrations curves of riboflavin and chlorpheniramine were linear in a range of 200-1000 ppm, giving a linear least square of 0.9944 and 0.9274, respectively. The detection limit of riboflavin and chlropheniramine determined from peak area were 285 and 283, respectively. Riboflavin and chlropheniramine in tablets were tested for the feasibility for real sample analysis by an injection of the methanolic extract of a tablet of each compound into the system. The C_8-monolith microfluidic chip was proved to be a new version of HPLC column for both ionisable and neutral compounds. Benefits of the microchip are low cost, handy and disposable.

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ACKNOWLEDGEMENTS

First and foremost, my deepest gratitude is to my dear parent, Napol and Cheunjidt Chunhakorn for their love, up-bringing and inspiration. Without them I would not be able to carry out the study.

I would like to extend my gratitude to my supervisor, Assoc. Prof. Dr. Orapin Chienthavorn for her valuable guidance, encouragement and kind supervision throughout the course of my postgraduate study. I appreciate Prof. Harry J. Whitlow for his help in setting up the laser induced fluorescence instrument. Assoc. Prof. Dr. Somsorn Singkarat, Nitipon Puttaraksa, and Somrit Unai are acknowledged for the support in microfluidic chip fabrication.

I would like to express my thanks the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education for providing a scholarship and financial support.

Further thank is extended to the Chemistry Department, Faculty of Science, Kasetsart University for facility support, and the Plasma and Beam Physics Research Facility, Department of Physics and Materials Science, Faculty of Science, Chiang Mai University, is also acknowledged for good collaboration.

My special thanks are to Sakdinak Wajvisut, my close friend, for all his helps, continuous encouragement, and true friendship. I also sincerely appreciate all of my friends at Kasetsart University, namely Holm, Por, Tha, Oar, Tee, Gift, Bow, Mew, Tan, Mook, Ying, and etc., for their help and support.

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September 2011

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LIST OF ABBREVIATIONS

C_8-TEOS	=	Octyltriethoxysilane
GaN	=	Gallium Nitrile
GC	=	Gas Chromatography
HPLC	=	High Performance Liquid Chromatography
LIF	=	Laser-induced Fluorescence
MPTMS	=	3-mercaptopropyltrimethoxysilane
PDMS	=	Plydimethylsiloxane
PMMA	=	Polymethylmetacrelate
PMT	=	Photomultiplier Tube
ppb	=	parts per billion
ppm	=	parts per million
SEM	=	Scanning Electron Microscopy
TEOS	=	Tetraethoxysilane

MONOLITHIC MATERIALS FOR MICROFLUIDIC SYSTEM

INTRODUCTION

A typical commercial high pressure liquid chromatography (HPLC) instrument is composed of a reciprocating pump, injection valve, a column, and a detector. The column is commercially available in the form of packed-bed silica-based particles. In generally two main kinds of forces driven mobile phase passing through the packed-bed particles are electrokinetic and pressure. The HPLC commonly used high pressure. The electrokinetic-driven flow utilized electrical potential to generate electroosmotic flow along a very narrow column or capillary. The instrument using such technique is widely-known as capillary electrophoresis (CE) where no packing particle is inside the capillary. For a packed-bed capillary the technique is called capillary electrochromatography (CEC). Demand in miniaturization of chemical analysis system has led to a technology in microfluidic device where analyte samples can be manipulated, separated and analyzed in one unit. The microdevice is very powerful to study chemicals and their reactions consequently, providing several advantages, i.e. minimal sample size, fast reaction time, low cost, and portability. Several applications of the microfabricated device have been focused onto a number of instrumental methods. For example, to miniaturize a flow injection analysis system, a microfluidic chip was simply fabricated, composing micro-valve, reservoirs, injectors, and several channels. A number of applications of analytical microchips in capillary electrophoresis and capillary electrochromatography have already been reported. The first microfluidic chip have been now been commercially available from Agilent Technologies, and it was designed for gel electrophoretic analysis of biopolymers.

Microfluidics has a meaning of a manipulation of fluids to flow through microchannels with a dimension of 10 to less than a thousand micrometers. In 1992 the first microchip of capillary electrophoresis was launched, and it was a result of successful laboratory-on-a-chip research efforts. Although a great advance in

development of separations in microfluidic platforms by electrokinetic driving force have been reported particularly in bio-separation and biomedical applications, a research in miniaturization of pressure-driven separation instrumental system, namely HPLC, has not received much attention. A highly reliable and versatile HPLC technique is based on silica-based columns, while silica chips are not likely used for biomedical separation. Compared to that of the electrokinetic separation in CE or CEC, flow rate in HPLC is not sensitive to surface properties of microchannel wall along the separation path. Sample introduction in microfluidic HPLC must be well-designed to fit the reduced volume injected to the microchannel. Microfabrication of HPLC on-a-chip can offer a number of advantages, for example, low cost micro-LC. Simplifying HPLC instrumentation, multiple separation, multiple columns in microchannels that lead to more complicated chromatographic techniques, such as 2D-chromatography, as well as leading to a higher dimension chromatographic technique, high-throughput parallel analysis.

Following the introduction of microfabricated silicon LC chips by Manz *et. al*. in 1990, research on microfabricated pressure-driven HPLC remained scarce until 2000, when Erickson *et al.* demonstrated rapid anion-exchange LC separation of four model proteins within 1 min using a quartz HPLC microchip. Seki *et al.* later separated bovine serum albumin (BSA) and immunoglobulin G (IgG) by anion-exchange chromatography using a polydimethylsiloxane (PDMS) microchip, while Vahey *et al.* fabricated a PDMS pressure-driven open tubular liquid chromatography (OTLC) microchip to analyse salt, indicator, and dyes. Schlund *et al.* reported an OTLC microchip driven by compressed air to separate phenols and vitamins. The development of high pressure on-chip valve and injector components was done by researchers at Sandia National Laboratories using an in situ photopolymerization technique. Reichmuth *et al.* integrated these components for a reversed-phase liquid chromatography (RPLC) microchip for a separationof peptides and proteins. Agilent has a more recently developed an HPLC chip technology integrating a sample enrichment column, a packed separation column, and a nanoelectrospray tip for mass spectrometry interfacing, which has been applied to separate biological samples and coupled it with mass spectrometric detection. Other researchers have reported fully

integrated pressure-driven HPLC microchips with major components such as pumps, mixers, sample injectors, and separation columns fabricated in a single device.

Significant efforts have continuously focused on fabricating efficient chromatographic columns on pressure-driven HPLC microchips. Microfluidic open-tubular columns, packed-bed columns, micromachined pillar array columns, and continuous-bed or monolithic columns have been reported. While open-tubular columns of initial chip-based HLPC were developing as they provided low hydraulic resistance, moderate separation performance, and ease of fabrication, they suffered from low sample loading capacity resulting from limited total surface area. The use of packed-bed columns employing derivatised particles, such as silica beads adopted from capillary separations, can significantly increase the surface area and loading capacity. Packing and retaining particles at specific locations within microfluidic devices is no longer problematic, as we can introduce desirable fabrication and design. As an extension of He and Regnier's early work, Malsche *et al.* used a deep reactive ion etch (DRIE) process to create a nonporous pillar array within a microchannel and demonstrated fast separation of three coumarin dyes using a pressure-driven RPLC separation. In theory, the ordered pillar microstructure can minimize eddy diffusion and afford higher efficiencies than other HPLC columns, while small loading capacity of the nonporous columns may be overcome by introducing a porous structure on the micro-machined pillars. However, obtaining a uniform coating on the pillar surface remains a major challenge, and the high fabrication cost associated with DRIE limits the possibility of this approach for disposable applications.

To introduce particles in the small channels two practical techniques have been applied. The classical one employs a high pressure driving slurry of particles in liquid solvent into the channel where a filter frit was initially made. The particles remain in the channel, leaving the solvent passing through the frit. A new filter frit formation is needed to keep particles within the channel. The difficulties of such method are retaining the frits within the small channel, and the need of very high pressure for packing particles. A particularly effective technique to introduce

separation media within defined regions of a microfluidic system is to synthesise continuous media beds, or monoliths, by an in situ polymerization. This eliminates the need for packing, and the monolith can be designed to have pore sizes in the order of 1 mm ensuring good flow-through properties.

Monoliths are highly porous inorganic or organic materials originally developed for conventional capillary HPLC separations. Inorganic monolith is mainly based on silica, alumina, zirconia, and etc, while the organic material employs polymers. A polymer monolith can be formed by polymerization in the presence of a high proportion of cross-linking monomer and a porogen. The physical properties (pore size and surface area) of the monolith are predominantly determined by the solubility of the forming polymer and the nature of the porogen, whereas its chemical properties are determined predominantly by the monomer. Polymer monoliths can be readily adapted for different separation mechanisms without further treatments by tuning the prepolymer composition and fabrication process. Two polymerization processes of monolith fabrication have been introduced. Thermal polymerization of monoliths has been reported within a polymer microfluidic LC chip integrating a rotary injection valve, separation channel, and on-chip UV detection. Photopolymerization offers substantial advantages by enabling the preparation of well-defined monolith geometries within specific regions of complex microchannel networks. The later feature has been applied in the development of polymethacrylate/acrylate monoliths in microfluidic systems as separation media, mixers, fluidic controls, reactors, and electrospray emitters, as well as the on-chip solid-phase extraction (SPE). However, in the development of microfluidic devices employing photopolymerised monolithic separation media, a small number of works have been reported toward integrated HPLC systems.

Silica-based monoliths 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. Silica monolith was first introduced into

a microfluidic chip by Chen *et al.*(2005). Just only a few researchers reported silica monolithic material synthesized in microchannels because the problem in cracking of silica monolith after the sol-gel process also occurred in the microfluidic channel. However, some successful works were reported in the use of silica monolith in the microchannel for sample preparation or preconcentration. Its direct application in chromatography has not been achieved.

The chip interfaces also constrain the maximum pressures and solvent flow rates which may be employed in chip-LC systems. The intrinsic dead volumes in these interfaces can be large and lead to band broadening when off-chip sample loading is required. Additionally, low-pressure interfaces limit the achievement of homogeneous monoliths within long separation columns. The pressure required to drive mobile phases through long columns may induce leakage or physical damage at these interfaces, constraining further improvements in separation performance of microfluidic HPLC systems. In this approach the main focus is on the separation on a channel, therefore the world-to chip interface is not of purpose.

OBJECTIVES

1. To fabricate silica based monolith in microfluidic chip and characterize its morphology.

2. To get a prototype of microfluidic chip with silica monolithic material in its channel.

3. To set up a home-made laser-induced fluorescence (LIF) instrument and connect it to a computer with a LabVIEW programme for detection and monitoring.

4. To investigate the capability of the fabricated monolith in the channel for separation by coupling the microchip with the LIF detection. This can be done by fluorescein dye tester.

5. To demonstrate a separation on the microchip by using amine mixture, chlorpheniramine, riboflavin.

6. To investigate the sensitivity, reusability and efficiency of the microfluidic chip with monolithic material with laser-induced fluorescence (LIF) detection.

LITERATURE REVIEW

In this work some backgrounds must be provided, since the research was involved with a compilation of fundamental concepts in microfluidic, monolith, and their combination. Apart from those, a laser induced fluorescence (LIF) measurement set-up with a simple light source was also integrated. This section is then composed of separated parts. The first one provides a background on microfluidics and how to construct a microfluidic device nowadays. This includes brief definitions of concepts that are fabrication of microfluidic device, materials, and its applications.

The second part of this chapter will focus on preparation, properties and applications of monolith. The next part will look at silica-based monolith on microfluidic chip. Method and technologies were interviewed in this section and the last part of this chapter will be a brief overview of detector that used in this research, laser induced fluorescence or LIF.

Microfluidic device

Microfludic device is a small device that encompasses one or more channels with at least one dimension less than 1 mm. Basic principle of microfluidics is the flow of a fluid through the very small channels that can be characterized by the Reynolds number (Re)

Reynolds number is a dimensionless number that gives a measure of the ratio of inertial forces to viscous forces and consequently quantifies the relative importance of these two types of forces for given flow conditions, source: http://en. Wikipedia.org/wiki/Reynolds_number, September 25, 2011.

$Re = (\rho v L) / \mu$

ρ = density of fluid (kg m^{-3}, lb ft^{-3})

v = average velocity of the flow (m s^{-1}, ft s^{-1})

L = characteristic length (m, ft)

μ = viscosity (Ns m^{-2}, lb s^{-1} ft^{-1})

Microfluidic devices have been used for applications in many fields, such as biomedicine, biology, chemistry, physics, and engineering, etc. The use of microfluidic devices has a significant advantage that is the small amount of reagents an analytes used because of low volume of fluid in the channels, in nanoliters. It is significant for expensive reagents.

To make a microfluidic device there have been a number of standard master mold microfabrications; these processes included film deposition, etching, photolithography, and soft lithography. Among these processes two important techniques mostly used for microfludic channel construction were photolithography, and soft lithography. Before going into the details of microfabrication, a definition of "photoresist" material must be given. A photoresist is a light-sensitive material used to form a pattern on a surface. Photoresist can be classified into two groups: positive resist and negative resist. When an area of photoresist material exposes to the light and the area is later soluble in a developer, remaining the unexposed area insoluble, the photoresist material is called "positive resist". On the other hand when an area of photoresist material exposes to the light and becomes insoluble in a developer, while the other area is dissolved by the developer, the photoresist material is called "negative resist".

The available technology, limitations, and breakthroughs of microfluidic fabrication techniques were given briefly below.

Film deposition is a deposition of materials on a silicon wafer. Examples of the materials are photoresist and metal. Both can act as a master mold. For metal sputtering or vacuum deposition are techniques used to deposit metal layers on the mold. For photoresist spin coating is used to deposit a photoresist material onto the surface at a desired thickness. All of methods require cleanroom conditions and expanded instrumentation to provide the protective surface. Once the photoresist has been deposited, the channel geometry required for the final device is "imprinted" onto

the photoresist. This is performed using a photomask containing the designed microchip, through which an ultraviolet (UV) light is passed, exposing the photoresist in the appropriate areas, to transfer the pattern from the mask onto the photoresist.

Etching is a traditional method for fabricating glass or silicon devices that desired pattern is transferred to the photoresist by exposure of the photoresist to UV light. The photoresist was removed by UV-induced damage with a buffered alkaline solution, a developer, or exposing the metal layers that cover the wafer. Once the layers are removed, a rate of exposure to hydrofluoric acid (HF) etching the glass or silicon can be predetermined based on the substrate that used and the concentration of HF used in the etch solution.

Photolithography is the most widely-used technique for the fabrication of microfluidic devices. The technique facilitates an industrial processing of microchip where several chips must be produced at a time by using a high-resolution imagesetter. The formation process with photolithography is limited only to two-dimensional lateral structure. This technique used a photoresist emulsion layer to transfer a desired pattern from a mask to the substrate. The mask is a transparent glass plate with metal (chromium) patterns on it.

In soft lithography, a patterned elastomer is used as a mold or mask to generate micropatterns and microstructures instead of using a rigid photomask. As the technique is low cost, the elastomer can be any kind of polymers that can be prepared from polymeric precursors. This method includes replica molding, micro-contact printing, micromolding in capillaries, and micro-transfer molding. The advantage of soft lithography is its short turnaround time, possible to go from design to production of replicated structures in a short period. Deformation of the stamp or mold, low reproducibility, and defects are problematic, according to the use of soft materials, and these prevent the feasibilty in manufacturing process, but it is widely used in research work.

Polydimethylsiloxane (PDMS), a silicone elastomer, is the popular material choice for replication as easily molding in soft lithography because it is clear, inert, non-toxic and non-flammable. Most of these studies used PDMS stamps fabricated from Sylgard-184 component kit (Dow Corning, USA). In soft lithography, a PDMS stamp on its surface is employed to generate designs and structures with different sizes ranging from nanometers to centimeters. Five molding techniques oftenly used are microcontact printing, replica molding, microtransfer molding, micromolding in capillaries, and solvent-assisted micromolding. Most applications of Sylgard-184 was used without any further modification, but in some cases the surface of PDMS produced from Sylgard-184 had to be physically and/or chemically altered to achieve its desired properties. For example, some researchers reported the combination of oxygen plasma and chemical attachment of chlorosilane molecules to tune the surface energy of the PDMS surface. Such modification facilitated the contact of hydrophilic materials to the hydrophobic PDMS surface. Physical or physicochemical surface modification of PDMS has been performed to fabricate PDMS nanostructures and fracture, and polymers, and to adjust the surface energies of microfluidic channels. To properly adjust the surface characteristics of PDMS in these applications, it is important to understand the changes the PDMS surface undergoes when exposed to various surface treatments.

An example of soft lithography frequently used in creating microfluidics is polydimethylsiloxane (PDMS) molding. In this method, high-aspect-ratio microfluidics can be fabricated using SU-8 negative photoresist molds. SU-8 shown in appendix B1 is a chemically amplified negative photoresist with high transparency. The high transparency allows light to penetrate through thick layers of photoresist, thus creating near vertical sidewall profiles. As a form of soft lithography, SU-8 structures have been used as molds for microfluidic applications. In some applications, PDMS is first poured onto the patterned SU-8 mold. The PDMS is cured, removed, and then pressed or bonded onto a flat substrate to create microchannels.

In another approaches, microfluidics has become a component of microelectromechanical systems (MEMS) technology with polymer devices in order to develop disposable microfluidic systems. Microfluidic devices and electrophoresis separation of biomolecules has become a dominant diagnostic tool for clinical diagnosis, and for rapid screening of health conditions and diseases. Conventional microfluidic devices are prepared from glass and silicon, but nowadays polymers such as polymethylmethacrylate (PMMA or Plexiglas) become more popular. PMMA have several advantages over traditional materials, such as lower fabrication costs and complexity, excellent optical transparency, and attractive mechanical/chemical properties. An effective PMMA is used in microfluidic devices, blood filters, and waveguide devices. The fabrication of PMMA microfluidic system may be performed by using a wide range of techniques, including injection molding, hot embossing, laser photo-ablation, and X-ray lithography.

An attempt in chromatographic separation to make a single piece monolithic material has been published in literature for more than 50 years. Monolith by definition is a single piece skeleton of material. In chromatography and separation monolithic material is a single piece skeleton comprising of interconnected particles and flow paths. Chromatographers are interested in the monolithic structure, as it can be used in various techniques in chromatography. Synge *et al.* (1952), a Nobel Prize laureate, launched the theoretical discussions in a single piece skeleton material for chromatography, but soon disappeared because when flowed with an eluent gel-like materials available at that time collapsed under hydrostatic pressure. Later the synthetic hydrogels were carried out, and confirmed these theoretical assumptions. Foams of rigid open pore polyurethane gave good separations in both LC and GC, but for years thereafter, no other similar works in the chromatography was followed. Thus, the genuine “monolithic era” starts in the late 1980s. Nowadays monolithic materials can be divided into two categories: organic-based monolithic column and inorganic-based monolithic column. Organic based monolith is generally made of polymer and co-polymers. The inorganic based monolith mainly focuses on silica material where sol-gel process is involved with the formation of monolith. In this work the silica monolith will be synthesized in a microfluidic channel. A number of

reviews and scientific papers about silica monolith applications and usage have been given elsewhere, but because the number of publications is rather large, only some reports mostly involved with preparation method of silica monolith and some applications were given below.

Fields *et al.* (1996) was the first one who prepared a silica-based monolithic capillary column via in situ solidification of formamide solution of potassium silicate. The fused silica capillary was not pretreated before the xerogel was cast. The xerogel was formed by a method similar to that used to cast column end frits in fused silica tubing for packed capillary HPLC. A 10% solution of formamide in potassium silicate solution was vortexed until the formamide was completely dispersed The fused silica column was filled with the solution and kept at 100°C for 1 h. The column was then washed with water, 50% water-methanol, methanol, and tetrahydrofuran. The column was purged with dry helium for 24 h at 120°C and then filled with a 10% dimethyloctadecylchlorosilane (ODS) in dry toluene through an empty length of the same tubing. The column and empty tubing ends were connected to form a circle and then heated at 70°C for 5 h. The column was then washed with toluene, tetrahydrofuran, methanol, 50% water-methanol, and water. Short lengths of the column were cut off and vapor deposited with gold for observation with a scanning electron microscope. For chromatographic evaluation, the column void volume was measured as the elution time of sodium nitrate, from which the mean linear velocity was calculated. Gravimetric determination of the column void volume using methanol and chloroform yielded a value about 10% lower than that obtained with the sodium nitrate method.

The final contribution to the family of different monolithic technologies has been added by Tanaka in the mid-1990s. The most popular silica inorganic materials are well-known and widely used in liquid chromatography. In collaboration with materials scientists in Kyoto, they designed a process that afforded silica-based monoliths with controlled porous properties. The *in situ* preparation of typical analytical size columns is difficult due to the significant shrinkage of silica occurring

during the polycondensation reaction, aging, and heat treatment. Therefore, they encased the preformed monolith in a plastic holder to obtain chromatographic column.

Nakanishi *et al.* (2000) reported the preparation method of mesopores that can be tailored by aging in ammonia solution formed by the hydrolysis of urea. The addition of urea in the starting preparation mixture made the process simpler, since the mesopores were prepared just by heating the whole reaction mixture in the capillary. The monolithic silica column in a capillary was used after on-column chemical modification with *N,N*-diethylaminodomethyloctadecylsilane without retaining frits.

Tanaka *et al.* (2002) studied the structural and chromatographic properties of monolithic silica columns. Monolithic silica columns prepared from tetraalkoxysilane by a sol–gel method showed high efficiency and high permeability on the basis of the small-sized silica skeletons, large-sized through-pores, and resulting through-pore size per skeleton size ratios much larger than those found in a particle-packed column. Monolithic silica columns could be prepared either in a mold of a 6-9 mm i.d. glass test tube or in a fused-silica capillary. Silanes, such as tetramethoxysilane (TMOS) or tetraethoxysilane, underwent hydrolytic polymerization in aqueous acetic acid in the presence of polyethylene glycol (PEG) to form monolithic silica having network structures. The preparation in a mold is accompanied by the volume reduction of the whole structure. The diameters of products were ca. 4.6 or 7 mm when molds with 6 and 9 mm i.d. were used respectively. The resulting silica monoliths were covered with PTFE tubing or with PEEK resin to fabricate a column for HPLC. Long columns could not be prepared to be straight, limiting the column length to about 15 cm or shorter. The PTFE-covered monolith (MS-PTFE) was used in an external pressurizing device. The PEEK-covered column (MS-PEEK) could withstand inlet pressure of up to 120 kg cm^{-2}. This type of monolithic silica columns, Chromolith, is commercially available at 5- or 10- cm length.

Smatt *et al.* (2002) prepared monolithic silica exhibiting a trimodal, hierarchical pore structure via sol-gel processing. Monolithic bodies with

interconnected macropores in the micrometer range were a result of controlled phase separation and gelation kinetics, whereas textural mesopores in the 10-20 nm range originated from voids between particles. Furthermore, the particles exhibited internal mesoporosity with pore diameters in the 2-4 nm range originating from supramolecular templating by the surfactant. Poly(ethylene glycol) was used together with alkylammonium surfactants to control the particle aggregation and internal structure. The silica monoliths were prepared by adding TEOS to a mixture of PEG (MW 35,000) dissolved in an aqueous nitric acid solution. The sol was subsequently stirred at room temperature, then added CTAB, and continued stirring until the surfactant dissolved. The sols were left to gel at 40°C for 6-10 h and aged for > 48 h at 40°C. Solvent exchange was performed to increase the degree of condensation and stability of silica gels. An efficient procedure for obtaining large textural pores was to keep the monoliths in a 1 M NH4OH solution for 9 h at 90°C. The volume of the solvent exchange solution was 10 times the volume of the monoliths. The monoliths were then acidified with dilute nitric acid solution and washed with 25% ethanol, then dried for 3 days at 60°C and subsequently calcined at 550°C for 5 h.

Leinwebwer *et al.* (2002) demonstrated the fabrication of silica-based monoliths that possessed a rather discrete bimodal pore size distribution and a high correlation of interconnectivity between these two sets of pores. The large macropores formed a flow-channel network outside the monolith skeleton that rapidly transports solute molecules. The porous skeleton provides a relatively large surface area compared to the interskeleton pore space but needs only very short diffusion lengths and does not contain micropores to any significant amount. The macropore size was varied independently from the skeleton size. Thus, a careful control of the macropore permeability relative to mesopore residence times was used to optimize the performance of this monolithic support in terms of throughput, dispersion, and capacity. For particulate structures, adjustment of permeability and dispersion length scales includes the transition from totally porous via pellicular to nonporous particles with a decrease in surface area. In this study, the retention thermodynamics as column pressure drop spans was more than 2 orders of magnitude, then band dispersion was

characterized, and finally sphere dimensions was determined to interpret the observed behavior in view of particulate structures.

Nakanishi *et al.* (2004) reported the synthesis of monolithic ethane-silica gels with well-defined co-continuous macropores and highly ordered mesopores via a spontaneous route from silicon alkoxide with the aid of a structure-directing agent. Poly(ethyleneoxide)-block-poly(propyleneoxide)-block-poly(ethyleneoxide) with an average molecular mass of 5800 (P123) was homogeneously dissolved in 0.1 M nitric acid. 1,2-Bis(trimethoxysilyl)ethane was added, the resultant homogeneous solution was poured into a glass tube, and it was sealed and kept at 60°C for gelation. The wet gel was aged at 60°C for about 5 times of the gelation time and then dried at 60 °C. While the macropores were formed by the concurrent phase separation and sol-gel transition induced by the polymerization, the mesopores were templated by the self-organization of the structure-directing agent. Starting from a homogeneous mixture of the starting components, all the structure formation processes take place spontaneously in closed conditions at a constant temperature. Residual volatile, organic substances, and P123 were removed by heat-treating the sample under air from room temperature to 350°C in 2.5 h and holding at the same temperature for 5 h. Subsequent evaporation drying and heat treatment resulted in gels with pores in discrete size ranges of micrometer and nanometer. The local alignment of the mesopores was confirmed by FE-SEM observation, and the long-range mesoscale order over the whole sample was evidenced by X-ray diffraction measurements. With the addition of a micelleswelling agent, the mesostructural transition from 2D-hexagonal order to mesostructured cellular foam was observed without disturbing the macroporous framework structure.

Chang *et al.* (2006) prepared monolithic silica capillary by sol-gel process and investigated it as an adsorbent for a pre-concentration step 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 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, and

allowed to react at 40°C for 24 h., and heated to 120°C for 3 h, and then at 350°C for 24 h to decompose of organic moieties. The detection limits by GC-MS analysis 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%.

Nunez *et al*. (2007) polymerized octadecyl methacrylate using α,ά-azobis-isobutyronitrile (AIBN) as a free radical initiator for high efficiency and highly retentive monolithic silica capillary columns Hybrid type monolithic silica columns were prepared from a mixture of tetramethoxysilane and methyltrimethoxysilane were used as a support. The effects of the monomer and the radical initiator concentrations in the reaction mixture were examined. The performance of the columns was tested in terms of column efficiency and retention behavior by using alkylbenzenes and a few other compounds as solutes and compared with that of hybrid monolithic silica columns modified with octadecylsilyl-(N,N-diethylamino)silane (ODS-DEA). Highly retentive monolithic silica columns were obtained by polymerization at high monomer concentrations. Although a decrease in column efficiency was observed with the increase in the monomer concentration in a feed mixture, an improvement in efficiency was achieved (a plate height value lower than 10 µm) by increasing an initiator concentration without significant variations in column retention properties. Results obtained by polymerization using other monomers were also presented to demonstrate the applicability of the preparation method.

The bare monolithic silica capillary column was prepared according to the procedures previously reported by Ye *et al*. (2007). The post-modification was finished through silanization reaction on the surface of prepared bare monolithic silica capillary column with HDTMS and APTMS molecules. A solution consisting of different ratios of HDTMS to APTMS in toluene was pushed through the bare monolithic silica capillary column by an HPLC pump at 0.1 mL min^{-1} for 1 h, and then the capillary column was reacted at 110◦C for 1 h. This step was repeated three times and the final reaction was done overnight. The columns were then rinsed with toluene and methanol. A detection window was made behind the continuous bed by

removing the polyimide coating of the fused-silica capillary using a thermal wire stripper. The tested column had a final total length of 42 cm and an effective length of 30 cm.

A novel kind of immobilized trypsin reactor based on organic−inorganic hybrid silica monoliths has been developed by Ma *et al.* (2008). With the presence of cetyltrimethyl ammonium bromide (CTAB) in the polymerization mixture, the hybrid silica monolithic support was prepared in a 100 μm i.d. capillary by the sol−gel method with tetraethoxysilane (TEOS) and 3-aminopropyltriethoxysilane (APTES) as precursors. Subsequently, the monolith was activated by glutaraldehyde, and trypsin was covalently immobilized. By monitoring the reaction of a decapeptide, C-myc (EQKLISEEDL), the enzymatic activity of the immobilized trypsin was calculated, and the results showed that the digestion speed was about 6600 times faster than that performed in free solution. The performance of such a microreactor was further demonstrated by digesting myoglobin, with the digested products analyzed by microflow reversed-phase liquid chromatography coupled with tandem mass spectrometry (μRPLC−MS/MS). With a stringent threshold for the unambiguous identification of the digests, the yielding sequence coverage for on-column digestion was 92%, the same as that obtained by in-solution digestion.

Miyamoto *et al.* (2008) prepared monolithic silica capillary columns from a mixture of (3:1) MTMS-TMOS to form a hybrid structure, or from TMOS only. The use of TMOS as a starting material allowed the preparation of 50-100 μm i.d. columns, although a 200 μm column could be prepared under certain conditions. Under typical conditions, the fused-silica capillary tube was first treated with 1 M NaOH at 40°C for 3 h, followed by a flush with water, and then kept with 1 M HCl at 40°C for 2 h. After that it was flushed with water, and then acetone, the capillary tube was air-dried at 40°C. A MTMS-TMOS mixture was added to 1.80 g PEG and 4.05 g urea in 0.01 M acetic acid at 0 °C and stirred for 30 min. The homogeneous solution was then filtered at 40°C with a 0.45 μm PTFE filter, and put into a fused-silica capillary tube, and allowed to react at 40°C. The resultant gel was subsequently aged in the capillary overnight at the same temperature. The monolithic silica columns

were treated at 120 °C for 4 h to form mesopores with the ammonia generated by the hydrolysis of urea, then they were cooled and washed with methanol. After air-drying, the columns were heated at 330°C for 25 h causing the pyrolysis of all organic moieties in the column.

Ye *et al.* (2009) prepared a silica-based monolithic stationary phase with mixed-mode of reversed phase (RP) and weak anion-exchange (WAX) for capillary electrochromatography (CEC). The mixed-mode monolithic silica column was prepared using the sol–gel technique and followed by a post-modification with hexadecyltrimethoxysilane (HDTMS) and aminopropyltrimethoxysilane (APTMS). The amino groups on the surface of the stationary phase were used to generate a substantial anodic EOF as well as to provide electrostatic interaction sites for charged compounds at low pH. A variety of analytes were used to evaluate the electrochromatographic characterization and column performance. The monolithic stationary phase exhibited RP chromatographic behaviour toward neutral solutes. The model anionic solutes were separated by the mixed-mode mechanism, which comprised RP interaction, WAX, and electrophoresis. Symmetrical peaks were obtained for basic solutes because positively charged amino groups minimized the adsorption of positively charged analytes to the stationary phase.

Lu *et al.* (2010) prepared a novel chitosan functionalized monolithic silica capillary column using carboxymethylchitosan as chemical modification reagents. Its performance was investigated through the separation of polar compounds including nucleosides, nucleotides, aromatic acids, and aliphatic acids. The monolithic silica column was prepared as described by Miyamoto *et al.* (2008) with some modifications. A (3:1) TMOS-MTMS mixture, PEG, urea and acetic acid were mixed thoroughly, then the homogeneous solution was stirred for 10 min at 40◦C. The transparent sol was injected into a pretreated capillary which was later heated at 40 ◦C overnight. Then, the column was treated for 3 h at 120 ◦C to form mesopores. Next, the column was washed with water and methanol and then dried at 60 ◦C overnight. After drying, the column was treated at 330 ◦C for 25 h to complete the formation of silica monolith.

Chao *et al.* (2010) prepared a novel inorganic silica-organic polymer mixed-based capillary electrochromatography monolithic column by using precursor 3-(trimethoxysilyl)propyl methacrylate with double-functional group hydrolysis/polycondensation in acidic aqueous solution, followed by double-bond co-polymerization with organic monomer hexadecyl mathacrylate under the thermal initiated condition. The combination of preparation of silica-based monolithic column and organic polymer monolithic column was suggested. A simple operation of controlling sol mixture using mercury infusion when the mixture infused into capillary was raised to acquire smooth interface of stationary phase. The preparation conditions of monolithic column were optimized. The structure of the monolithic columns was characterized by SEM

Gui *et al.* (2010) presented a novel silica monolithic stationary phase functionalized with butylaminopropyl ligands for capillary electrochromatography (CEC). The monolithic capillary columns were prepared by a sol-gel process and a subsequent chemical modification was followed. The amino groups on the surface of the stationary phase generated an anodic electroosmotic flow (EOF) and the butyl groups together with propyl groups provided sufficient hydrophobic properties. To evaluate the column performance, effects of buffer pH on the EOF and electrochromatographic retention behavior of alkylbenzenes, organic acids, and anilines were investigated. The monolithic stationary phase exhibited reversed-phase (RP) chromatographic behavior toward neutral solutes. The standard organic acid anion solutes were separated by a mixed mode mechanism, which comprised of RP interaction, weak anion-exchange, and electrophoresis. Basic compounds such as anilines were well separated on the butylaminopropyl silica monolithic column without peak tailing.

Hara *et al.* (2010) studied the effect of a ratio of methyltrimethoxysilane (MTMS) to tetramethoxysilane (TMOS) to improve the performance of a hybrid monolithic silica capillary column with 100 µm i.d. in HPLC in a range MTMS/TMOS of (10:90) to (25:75). The domain size was also varied by adjusting the amount of PEG to control permeability ($K = 2.8\times10^{-14} – 6.9\times10^{-14}$ m^2). Evaluation

of the performance for those capillary columns following octadecylsilylation proved an increase in retention factor and a decrease in steric selectivity α (triphenylene/orthoterphenyl) with the increase in MTMS content. The effect of the ratio was also observed in porosity and hydrophobic property of the C_{18} stationary phase from the results of size exclusion chromatography (SEC) and reversed phase characterization. The monolithic silica capillary columns prepared under new preparation conditions were able to produce a plate height of 4.6–6.0 μm for hexylbenzene in 80% acetonitrile-water at a linear velocity of 2 mm/s. Consequently, it was possible to prepare hybrid monolithic silica capillary columns with higher performance than those reported previously while maintaining the retention factors in a similar range by reducing the MTMS/TMOS ratio and increasing the total silane concentration.

Hou *et al.* (2010) deveploped an immobilized metal affinity chromatography (IMAC) column based on organic−inorganic hybrid silica monolith. The monolithic support was prepared in a 250 μm i.d. capillary by the sol−gel method with tetraethoxysilane (TEOS) and 3-aminopropyltriethoxysilane (APTES) as precursors. Subsequently, amine groups were functionalized by glutaraldehyde, and then activated with (aminomethyl) phosphonic acid, followed by Ti^{4+} chelation. By such a hybrid silica monolithic Ti^{4+}-IMAC column, 15 phosphopeptides were effectively isolated from the digest mixture of α-casein and BSA with the molar ratio as low as 1:200, illustrating its superior selectivity. With a synthetic phosphorylated peptide sample, the loading capacity and recovery of the Ti^{4+}-IMAC monolithic column were measured to be 1.4 μmol mL^{-1} and 69%, respectively. These results demonstrate that the hybrid silica monolith based Ti^{4+}-IMAC column might provide a promising tool for large-scale phosphopeptide enrichment, facilitating the in-depth understanding of the biological functions of phosphoproteomes.

Bruns *et al.* (2010) presented a fast, nondestructive, and quantitative approach to characterize the morphology of capillary silica-based monolithic columns by reconstruction from confocal laser scanning microscopy images. The method comprised column pretreatment, image acquisition, image processing, and statistical

analysis of the image data. The received morphological data are recorded length distributions for the bulk macropore space and skeleton of the silica monolith. The morphological information was shown to be comparable to that derived from transmission electron microscopy, but far easier to access. The approach was applicable to silica-based capillary columns, monolithic or particulate, allowing the rapid acquisition of hundreds of longitudinal and cross-sectional images in a single session, and resolving a multitude of morphological details in the column.

The microfluidic and monolith technology has been merged and some successful applications were summarized chronologically below. It should be noted that most success in the combination of both technology were mostly polymer monolith on a microfluidic chip, and the applications were mostly in biomedicine and biology.

Yu *et al.* (2001) prepared monolithic porous polymers by photoinitiated polymerization within the channels of a microfluidic device and used for on-chip solid-phase extraction and preconcentration. The preparation of the monolithic material with hydrophobic and ionizable surface chemistries was easily achieved by copolymerization of butyl methacrylate with ethylene dimethacrylate, or 2-hydroxyethyl methacrylate and [2-(methacryloyloxy)ethyl]trimethylammonium chloride with ethylene dimethacrylate, respectively. The porous properties, and consequently the flow resistance, of the monolithic device were controlled by the use of a mixture of hexane and methanol as a porogenic mixture.

Gao *et al.* (2003) prepared a biporous monolithic silica gel column possessing both micrometer sized through-pores and nanometer sized mesopores located in the silica skeletons and the high concentration porogen was used to increase mesopore size. The mechanism for the preparation of the monolithic column was investigated in detail. A lower concentration range of polyethylene glycol starting solution was suitable for forming the interconnected porous structure for the monolithic silica gel column. At lower concentrations, however, the phase separation mechanisms and experimental results were different.

Le Gac *et al.* (2004) reported the preparation of monolithic capillary columns and integrated it in a microsystem for on-chip sample preparation before on-line analysis by electrospray and mass spectrometry (ESI–MS). These monolithic columns were based on polymer materials and consisted of reversed phases for peptide separation and/or desalting. They were prepared using lauryl methacrylate (LMA), ethylene dimethacrylate (EDMA) as well as a suitable porogenic mixture composed of cyclohexanol and ethylene glycol. The resulting stationary phases presented a C_{12}-functionality. The LMA-based columns were first prepared in a capillary format using capillary tubing of 75 µm i.d. and tested in nano LC–MS experiments for the separation of a commercial Cytochrome *C* digest composed of 12 peptidic fragments. Monolithic LMA-based phases were then successfully polymerized in microchannels fabricated using the negative photoresist SU-8. The systems withstanded the pressures applied during the nano LC–MS separation tests that were carried out in the same conditions as for the monolithic capillary columns. However, the separation was not as good as for a capillary format which could be accounted for by the monolith dimensions.

Bedair *et al.* (2006) reported a coupling of polymeric microfluidic devices to mass spectrometry using porous polymer monoliths (PPM) as nanoelectrospray emitters. Lauryl acrylate-*co*-ethylene dimethacrylate porous polymer monolith was photopatterned for 5 mm at the end of the channel of microfluidic devices that were fabricated from three different polymeric substrate materials, including the following: poly(dimethylsiloxane) (PDMS), poly(methyl methacrylate) (PMMA), and cyclic olefin copolymer (COC). Spraying directly from the end of the chip removed any dead volume associated with inserted emitters or transfer lines, and the presence of multiple pathways in the PPM prevented the clogging of the channels. Spraying from a microfluidic channel having a PPM emitter produced a substantial increase in TIC stability and increased sensitivity by as much as 70× compared to spraying from an open end chip with no PPM. The performance of PPM emitter in COC, PMMA, and PDMS chips was compared in terms of stability and reproducibility of the electrospray. COC chips showed the highest reproducibility in terms of chip-to-chip

performance, which could be attributed to the ease and reproducibility of the PPM formation due to the favorable optical and chemical properties of COC. The PPM sprayer was also used for protein preconcentration and desalting prior to mass spectrometric detection, and results were comparable with a chip spraying from an electrospray tip.

Wu *et al.* (2006) demonstrated an effective microchip extraction of deoxyribonucleic acid (DNA) from crude biological matrixes using silica beads or hybrid phases composed of beads and sol−gel. However, the use of monolithic sol−gels alone for extraction of human genomic DNA was difficult to define. For the first time, the successful use of monolithic tetramethyl orthosilicate-based sol−gels for effective micro-solid-phase extraction (μSPE) of DNA in a glass microchip format was described. A functional monolithic silica phase with micrometer-scale pores in the silica matrix was produced from an addition of poly(ethylene glycol), a porogen, to the precursor mixture. This allowed a monolithic sol−gel bed to be established in a microchip channel that provided large surface area for DNA extraction with little flow-induced back pressure. The developed μSPE protocol was further evaluated to show applicability to clinical samples and bacterial cultures, through extraction of PCR-amplifiable DNA.

Logan *et al.* (2007) developed a method for photopatterning multiple enzymes on porous polymer monoliths within microfluidic devices and used to perform spatially separated multienzymatic reactions. To reduce nonspecific adsorption of enzymes on the monolith, its pore surface was modified by grafting poly(ethylene glycol), followed by surface photoactivation and enzyme immobilization in the presence of a nonionic surfactant. Characterization of bound horseradish peroxidase (HRP) was carried out using a reaction in which the steady-state profiles of the fluorescent reaction product could be measured in situ. Mass-transfer limitations were evident at relatively low flow rates but were absent at higher flow rates. Sequential multienzymatic reactions were demonstrated using the assembly of two- and three-enzyme systems. Photopatterning enzymes on polymer monoliths provides a simple

technique for preparing spatially localized multiple-enzyme microreactors capable of directional synthesis.

Satterfield *et al.* (2007) developed UV-initiated methacrylate-based porous polymer monoliths (PPM) for microfluidic trapping and concentration of eukaryotic mRNA. Efficient isolation of eukaryotic mRNA from total RNA was first mathematically modeled and then achieved using PPM in capillaries. mRNA yield and purity were compared with mRNA isolated by commercial kits with statistically equivalent yields and purities. Even after extracting mgof mRNA from 315 mg of total RNA, the 0.4 mL volume monolith showed no signs of saturation. Elution volumes were below 20 mL with concentrations up to 1 mg mL^{-1} In addition, the polymeric material exhibited exceptional stability in a range of conditions (pH, temperature, dryness) and was stable for a period of months. All of these characteristics make porous polymer monoliths good candidates for potential microfluidic sample preconcentrators and purifiers.

Sun *et al.* (2008) designed the use of immunoaffinity monolith pretreatment columns coupled with capillary electrophoresis separation in PMMA microchips. Microdevices were designed with eight reservoirs to enable the electrically controlled transport of selected analytes and solutions to carry out integrated immunoaffinity extraction and electrophoretic separation. The microdevices were fabricated reproducibly and with high fidelity by solvent imprinting and thermal bonding methods. Monoliths with epoxy groups for antibody immobilization were prepared by direct in situ photopolymerization of glycidyl methacrylate and ethylene glycol dimethacrylate in a porogenic solvent. Antifluorescein isothiocyanate was utilized as a model affinity group in the monoliths, and the immobilization process was optimized. The developed immunoaffinity column/capillary electrophoresis microdevices showed great promise for combining sample pretreatment and separation in biomolecular analysis.

Liu *et al.* (2009) reported a polymer microfluidic chips employing in situ photopolymerized polymethacrylate monoliths for HPLC separations of peptides. The

integrated chip design employed a 15 cm long separation column containing a reversed-phase polymethacrylate monolith as a stationary phase, with its front end coupled to a 5 mm long methacrylate monolith which functioned as a solid-phase extraction (SPE) element for sample cleanup and enrichment, serving to increase both detection sensitivity and separation performance.

Only a few researchers reported silica monolithic material synthesized in microchannels for chemical separation. However, A high-pressure electro-osmotic (EO) micro-pump fabricated by a sol–gel process was shown to be potentially effective as a fluid-driving unit on chip-scale analytical systems. The works were summarized below.

Wang *et al.* (2004) synthesized a silica monolithic matrix with a morphology of micron-scaled through pores within the 100 m i.d. fused-silica capillary of the micro-pump. The monolith bonds directly with the capillary wall such that frits with large pressure loss are unnecessary. This pump used electro-osmotic flow to propel liquid solution with no moving parts. The Nafion® housing design in the cathode chamber prevented flow leakage into the electrode reservoir from the flow channel and hence maximized the pressure build-up. It also eliminated electrolytic bubble interference from the flow channels and provides ionic channels for current penetration simultaneously. As the monolith was silica-based, this pump could be used for a variety of fluids, especially for organic solvents, such as acetonitrile and methanol, without swelling and shrinking problems. The maximum flow rate and maximum pressure generated by the 100 μm i.d. monolithic pump were 2.9 μL min^{-1} and 3 atm for deionized water at 6 kV applied voltage. These results indicated that the pump could provide sufficient pressure and flow for miniaturized HPLC and micro-total-analysis systems (μ-TAS).

Wang *et al.* (2006) prepared the monolithic silica matrix from 0.5 mL of 0.01 M acetic acid, 54 mg of PEG and 0.2 mL of TMOS mixture, and the solution was stirred for about 30 min at 0ºC. When all PEG has dissolved and a transparent and single-phase solution was observed, the solution was then introduced into a 40 cm

long capillary (100 µm i.d.). Two ends of the capillary were then connected with a Teflon® tube (Alltech, IL) to form a circle and then placed in a 40ºC oven for 12 h. The Teflon® connector was removed and the whole capillary was heated with a programmed temperature ramp. While the temperature was increased from 40 to 300ºC at a rate of 1ºC min^{-1}, it was soaked for 4 h at 80, 120, 180 and 300ºC, respectively. Finally, the capillary was cooled down to room temperature.

MATERIALS AND METHODS

Materials

1. Reagents

For C_8-silica monolithic materials preparation, tetraethoxysilane (TEOS), n-octyltriethoxysilane (C_8-TEOS) and 3-mercaptopropyltrimethoxysilane (MPTMS) of 98%, 97%, and 95% purity, respectively, were purchased from Sigma-Aldrich and used without further purification. *N*-Dodecylamine of 98% purity was obtained from Fluka (USA). The fabricated channel was flushed with analytical grade ethanol obtained from Merck (USA).

The microfluidic chip was made of poly(dimethylsiloxane) (PDMS) from a PDMS mixture (Sylgard 184 silicone elastomer kit, Dow Corning, Midland, MI, USA). The details of the composition of Sylgard 184 is given in Appendix D. Microfluidic chip is based on replication of PDMS from a master mold fabricated by UV lithography; the master mold is shown in Figure 1.

Methylamine, ethylamine and trimethylamine were used as model compounds for chromatographic separation. Chlorpheniramine maleate salt was purchased from Sigma-Aldrich, India. Riboflavin was purchased from Fluka Analytical (Germany). The 4-(2-Aminoethylamino)-7-(N,N-dimethylsulfamoyl) benzofurazan was obtained from Fluka, Japan. It was used as a fluorescing agent for all amine compounds, giving the emission wavelength at 518 nm. The detail of the compound is given in Appendix E. Phosphate buffer solution (PBS) was prepared according to a standard laboratory method. The mobile phase was either HPLC grade methanol or a mixture of methanol and water. Chlorpheniramine tablets were purchased from ST group pharma & Health care Co., Ltd. Vitamin B2 tablets were obtained from Union Drug Laboratories Co.,Ltd.

2. Apparatus

A HPLC reciprocating pump (LC-10ADVB, Shimadzu, Japan) and an oven (Binder, Germany) were used to fabricate a C_8-monolith in the microfluidic channel and to flow a mobile phase for chromatography**.**

Chromatographic separation was carried out using the HPLC pump flowing a mobile phase passing to an injection valve where a bare fused silica capillary was connected at one end and the other end to the fabricated microfluidic chip. At the outlet of the microchannel a second fused silica tubing was connected, leading to the sheath flow-cell of the LIF system, and the polyimide coating was removed 2-3 cm from the end of the tubing.

Methods

1. Reagent and sample preparation

1.1 0.5 M aqueous acetic acid

A 6.00 g of glacial acetic acid was dissolved with deionised water in a 100.0 mL volumetric flask and the solution was half-diluted with deionised water to make 0.5 M acetic acid solution.

1.2 1.00 M sodium hydroxide solution

A 1.00 M sodium hydroxide solution was prepared by weighing 2 g of sodium hydroxide and dissolved in 50 mL deionized water.

1.3 5,000 ppm stock standard solution of model compounds

Each 0.025 g of standard chemicals, namely chlorpheniramine and riboflavin, was weight in a 5.0 mL volumetric flask and the volume was adjusted by using methanol.

1.4 200 ppm fluorescencing agent

The fluorescencing agent solution was prepared by weighing 0.0010 g of 4-(2-aminoethylamino)-7-(N,N-dimethylsulfamoyl) benzofurazan (DBD-ED) and dissolved in 5.0 mL methanol. If not immediately used, the solution was kept in the refrigerator for long shelf-life. However, it is usually discarded one week after preparation.

1.5 0.1 M Phosphate buffer (PBS)

A 0.1 M phosphate buffer solution was prepared from a mixture of 30.5 mL 0.2 M $Na_2HPO_4 \cdot 2H_2O$ and 19.5 mL 0.2 M $NaH_2PO_4 \cdot H_2O$ in 50 mL volumetric flask. The solution was then adjusted to pH 7.0 with diluted NaOH or H_3PO_4.

1.6 Amine mixture solution

The amine solution was prepared by adding 200 µL of each methylamine, ethylamine and trimethylamine in 200 µL (50:50) methanol-phosphate buffer solution pH 7.0. The solution was then mixed with 200 µL 200 ppm DBD-ED derivatising agent and left for 30 seconds before an introduction of 20 µL to the C8-monolithic microchannel. At this step the derivatisation was occurred before the separation on the monolithic channel.

2. PDMS microchip fabrication

The microchip fabrication was followed by Puttaraksa (2011) . In brief, initially the master mold was fabricated by soft-lithography, the picture of the master mold is shown in Figure 1. The base and curing agent of PDMS were mixed in a ratio of 10:1 (Sylgard 184 silicone elastomer kit, Dow Corning, Midland, MI, USA). The PDMS mixture is degassed under vacuum for 20 minutes, and poured over the master mold subsequently. The sample is cured at 70ºC for 30-45 minutes. Next, the replica is peeled off the master. Then, the micro-channels are enclosed by sealing the replica with a PDMS flim, which is spin coated on a clean glass substrate and precured at 70ºC for 4-5 minutes. This bonding process is made under a coarse vacuum. To completely harden the PDMS, the microfluidic chip is cured again at 70ºC for 30 minutes.

Completely PDMS chip was connected with fused silica capillary for flow the testing mixture and solution. Microchip was flushed by methanol after using, sealed at both end with silicon rubber and preserved in refrigerator.

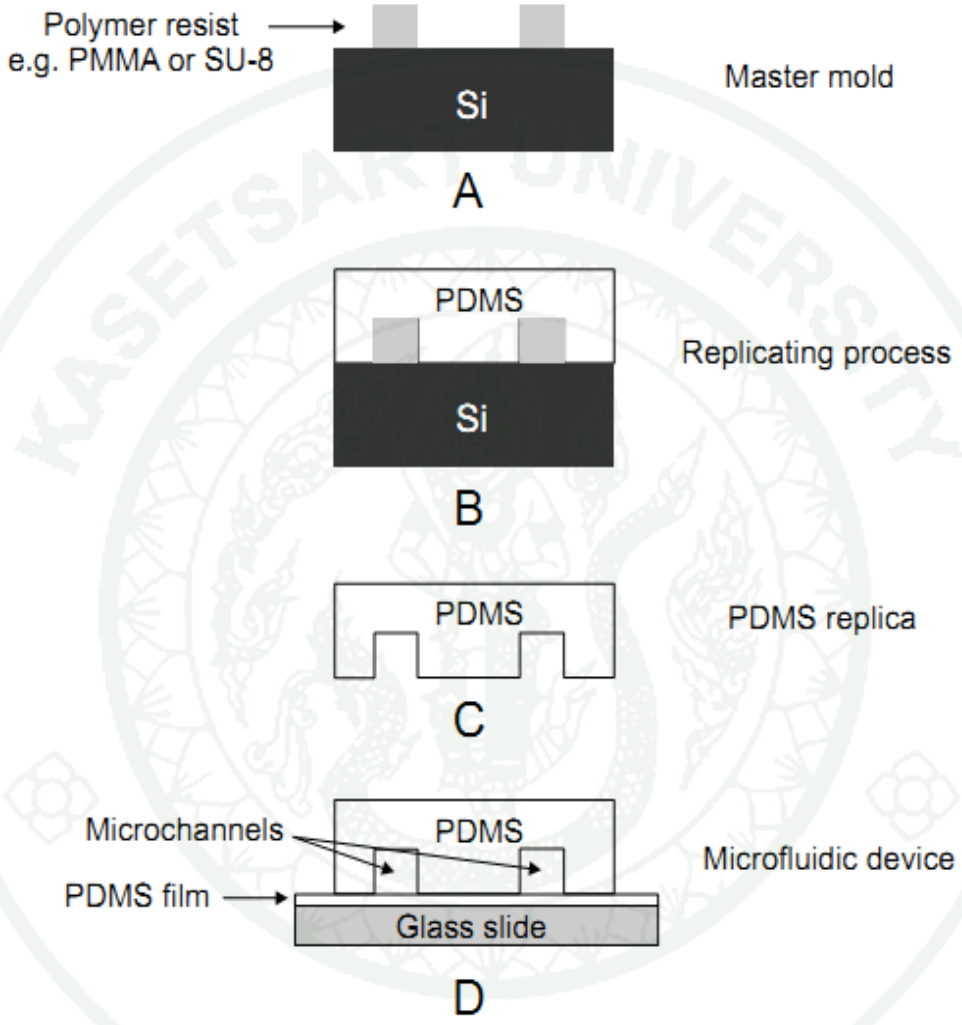

Figure 1 Fabrication of a microfluidic device by soft-lithography: (A) lithography of master mold, (B) PDMS replica molding, (C) separation of PDMS from the master mold, and (D) sealing (With permission from Puttaraksa (2011)).

3. Synthesis of monolithic column

3.1 Synthesis of C_8-silica monolith in microfluidic chip

3.1.1 Pre-treating a PDMS microfluidic channel

To be used as a chromatographic column, a channel in the PDMS microfluidic chip was pre-treated with NaOH solution which was flushed by connecting the chip to a syringe through a hypodermic needle that previously connected to the microchip. The needle was used as a tubing in conjunction with silica capillary tubing. The 1.0 M NaOH solution flushed through the channel for 4 h. The chip was washed by deionized water subsequently until the pH value of the outlet solution was 7.0, and dried by baking on a hotplate at 100°C for 30 min.

3.2.2 In-*situ* polymerization of silica monolith column

Formulation of monolithic silica was adapted from the method of Chen (2010) described as follows. All in all the method of preparation was described as follows.

In a 1 dram vial, 180 µL of methanol, 23 µL of 0.5 M HCl, 65 µL of C_8-TEOS, 35 µL of MPTMS, and 150 µL of TEOS were mixed together. After mixing, the mixture was left for hydrolysis at 60°C for 5 h. After the solution was cooled to room temperature, 10 mg of *n*-dodecylamine was added into the solution. Then the sol-mixture was filled in the prepared microchannel to a certain length by using a syringe. The filled microchannel was sealed with a silicone rubber and then allowed to react at 40°C for 15 h. The microchannel was rinsed with ethanol to remove *n*-dodecylamine and any soluble hydrolysis products. The fabricated microchip was then dried at 40°C for 48 h.

4. Laser induced fluorescence (LIF) system

4.1 Laser source setting-up and beam line alignment

A laser induced fluorescence detection system consists of a laser source, optical lens, sheath flow cell and photomultiplier tube (PMT) for signal detection. A schematic diagram of the system is shown in Figure 2.

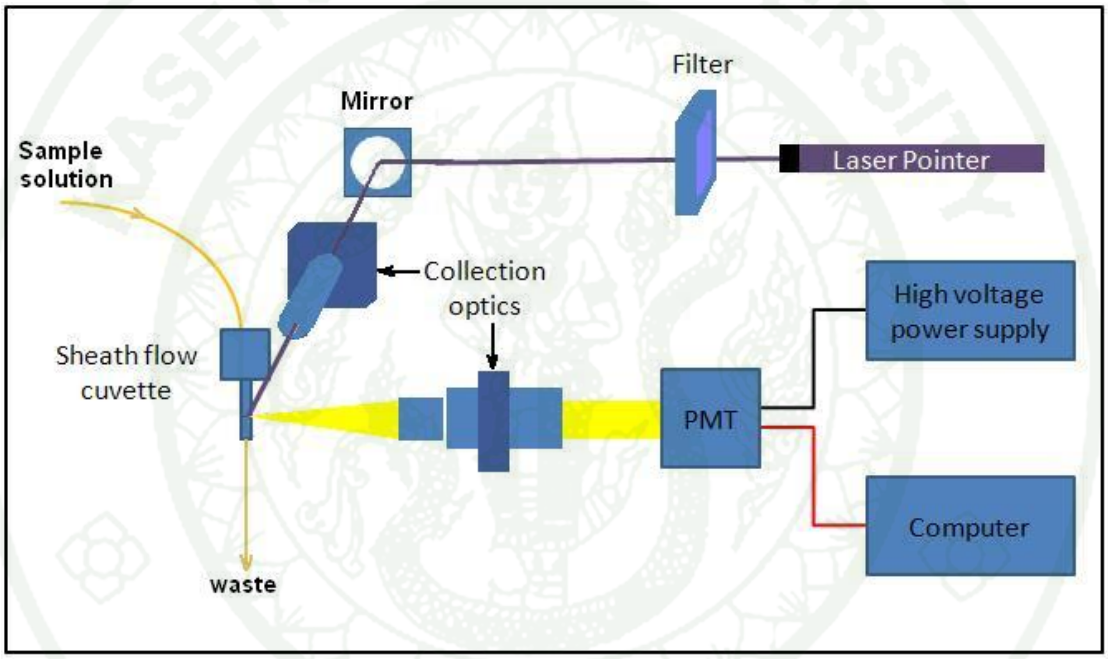

Figure 2 Schematic diagram of the laser induced fluorescence system

The high power 50 mW blue-violet laser pointer utilised GaN (gallium nitride) semiconductors to produce blue light without frequency-doubling. The laser emits a blue light beam at 405 nm wavelength which caused bright blue fluorescence on any white surface. The laser source was connected with 150 mW AC-to-DC adapter to apply a voltage of 3.0 V. The blue laser beam line was directly projected to a UV mirror, which was orthogonally reflected to a collection of focusing lenz (Newport, Irvine, CA, USA) that focused the laser beam onto the center of the cuvette. The fluorescence signal was collected with a collection optics assembly and monitored by a photomultiplier (PMT) module (HC-135–01, Hamamatsu, Middlesex, NJ). The data

collection and acquisition was controlled by a custom-tailored program written in LabVIEW 7.1 (National Instruments, USA), resulting a real-time signal monitoring.

4.2 Connection of the fabricated microchip to the LIF detector

A mobile phase was driven by using a reciprocating pump (LC-10ADVB, Shimadzu, Japan) passed a 6-port Rheodyne injection valve. A 0.32 mm i.d. x 10 cm bare fused silica capillary was connected at one end to the injection valve and the other end to the fabricated microfluidic chip. At the outlet of the microchannel a second 0.32 mm i.d. x 10 cm fused silica tubing was connected at one end. The polyimide coating was removed 2-3 cm from the other end of the tubing that was connected to the shealth flow-cell of the LIF system. The polyimide-free outlet tubing was aligned at the centre of a 1×1×20 mm quartz cuvette (NSG precision cells, Farmingdale, NY, USA) serving as the sheath flow chamber. The sheath flow was driven by the gravity force causing a water flow from the water reservoir that was placed in a higher position than the sheath flow cell, and the linear sheath flow speed was approximately 1 mm/s. The schematic diagram of the integrated monolith microfluidic chip with LIF instrument set up is shown in Figure 3.

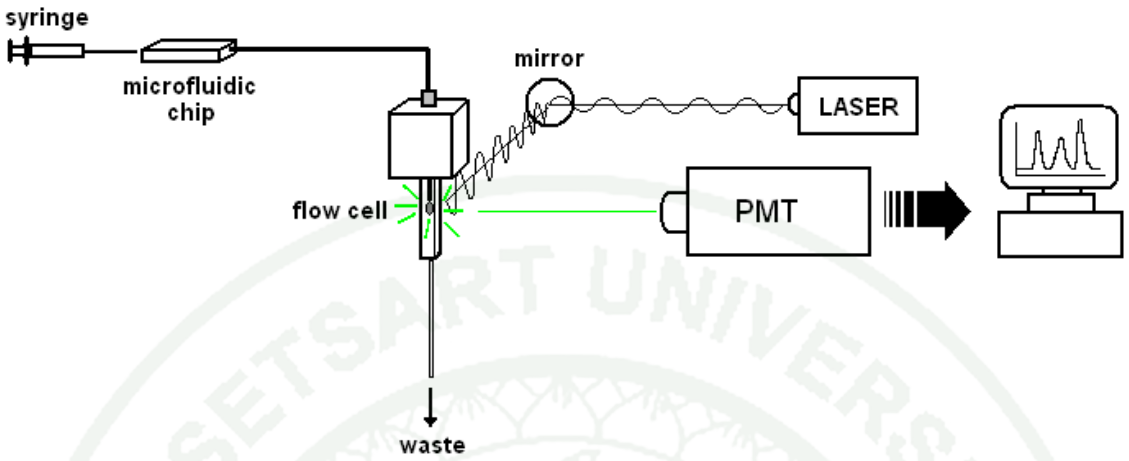

Figure 3 Schematic diagram of the integrated monolith microfluidic chip with LIF instrument

4.3 System operation

The background signal of the LIF system was monitored by turning on the PMT power supply which was indicated by a red light on the supply box, while the LabVIEW program is running. The data collected by using the LabVIEW program could also be computerised by using Excel 2007 software. A chromatographic separation was also monitored by the LabVIEW in a similar way to the background signal data collection.

4.4 Varying the water flow rate through the cell

The effect of flow rate of the microchannel on the intensity of the peaks was studied and the flow rate of flow cell system was controlled.

5. Scanning Electron Microscope (SEM)

Well-fabricated C_8-silica monolithic in microchannel was cross-sectional cut into 0.2 cm by a cutter. The specimens were stuck on an aluminium stud cell by using a double-sided carbon tape and then coated with gold 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 10 kV.

6. Separation of amine mixture

Standard amines, namely methylamine, ethylamine, and trimethylamine was prepared at a concentration of 200 ppm in methanol and 20 µL were injected via a 20 µL injection loop connected to the Rheodyne injection valve to separate the analytes in the fabricated microchannel. The mobile phase was a solution of (50:50) 0.1M PBS - methanol, and it was flowed at 0.5 mL min^{-1} through the channel. With such flow rate the back pressure monitored by the reciprocating pump was between 300-350 psi. The excitation and emission wavelength were set at 405 and 518 nm, respectively. The water sheath flow was controlled at about 0.5 mL min^{-1}.

7. Separation of chlorpheniramine

7.1 Conditions of chlorpheniramine

Standard chlorpheniramine was prepared at a concentration of 5000 ppm in methanol and 20 µL were injected via an injection valve. The mobile phase was a solution of pure methanol, and it was flowed at 0.50 mL min^{-1} through the channel. The back pressure monitored by the reciprocating pump was between 200-300 psi. The excitation wavelength of chlorpheniramine was given at 405 and the emission was monitored at 518 nm. The water sheath flow was controlled at about 0.5 mL min^{-1}.

7.2 Determination of chlorpheniramine in tablets

Chlorpheniramine tablet was accurately weighed, and mechanically blended to powder. A 0.05 g of the solid powder was weighed accurately and dissolved in 1.0 mL methanol. The solution was filtered through 0.45 μm Nylon filter. The extraction was repeated three times (n=3). A calibration curve of chlorpheniramine standard solution was prepared from standard chlorpheniramine concentration in a range between 200-1000 ppm. The standard chlorpheniramine solutions were prepared by mixing 500 ppm chlorpheniramine at different volume to 200 μL fluorescencing agent (DBD-ED), and the solutions were made up to 1.00 mL with methanol. A 20 μL of each derivatised solution was injected to the monolithic microchannel. After the chromatographic analysis, the area of each extracted compounds were averaged and the concentration of chlorpheniramine extract solution were subsequently calculated by comparing with the calibration curve.

7.3 Detection limit of chlorpheniramine by using the monolith microchip with LIF detection

Standard solutions of chlorpheniramine were prepared at a concentration ranged between 200 -1000 ppm. A concentration range close to the detection limit was repeatedly injected to find the lowest concentration.

8. Separation of riboflavin

8.1 Conditions of riboflavin

Standard riboflavin was prepared at a concentration of 5000 ppm in methanol and 20 μL were injected via an injection valve. The mobile phase was a solution of methanol, and it was flowed at 0.5 mL min^{-1} through the channel. The back pressure monitored by the reciprocating pump was between 200-300 psi. The excitation wavelength of riboflavin was at 405 and the emission was monitored at 520 nm. The water sheath flow was kept at about 0.5 mL min^{-1}.

8.2 Determination of riboflavin in tablets

Riboflavin tablet was accurately weighed, and mechanically blended to powder. A 0.05 g of the solid powder was weighed accurately and dissolved in methanol. The solution was filtered through 0.45 μm Nylon filter. The extraction was repeated three times (n=3). A calibration curve of riboflavin standard solution was prepared from standard riboflavin concentration ranged between 200-1000 ppm. A 20 μL of each solution was injected to the monolithic microchannel. After the chromatographic analysis, the area of each extracted compounds were averaged and the concentration of riboflavin extract solution were subsequently calculated by comparing with the calibration curve.

8.3 Detection limit of riboflavin by using the monolith microchip with LIF detection

Standard solutions of riboflavin was prepared at a concentration range between 100 -1500 ppm. A concentration range close to the detection limit was repeatedly injected to find the lowest concentration.

RESULTS AND DISCUSSION

In this work there are three main components to be assembled for chromatography on the chip, namely microfluidic chip, fabricated monolith and the laser induced florescence detection. The microchip is comparable to a column hardwater of high performance liquid chromatography (HPLC). Fabricated monolith in the microchip channel was used for separation and imitated packing particles in a HPLC column. Although the As the mixture amines, riboflavin, and chlorpheniramine will be analysed as a samples, it is then needed to separate by using microfluidic chip combined with laser-induced fluorescence (LIF). The laser-induced fluorescence equipment then must be set-up and optimized for separation.

1. Laser-induced fluorescence set-up and optimization

At the initial of the study the laser induced fluorescence compartment had been donated, but the light source was broken. As a replacement of the light source, a commercial diode laser light source was of our interest, however, it was of high cost, and gave less simplification to the overall technique. A laser source was then attractive, and fortunately a “blue” GaN laser was in the local market.

1.1 Laser set-up and stability testing

1.1.1 Feasibility of a new laser source

The blue emitting light of GaN was tested for a suitable wavelength for being a near UV light source by using it as a light source in UV visible spectrometer, resulting a light absorption at 422.9 nm. When compared with the GaN from the literature (see Appendix C), which gives UV at 405 nm. It was proved that the blue laser pointer could be used as the light source.

Consequently the blue laser pointer or laser pen was adapted to use as a laser source in this LIF system. The source was supplied by a direct voltage

power supply, giving a continuous voltage of 3 V. With such adaptation the laser source gave continuous laser light. The laser beam was projected directly to the mirror for reflecting and then passing through the focus lens and to the sheath flow cuvette. The beam spot must be adjusted to the center of cuvette. This spot could be observed on the surface of cuvette.

1.1.2 Stability of the laser source

Although it has been proved that the “cheap” laser source could be utilized, the stability of the light emitting from the laser source and the long-term used were doubted. A few experiments were carried out for reliability of the laser. The LabVIEW data collection and acquisition was used to support the monitoring of the beamline stability. An intra-day and inter-day monitoring signal was recorded and compared, resulting the background signal monitoring as shown in Figure 4 and 5, respectively.

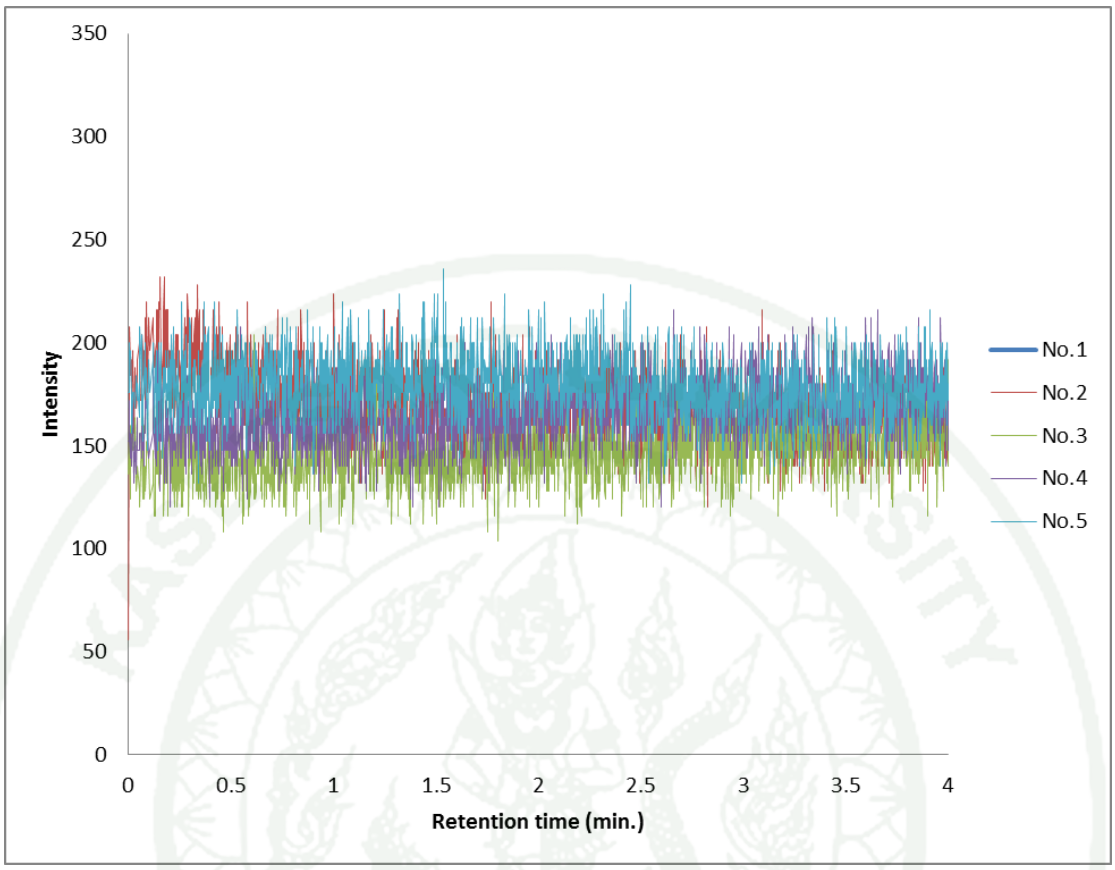

Figure 4 Intra-day background signal from the laser-induced fluorescence detector recorded in 20 July 2011, with 5 time repetition.

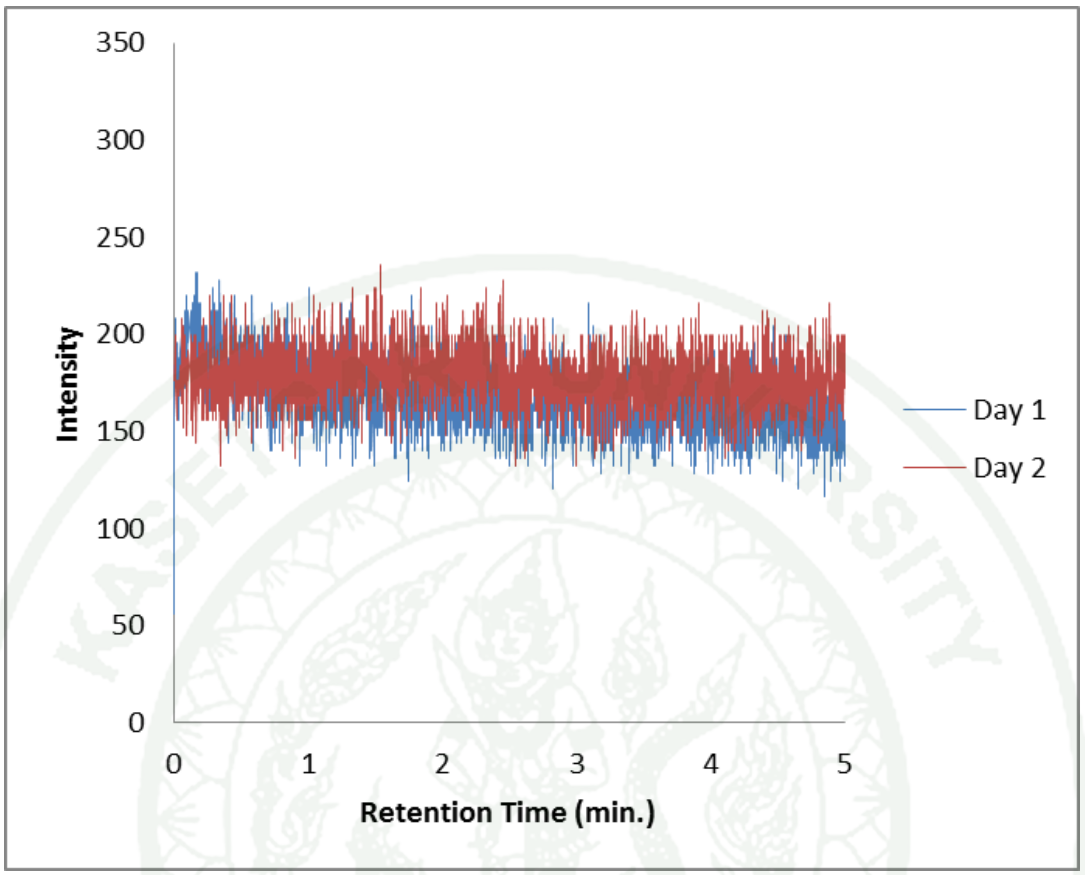

Figure 5 Comparison of background of laser-induced fluorescence detector in 20 July 2011 with 12 August 2011

Comparison of inter-day and intra-day background of laser-induced fluorescence was shown in Figure 1 and Figure 2, respectively. Inter- day background recording of the system was repeated 5 times, showing that the signal was stable with the intensity swinging between 150 – 200 for 4-5 min measurement In theory the background signal is as near zero as possible. However, a commercial instrument could have background intensity between 5-20, while a well-designed home-made instrument possibly affords a minimal intensity between 20-50. The between day backgrounds signal in figure 5 also shows swinging background between 150 – 200 in a period of measurement of 5 min. From my observation, the stability of laser intensity was satisfied, no drifting or other detrimental effect for a period of 3 hours. However, a typical laser generates heat after a long emitting period, an experiment

that needs a long period exposure for more than 1 hour is not recommended. In this work, the same laser pen was used for all experiments for prevention of possible signal errors. The testing of signal between lasers was not performed.

1.1.3 Mobile phase background

As mentioned earlier about minimizing the background signal, another possibility that contributed to the high background was the eluent flowing through the microchip to the LIF detector. As the work involved with the use of mobile phase with and without derivatising agent and the high concentration of derivatising agent could give high intensity background , the background signals of some mobile phases were compared, giving the result of background signals as shown in Figure 3.

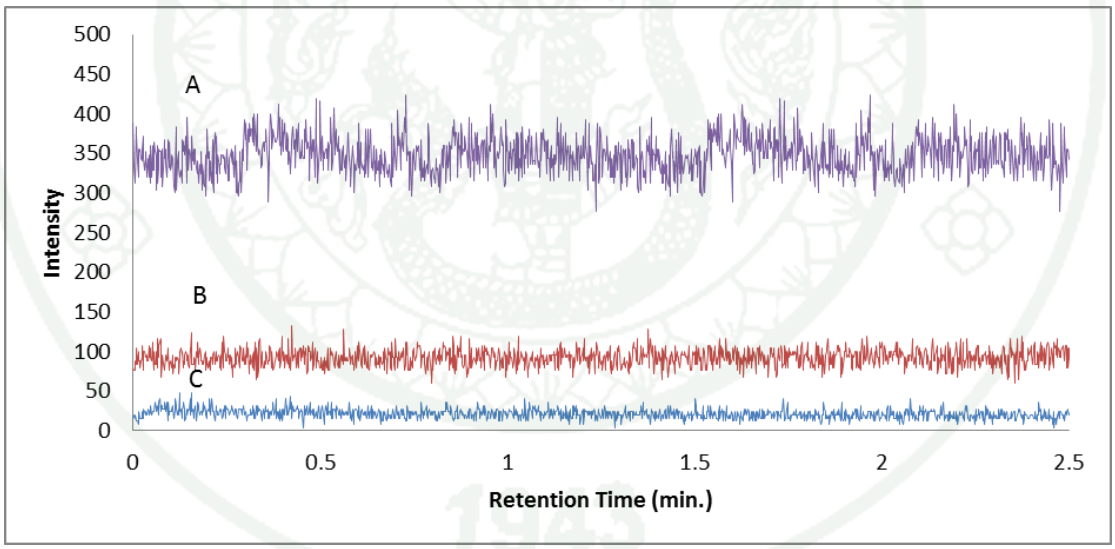

Figure 6 Comparison of background signals between (a) water, (b) pure methanol, and (c) methanol with derivatising agent.

2. C_8-monolith channel in polydimethylsiloxane (PDMS)-chip

2.1 PDMS configuration

The PDMS microchip was constructed as described previously in section of literature review. The two different configurations of microfluidic chips used in this research are shown in Figure 4.

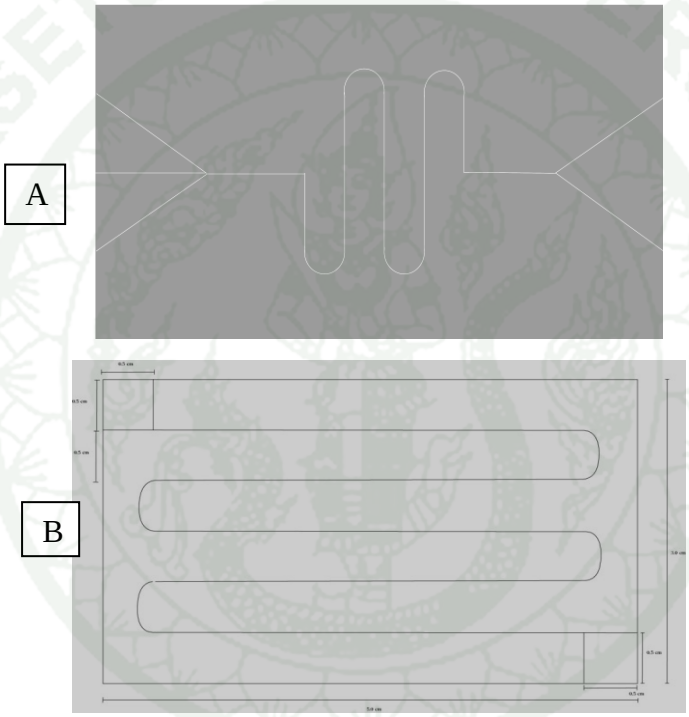

Figure 7 Two configurations of microfluidic chips, A and B.

Figure 7 A was used to separate amine model compounds namely, methylamine, ethylamine, and trimethylamine. The length of the serpentine channel for fabricating monolith is 10 cm and the width and depth of the channel is 100 x 100 micrometers. The size of the microchip is 3.0 cm wide and 5.0 cm long. The thickness of the PDMS initially made was 3.0 mm. It was found that thin microchip facilitated the control of the liquid flow along the channel, too thin microchip, however, could break the PDMS polymer when connected with a capillary prior to the microchip.

In theory the length of the channel affects the separation, giving higher plate number, a new configuration of microchip was designed to have longer channel. Figure 4 B was a new configuration that was adapted to separate other samples, such as riboflavin and chlorpheniramine. The length of the serpentine channel was 20 cm, more than twice of the previous design, and the width and depth of the channel were 250 and 250 micrometers, respectively.

After constructing a number of microchips, one microchip was sacrificed to investigate the real channel configuration. The microchip was side-cutting and subjected to the scanning electron microscopy (SEM), and the SEM photograph of the microchannel was shown in Figure 8. It was observed that the microchannel walls were smooth and the channel was rectangular with the width of 70 - 100 microns, and the depth of 35 microns.

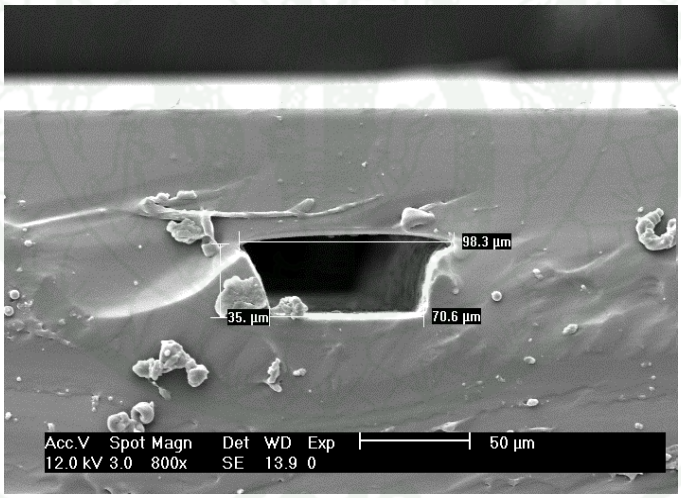

Figure 8 SEM photograph of microfluidic chip.

It must be pointed out that not every microchip was good enough for further fabrication. This was caused by the chemical lithography of the very small channels, therefore every single microchip quality was carefully controlled, all channels were seen under a microscope. To assemble microfluidic chip with a pump, and an inlet capillary was inserted to the side of microchip inlet, and the microchip

was also tested for the best quality by a flow of water. The good quality was indicated by the continuous flow of water along the microchip with almost no back pressure.

2.2 C_8-monolith fabrication in channel

C8-silica monolith was fabricated in a selected microchip by using sol-gel process. The channel was pretreated by using a 1.0 N NaOH solution and the fabrication was described in the material and methods section. The fabricated monolith in the channel was subjected to scanning electron microscopy (SEM), and the SEM image of the C8-silica monolith was shown in Figure 9. It was confirmed that the synthesized material was monolithic structure with a continuous skeleton of a uniform organic-silica hybrid monolith with the particle size of ca. 1 µm.

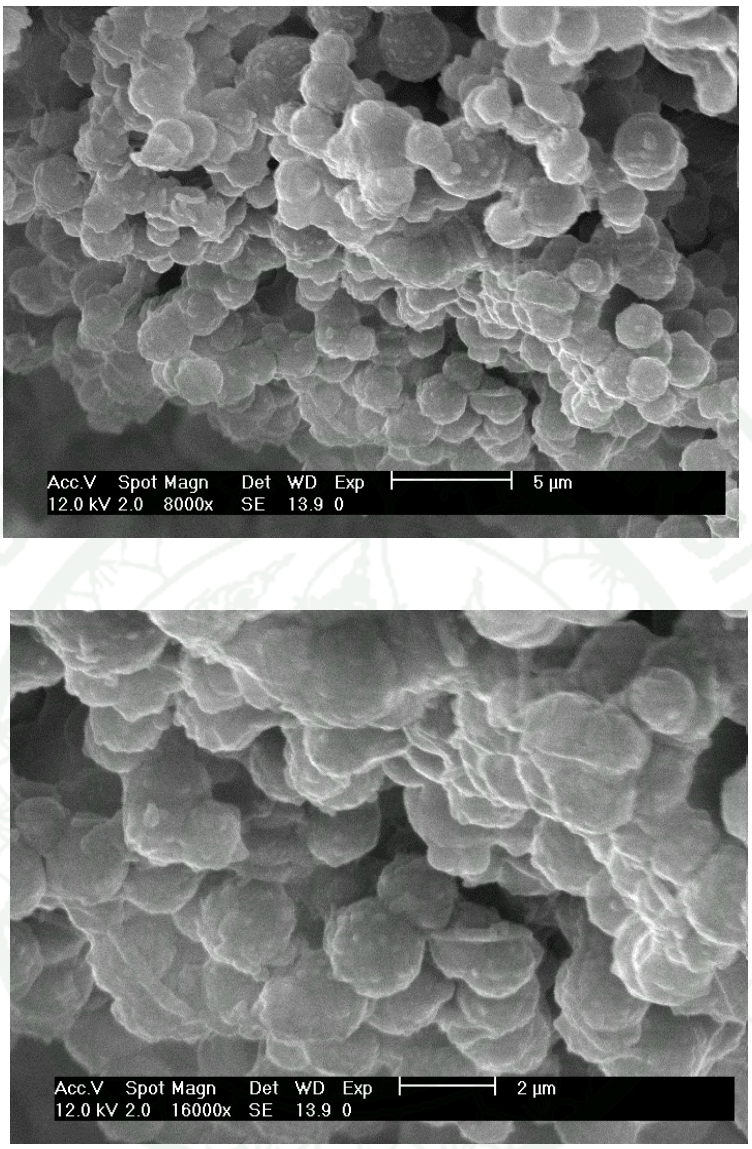

Figure 9 SEM images of cross-section of C_8-silica monolith in the microfluidic chip

3. Separation of amine model compounds

3.1 Optimisation of conditions

The C_8-monolithic channel microfluidic chip was used in conjunction with the laser-induced fluorescence system to separate a mixture of amines, namely methylamine, ethylamine, and trimethylamine. Some conditions for operating the integrated system were optimized to obtain the best separation of the analytes. Because the introduction of the derivatising agent into the stream of eluent after passing the monolith channel on the chip could not be carried out due to some limitations. Each 200 µL of each amine was mixed with 200 µL 200 ppm of derivatising agent and the solution was made up to 1.00 mL with (50:50) methanol-phosphate buffer pH 7.0. The solution was left for 30 seconds before an introduction to the monolithic microfluidic system. It was observed that the solution should not be left long before the injection into the system, otherwise the fluorescence intensity might reduce. A chromatogram was obtained from a direct injection of 20 µL of the mixture via a 20 µL loop injection. The amine model compounds were well-separated under the optimum conditions, as follows: the flow rate of 0.5 mL min^{-1} with pure methanol as a mobile phase.

A mixture of methylamine, ethylamine, and trimethylamine was well separated in the monolith channel. In this experiment a high backpressure (ca.300psi) occurred when using the flow of 0.5 mL min^{-1}. A flow of higher than 0.5 mL min^{-1} could not be carried out, because the high pressure could damage the microchip. The high pressure and the restricted flow presumably limited the chromatographic peak quality of the model compounds. The chromatogram of separated amines is shown in Figure 10.

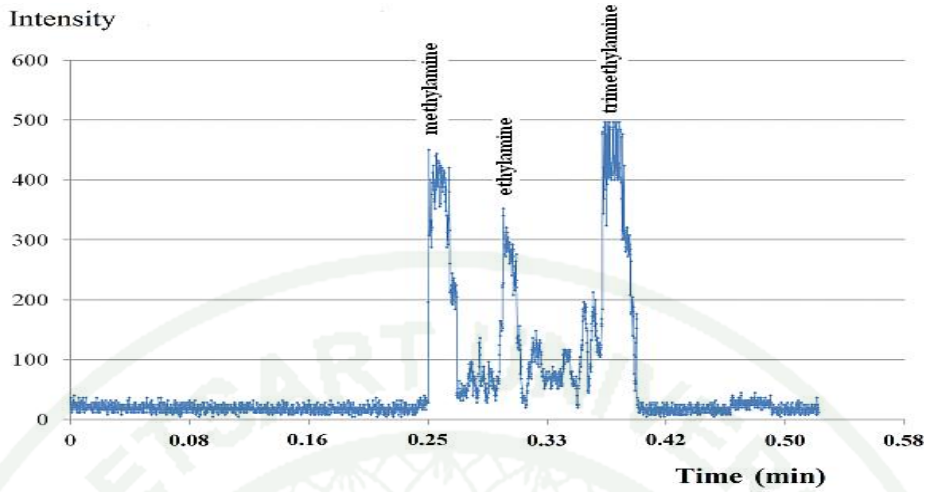

Figure 10 Separation of amine mixture on a 10 cm C8-monolithic microchannel of the microchip configuration A. Conditions: mobile phase, pure methanol; flow rate: 0.5 mL min^{-1}

When the mobile phase was changed to (50:50) methanol-phosphate buffer pH 7.0, the pressure of the system increased, and the intensity of all peaks greatly reduced. This was caused by the quenching of fluorescence by the water solvent.

3.2 Chromatograhic parameters

Since the C_8-monolith was synthesized in the microchip channel and was used to imitate the liquid chromatographic column, some chromatographic parameters were measured and calculated, according to the theory in chromatography. Table 1 shows some chromatographic parameters of the three amines separation on the monolithic channel.

Table 1 Chromatographic parameters of amine mixture separated by using the 10 cm C8-monolithic channel.

Parameters	**Methylamine(1º)**	**Ethylamine(2º)**	**Trimethylamine(3º)**
Retention time, t_R (min)	2.1784	2.7015	3.4055
Peak area	34093.94	17930.43	45947.93
Peak height	421.5851	293.5851	469.5851
Peak width,W (min)	0.3419	0.2595	0.4220
Peak width at half height, $W_{0.5}$ (min)	0.1495	0.0112	0.2595
Dead time, t_0 (min)	0.005	0.005	0.005
Retention factor (k′)	432.62	539.30	680.10
Tailing factor (TF)	5.0067	1.0790	1.8162
Plate number, N (plates)	224.8986	600.4059	360.783
Plate height, H (plate m^{-1})	0.0446	0.0167	0.0277
Selectivity factor (α)	1.2407 (for 1º-2º)		1.2611 (for 2º-3º)

From the chromatographic parameters in Table 1 to separate methylamine, dimethylamine, trimethylamine it took only less than 4 mins, and the monolith in the channel gave good selectivity factors of 1.21 and 1.26 for α (methylamine/ dimethylamine) and α(dimethylamine/trimethylamine). The plate number of the microchip was rather low (395.3625 plates per 10 cm), compared with the plate number of conventional HPLC. However, the amine peak was quite symmetry, giving tailing factors of averagely 3.1564

4. Application to riboflavin

Riboflavin was chosen as another model compounds, because it is naturally fluorescencing agent. A study in a real sample of riboflavin is also possible because riboflavin tablet is commercially available.

4.1 Separation of riboflavin

Riboflavin was dissolved in methanol and it was introduced to the C_8-monolithic microchannel for separation. A chromatogram of riboflavin obtained with a flow of methanol mobile phase at 0.50 mL min^{-1} is shown in Figure 11, and some chromatographic parameters were given in Table 2.

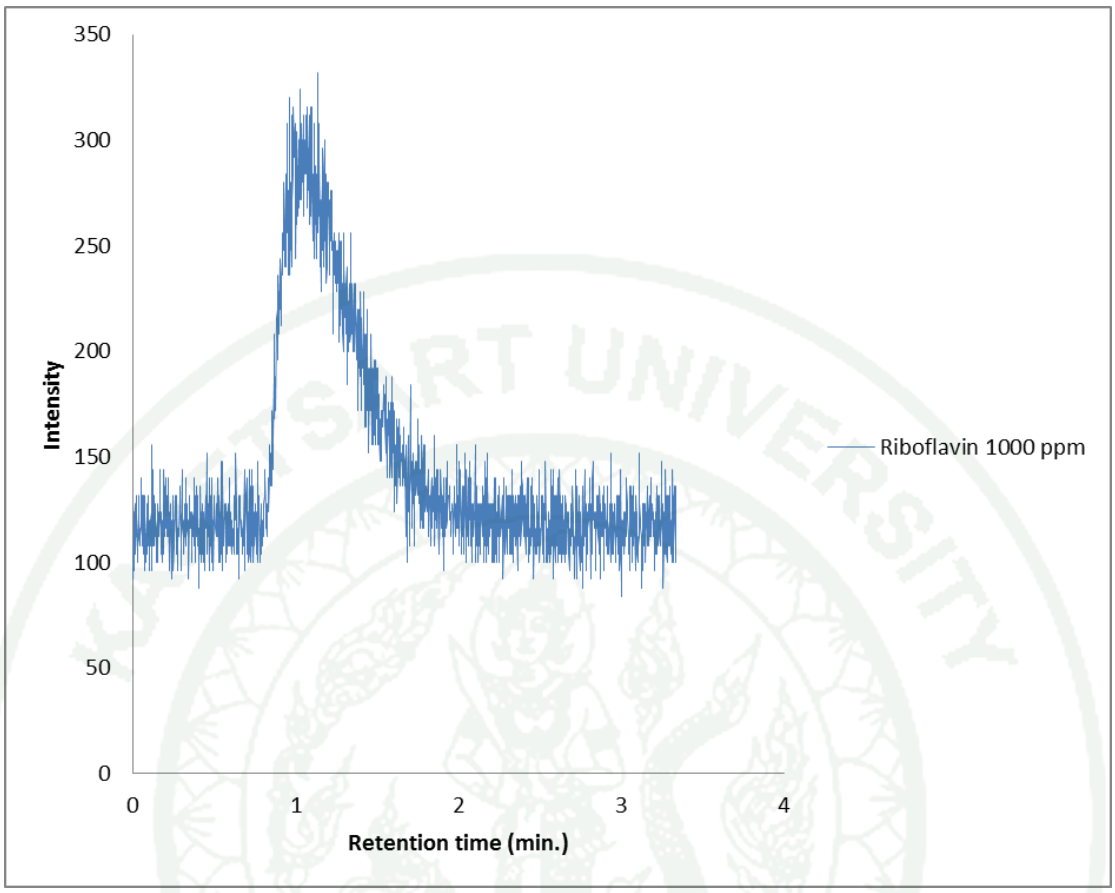

Figure 11 Separation of riboflavin on a 20 cm C_8-monolithic microchannel of the microchip configuration B. Conditions: mobile phase, pure methanol; flow rate: 0.5 mL min^{-1}

Table 2 Chromatographic parameters of riboflavin separated by using the 20 cm C_8-monolithic channel.

Parameters	Results
Retention time, t_R (min)	0.9627
Peak area	48538.6
Peak height	202.169
Peak width, W (min)	0.9558
Peak width at half height, $W_{0.5}$ (min)	0.4950
Dead time, t_0 (min)	0.007
Retention factor (k′)	126.53
Tailing factor (TF)	2.74
Plate number, N (plate)	5.62
Plate height, H (cm plate^{-1})	3.56

From the chromatographic parameters in Table 2 to separate riboflavin it took only less than 1 min. The plate number of the microchip was rather low, compared with the plate number of conventional HPLC. Since riboflavin peak was tailing, the tailing factor was rather high (2.74). The plate number of the monolithic channel was low (5.62 plates per 20 cm channel) , which could be a result of incomplete formation of monolith along the channel. Separation of the compound could be better by an improvement of the monolith synthesis.

From Figure 8 the riboflavin peak is quite tailing, giving the tailing factor of 2.7422. The plate number calculated from the riboflavin is 7.968, which is lower than that calculated from the separation of amine components (plate number = 170).

4.2 Calibration curve of riboflavin and sample determination

Since the peak of riboflavin is asymmetry, it was then doubted if a calibration of a range of concentration could be liner. A calibration curve of riboflavin was prepared from a concentration of standard of riboflavin between 200-1000 ppm. The

riboflavin solutions were injected at 20 µL and the peak areas of riboflavin was shown in Table 3 and they were plotted as shown in Figure 12. The result showed a linear calibration with a Pearson's correlation coefficient of 0.9944.

Table 3 Retention time and peak height of riboflavin concentrations of 200, 300, 500, 800, and 1000 ppm.

Concentration (ppm)	Retention time (min)	Peak height (Intensity)	Peak area
200	1.09987	164	4240.86
300	1.06132	180	7837.46
500	1.01645	224	21957.8
800	1.05233	288	40033.6
1000	0.962767	320	48538.6

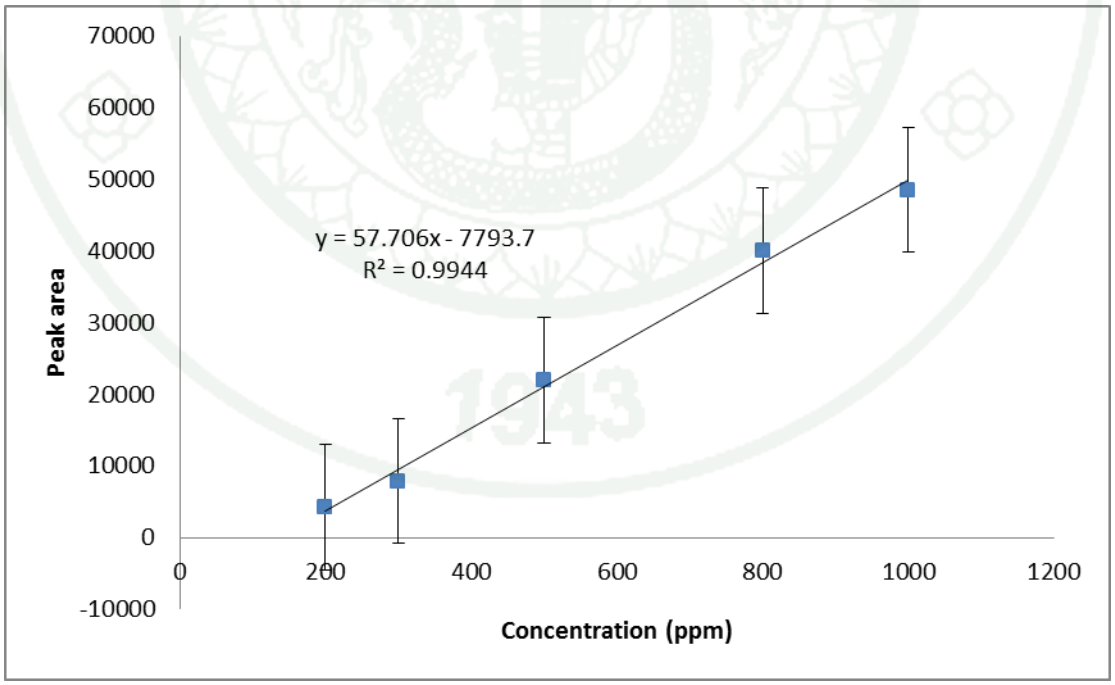

Figure 12 Calibration curve of riboflavin concentration range between 200-1000 ppm

Riboflavin tablet was extracted and injected into the monolithic microfluidic system, the peak height was taken and compared with the calibration curve. It was found that the riboflavin in the extract was 939.04 ppm, and when it was converted to percentage in tablet. The concentration result of riboflavin was 46.952 % compared to the declared concentration of 50 mg in the tablet. The low yield of riboflavin could be a result of incomplete dissolution during extraction, as it was noticed from the intense yellow solid of riboflavin on the Nylon filter after filtration.

4.3 Detection limit of riboflavin

To calculate the detection limit, the slope, intercept (y_B) and least square of correlation coefficient (r^2) were obtained from the calibration curve. Since the riboflavin calibration curve was calculated from peak height, the average peak height, standard deviation (SD) and three times of standard deviation (3SD) of riboflavin were determined by 20 injections of the lowest concentration of riboflavin of which peak was appeared on a chromatogram, which was 200 ppm. The 3SD was recalculated to examine the true detection limit, by substitution in the equation, $y\text{-}y_B$ = 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 285 ppm.

5. Application to chlorpheniramine

Chlorpheniramine was chosen because it is a secondary amine compounds and related to the amine mixture previously demonstrated for separation. The drug is very well-known antihistamine with minimal side-effect.

5.1 Separation of chlorpheniramine

A 800 μL of chlorpheniramine extract was mixed with 200 μL 200 ppm of derivatising agent and the solution was made up to 1.00 mL with (50:50) methanol-

phosphate buffer pH 7.0. The solution was left for 30 seconds before an introduction to the monolithic microfluidic system. A chromatogram of chlorpheniramine obtained with a flow of methanol mobile phase at 0.5 mL min^{-1} is shown in Figure 13, and some chromatographic parameters were given in Table 4.

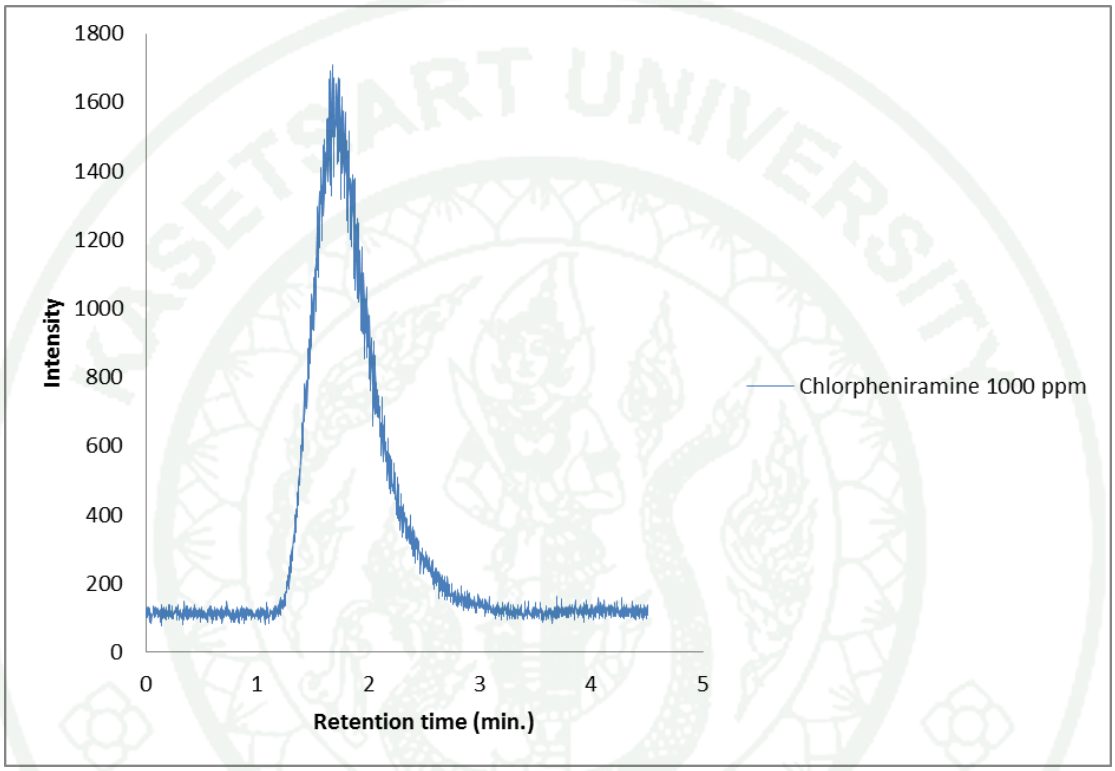

Figure 13 Separation of derivatised chlorpheniramine on a 20 cm C8-monolithic microchannel of the microchip configuration B. Conditions: mobile phase, pure methanol; flow rate: 0.5 mL min^{-1}

Table 4 Chromatographic parameters of derivatised chlorpheniramine separated by using the 20 cm C_8-monolithic channel.

Parameters	Results
Retention time, t_R (min)	1.6439 min
Peak area	611515
Peak height	11549.98
Peak width, W (min)	1.79825 min
Peak width at half height, $W_{0.5}$ (min)	0.5185
Dead time, t_0 (min)	0.007 min
Retention factor (k′)	1.64
Tailing factor (TF)	1.71
Plate number, N (plate)	4.63 plates
Plate height, H (cm plate^{-1})	4.32 plates m^{-1}

From the chromatographic parameters in Table 4 to separate chlorpheniramine it took about the same period as that for riboflavin. The plate number of the microchip was rather low (4.6298 plates per 20 cm) compared with the plate number of conventional HPLC. Since chlorpheniramine peak was tailing, the tailing factor was rather high (1.71), it was however lower than that of riboflavin. This could be due to the higher polarity of the compound, compared with that of riboflavin. The plate number of the monolithic channel was low (4.6298 plates per 20 cm channel). A comparison between the 10 cm and 20 cm monolith in different microchip showed that the two microchips gave different qualities, which could depend on the synthesis technique and process.

5.2 Calibration curve of chlorpheniramine and a determination in a tablet

A calibration curve of chlorpheniramine was prepared from a concentration of standard of chlorpheniramine between 200-1000 ppm. The chlorpheniramine solutions were injected at 20 µL and the peak areas of

chlorpheniramine was shown in Table 5 and they were plotted as shown in Figure 14. The result showed a linear calibration with a correlation coefficient of 0.9274.

Table 5 Retention time and peak height of chlorpheniramine concentrations of 200, 300, 500, 800, and 1000 ppm.

Concentration (ppm)	Retention time (min.)	Peak height (Intensity)	Peak area
200	1.4722	1220	390007.1
300	1.3061	1332	428173.3
500	1.5319	1432	528767.4
800	1.5506	1548	607199.0
1000	1.6439	1668	611514.8

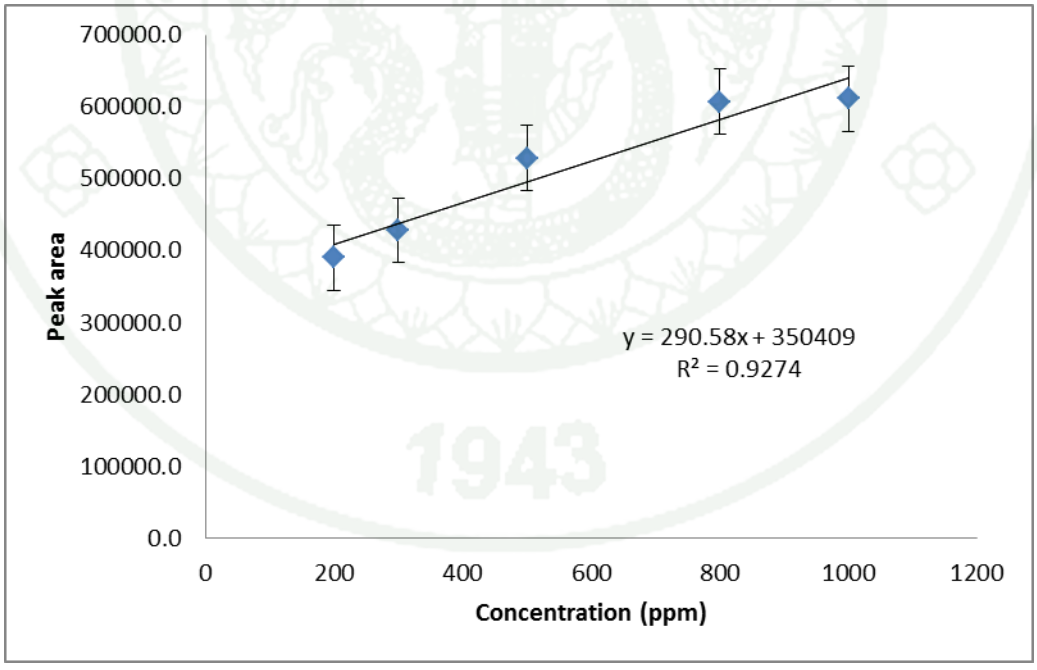

Figure 14 Calibration curve of chlorpheniramine concentration range between 200-1000 ppm

Chlorpheniramine tablet was extracted and injected into the monolithic microfluidic system, the peak height was taken and compared with the calibration curve. It was found that the chlorpheniramine in the extract was 859.181 ppm, and when it was converted to percentage in tablet. The concentration result of chlorpheniramine was 57.99 %, compared to the declared concentration of 0.004 g in the tablet. The low yield of chlorpheniramine could be a result of error during extraction, as it was noticed from the yellow solid of chlorpheniramine on the Nylon filter after filtration. Some errors might occur from incomplete dissolution and/or prederivatisation.

5.3 Detection limit of chlorpheniramine

To calculate the detection limit, the slope, intercept (y_B) and least square of correlation coefficient (r^2) were obtained from the calibration curve. Since the riboflavin calibration curve was calculated from peak height, the average peak height, standard deviation (SD) and three times of standard deviation (3SD) of chlorpheniramine were determined by 20 injections of the lowest concentration of chlorpheniramine of which peak was appeared on a chromatogram, which was 200 ppm. 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 283 ppm.

CONCLUSION AND RECOMMENDATIONS

An attempt of using a laser pointer for being the light source of laser-induced fluorescence was made possible. With the overall newly set up detection system, the signal was monitored by processing the data collected by using the photomultipler. A comparison of intraday and intraday signals showed that the detection system was reliable. The baselines of water as blank, methanol mobile phase with and without derivatising agent substantiated the reliability.

The synthesis of C_8-silica monolith in a microchannel of a microchip was subsequently demonstrated, and the length of the synthesized monolith in the channel as long as 10 cm was possible. The monolith structure and the configuration of wall channel were confirmed by scanning electron microscopic technique. The particle size of the C_8-silica monolith was averagely 1.0 micrometers, with interconnected skeleton and flow through pore. The separation of the model compounds was demonstrated by injecting riboflavin, chlorpheniramine and a mixture of amine homologous series, namely methylamine, ethylamine, and trimethylamine, via a typical loop injection of 20 μL. Since the laser induced fluorescence was coupled with the monolith microfluidic chip for using as the detector with an excitation and emission wavelength of 405 and 518 nm, a prederivatisation technique was performed for all amines prior to the detection. With the methanol mobile phase of 0.3-0.5 mL min^{-1}, the back pressure monitored by the reciprocating pump was not more than 300 psi. The peaks of all amines were separated and well-resolved, so were the peaks of riboflavin and chlropheniramine.

Riboflavin and chlorpheniramine were also tested for the linearity of calibration in a concentration range between 200-1000 ppm, and it showed satisfactory results with a linear correlation of 0.9944 and 0.9274 for riboflavin and chlorpheniramine, rescpectively. Both drug tablets were also examined and it was found that only 12.91% and 57.99% yield were achived, compared with declared tablet, for riboflavin and chlorpheniramine, respectively.

The C_8-monolith microfluidic chip could be a near future chip, replacing an HPLC column, for both ionisable and neutral compounds without modification of mobile phase or using supplementary techniques, provided that the detection is facilitated. For example, if the detection used mass spectrometric detector, the monolith microfluidic chip, could be integrated directly and simply. The main advantage was that the microchip is inexpensive and disposable, which makes the "HPLC column" disposable. This is suitable for complicated organic extract of which clean-up step is a problem. The microseparation is also beneficial in the reduction of the analyte, extract and, particularly, waste volume.

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APPENDICES

Appendix A

Liquid Chromatography with C_8-Silica Monolith in a Microchip

LIQUID CHROMATOGRAPHY WITH C_8-SILICA MONOLITH IN A MICROCHIP

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Abstract

An organic-inorganic hybrid silica with octyl group monolith was fabricated in a microfluidic chip for microchannel liquid chromatography. The structure of hybrid monolith was optimized by changing the composition of tetraethoxysilane (TEOS), octyltriethoxysilane (C_8-TEOS) and 3-mercaptopropyltrimethoxy-silane (MPTMS) in the mixture of precursors. The monolithic microchannel was characterised by scanning electron microscopy (SEM). The performance of microfluidic chip was evaluated by separating organic compounds in the microchannel connected to a pressure-driven pump and a laser-induced fluorescence detector. Primary and secondary amines were well-separated by the monolithic channel. Separation of a chlorpheniramine model compound using the chip will be also presented.

Keywords: Microfluidic chip, silica monolith, chromatography, fluorescent detection, chlorpheniramine

Introduction

Microfluidic chip technology has increasing attention and plays an important role, because of enhanced speed, economy, smaller analyte volumes, sensitivity and portability compared to conventional instruments. A number of microfluidic devices have been purpose-designed for bio- and biomedical separation. For majority of real world samples, microfluidic devices, present a major step forward in device functionality. As a result, microfluidic analysis is being extended to pharmaceutical chemistry where a very wide variety of chemical compounds or ions is involved.

Since the beginning of this century, monolithic materials have been developed and commercialised as chromatographic columns for separation of a broad range of compounds. Two types of widely used monolithic materials are polymers and silica. The effectiveness and performance of monoliths for separating various types of material, led to studies on fabricating monoliths in microchip channels. Fabrication of polymer monolith in a microfluidic channel has been achieved and has already been applied in some applications. However, the combination of silica monolith with a microfluidic channel on a die is much more challenging, because of several problems. For example, cracking of the silica monolith is similar to that which can can occur during silica monolith formation in a capillary. Formation of a gap between the wall and monolith, and the need of smooth microchip channel walls are also problematic. In our work we successfully constructed a C_8-silica monolith in a microfluidic channel and successfully demonstrated chromatographic separation of an amine mixture as well as chlorfenilamine with post-column derivatisation prior to detection.

Aims

To develop C_8-silica monolith on microfluidic die using laser-induced fluorescence (LIFs) detector for determining chlorpheniramine in pharmaceutical products.

Materials and methods

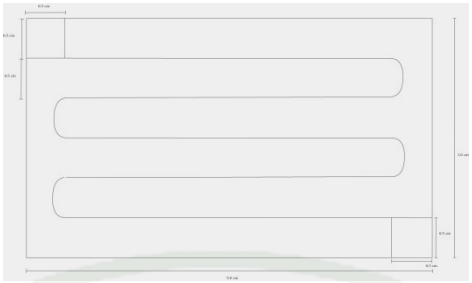
Chemicals and sample preparation

Tetraethoxysilane (98%, TEOS), *n*-octyltriethoxysilane (97%, C_8-TEOS) and 3-mercaptopropyltrimethoxysilane (95%, MPTMS) were purchase from Sigma-Aldrich and used without further purification. *N*-Dodecylamine (98%), were obtained from Fluka. Methylamine, ethylamine and trimethylamine was used as model compounds for chromatographic separation. Chlorpheniramine maleate salt was purchased from Sigma-Aldrich, India. The 4-(2-Aminoethylamino)-7-(N,N-dimethylsulfamoyl)benzofuraza was a fluorescing agent obtained from Fluka, Japan. Phosphate buffer solution (PBS) was prepared according to a standard laboratory method. The mobile phase was either HPLC grade methanol or a mixture of (70:30) methanol-water.

The chlorpheniramine tablet was purchased from ST Group Pharma &Health Care Co., Ltd. The tablet was ground, weighed then dissolved in 1 mL methanol. The mixture was filtered through 0.45 µm PTFE membrane.

Microfluidic chip preparation

Processing poly(dimethylsiloxane), (PDMS), microfluidic chip is based on replication of PDMS from a master mold fabricated by UV lithography, The master mold is shown in Figure 1. A (10:1) base:curing agent of PDMS mixture (Sylgard 184 silicone elastomer kit, Dow Corning, Midland, MI, USA) was vacuum degassed, and poured over the master mold. The sample was subsequently cured at 70 ˚C for 1h. Subsequently, the replica was peeled off the master. The microchannel was enclosed by sealing the replica with a PDMS film, which was earlier spin coated on a clean glass substrate and pre-cured at 70 ˚C for 5 minutes. To completely harden the PDMS, the microfluidic chip was cured again at 70 ˚C for 30 minutes [1,2].



Appendix Figure A1 Configuration of the microfluidic chip

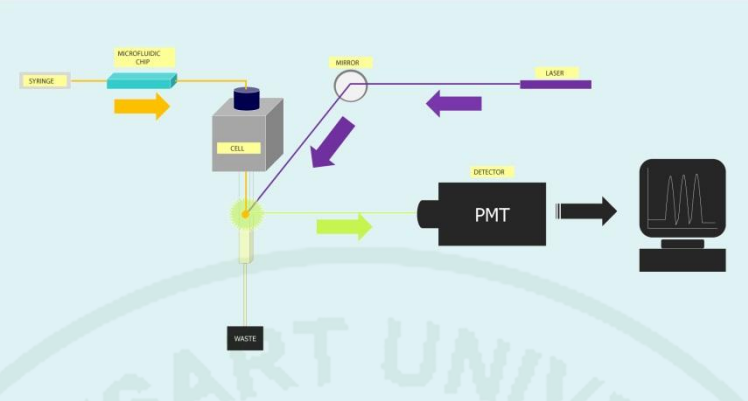
Fabrication of C8-silica monolith

In order to clean and activate the inner surface of microfluidic chip for effective attachment of silica skeletons, a 100 µm × 150 µm rectangular microchip channel was rinsed first with 1 M NaOH solution for 1h. A 180 µL of methanol, 23 µL of 0.5 M HCl, 65 µL C_8-TEOS, 35 µL MPTMS and 150 µL TEOS were mixed and left for hydrolysis at 60˚C for 5h. At room temperature, 10 mg of *n*-dodecylamine was added into the mixture. The mixture was filled into the pretreated microfluidic chip to the column length by a syringe. The filled chip was then tightly sealed, and allowed to stand at 40 ˚C for 15h for reaction. Afterwards, the chip was rinsed with ethanol to remove the *n*-dodecylamine and soluble hydrolysis products, and dried at 40ºC for 48h. The monolith formation in the channel was confirmed by scanning electron microscopy (SEM).

Instrumentation

The laser-induced fluorescence detector is based on a GaN laser source of 405 nm wavelength. The light was focused with an objective onto the sheath-flow cell. The light was collected with another lens, filtered to remove scattered exciting light and detected with a photomultiplier tube (Hamamatsu). The detection system was operated in photon-counting multi-scaling mode using a LabVIEW for data acquisition and control.

Chromatographic separation and analysis of chlorpheniramine from the tablet was carried out using pure methanol as a mobile phase in isocratic mode at a flow rate of 0.3 mL min^{-1}.

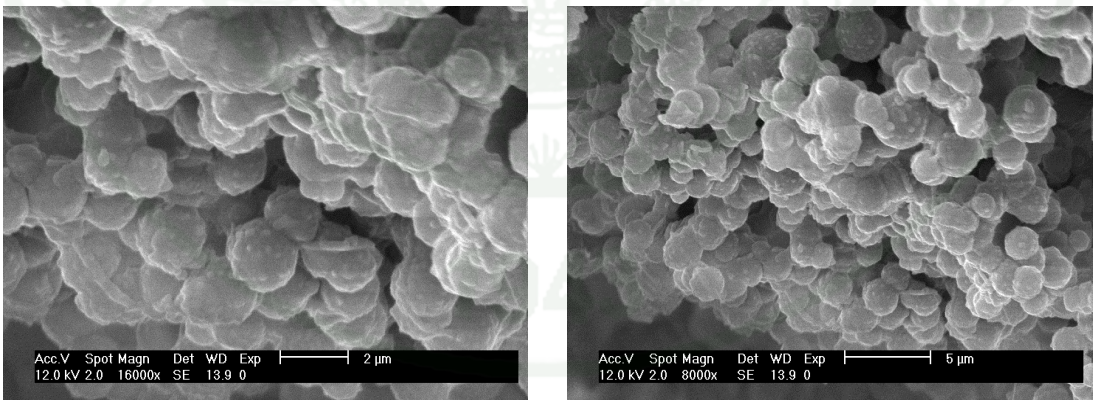


Appendix Figure A2 Schematic of laser-induced fluorescence using determine compounds

Results

Characterization of the silica based-monoliths

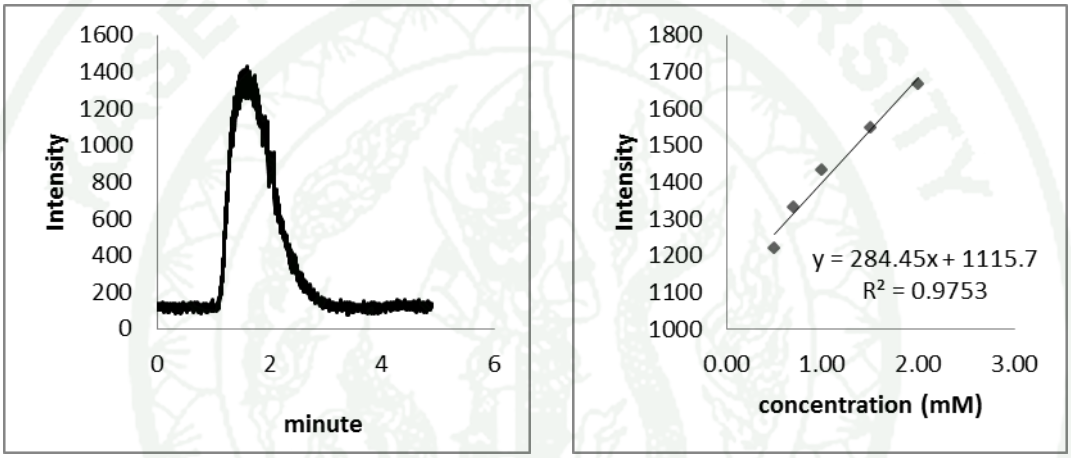
After fabrication the C_8-silica monolith in the channel was characterised by SEM. This revealed a continuous skeleton of a uniform organic-silica hybrid monolith with the particle size of ca. 1 µm. Figure 3 shows the SEM image of the C8-silica monolith. It was also observed that the microchannel walls were smooth (not shown).



Appendix Figure A3 SEM images of cross-section of C8-silica monolith in the microfluidic chip

Separation of chlorpheniramine and amine mixtures

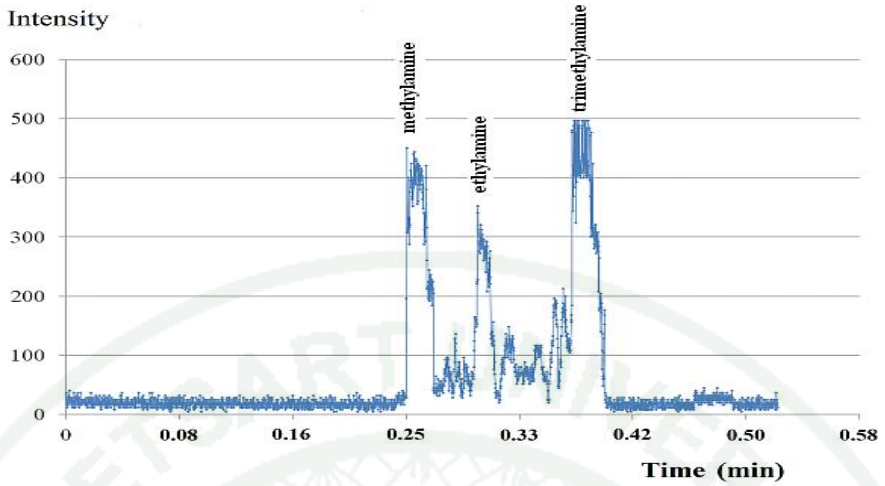
A standard concentration range of 0.5-2.0 mM chlorpheniramine in methanol was prepared, and 20 µL each was applied to the C8-monolith microchip system with LIF detection at 405 nm. Figure 4 shows the calibration data. Linear regression of intensity against concentration gave a coefficient of 0.9753. A solution of chlorpheniramine sample prepared from a tablet was also analysed and the chromatogram is shown in Figure 5.



Appendix Figure A4 Calibration curve for chlorpheniramine standard

Appendix Figure A5 Chromatogram of chlorpheniramine with a retention time of 1.44 min

A mixture of methylamine, ethylamine and trimethylamine was well separated in the monolith channel (Fig. 6). In this test a high backpressure occurred which presumably limited the chromatographic peak quality for the model compounds.



Appendix Figure A6 Separation of amine mixture

Conclusions and Discussion

A C_8-silica monolith in a microchip was successfully developed and the prototype reverse-phase LC chip demonstrated good chromatographic separation of chlorphenilamine as well as amine mixtures.

Acknowledgements

The Center of Excellence for Innovation in Chemistry, PERCH-CIC, for financial support and equipment is acknowledged. HJW's work was supported by the Academy of Finland Centre of Excellence in Nuclear and Accelerator Based Physics, ref. 213503 and the Schwartz Foundation.

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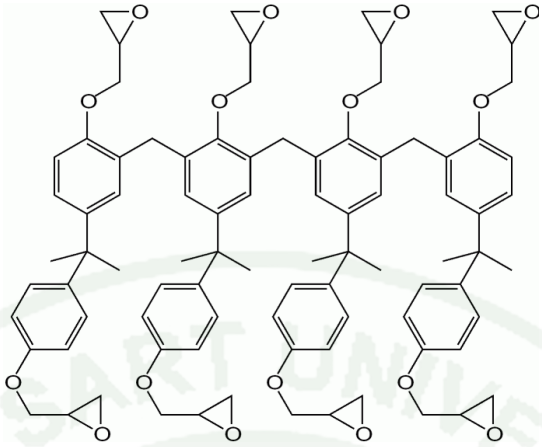
Appendix B

Composition and details of SU-8 photoresists



Appendix Figure B1 SU-8 molecule

SU-8 is a commonly used epoxy-based negative photoresist. Because of various polymer that can spun or spread over a thickness ranging from <1 micrometer up to >300 micrometer, SU-8 can be used to pattern high aspect ratio (>20) structure. The maximum absorption for UV light is 365 nm wavelengths. SU-8's long molecular chains cross-link causing the solidification of the material after exposed.

SU-8 was originally developed as a photoresist for the microelectronics industry, to provide a high-resolution mask for fabrication of semiconductor devices. It is now mainly used in the fabrication of microfluidics, and microelectromechanical system parts. It is also one of the most biocompatible materials known and is often used in bio-MEMS.

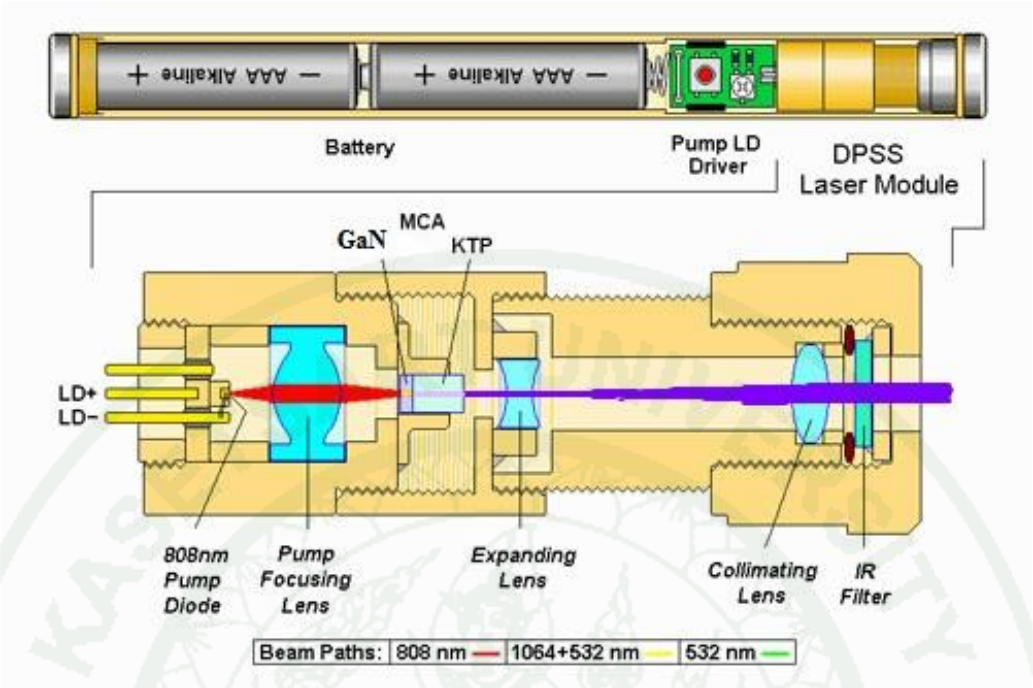
SU-8 is highly transparent in the ultraviolet region, allowing fabrication of relatively thick (hundreds of micrometers) structures with nearly vertical side walls. After exposition and developing, its highly cross-linked structure gives it high stability to chemicals and radiation damage. Cured cross-linked SU-8 shows very low levels of outgassing in a vacuum. However it is very difficult to remove, and tends to outgas in an unexposed state. The main developer for SU-8 is 1-methoxy-2-propanol acetate, source: http://en.wikipedia.org/Wiki/SU-8_photoresist

Appendix C

LASER: Light Amplification by Stimulated Emission of Radiation

Violet lasers may be constructed with GaN (gallium nitride) semiconductors. Gallium nitride lasers emit a violet light beam at 405 nm wavelength (close to ultraviolet, bordering on the very extreme of human vision) which can cause bright blue fluorescence (and thus a blue rather than violet spot) on many white surfaces, including white clothing, ordinary white sheet paper, and projection screens, due to widespread use of optical brighteners. On ordinary non-fluorescent materials, and also on fog or dust, the color appears as a shade of deep violet that cannot be reproduced on monitors and print. A GaN laser emits 405 nm directly without a frequency doubler, which means that accidental dangerous ultraviolet emission is impossible. These laser diodes are used in the reading and writing of data in Blu-ray drives (although the light emitted by the diodes is not blue, but distinctly violet). As of September 2011, 405 nm blue-violet laser diode modules with an optical power of 250 mW, based on GaN violet laser diodes made for Blu-ray disc readers, had reached the market from Chinese sources for prices of about $60 including delivery.

At the same time, a few higher-powered (120 mW) 404–405 nm "violet" laser pointers have become available which are not based on GaN, but use DPSS frequency-doubler technology from 1 watt 808 nm GaAlAs infrared diode lasers. As with infrared-driven green laser pointers above, such devices are able to pop balloons and light matches, but this is as a result of an unfiltered high-power infrared component in the beam. See the section on hazards below, for the difficulties with frequency-doubled IR-pumped lasers.

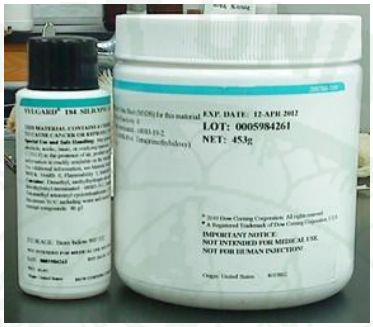


Appendix Figure C1 Diagram of violet laser pointer

Appendix D

Sylgard 184®

Sylgard 184®, a Dow Corning Corporation product, is a silicone elastomer kit. The kit contains two chemicals. Base (part A) and Curing Agent (part B), that are mixed in 10:1 mass ratio. Both the chemicals are transparent, but quite viscous in nature. Please don't use pipettes to draw the chemicals as they are pretty thick. Also, wear gloves when handling these chemicals.



Appendix Figure D1 Sylgard 184®: curing agent (left) and base (right)

The Base (part A) contains the followings:

1. Dimethyl siloxane, dimethylivinyl terminated - 68083-19-2
2. Dimethylvinylated and trimethylated silica - 68988-89-6
3. Tetra (trimethoxysiloxy) silane - 3555-47-3
4. Ethyl benzene - 100-41-4

VOC content: 8 grams/liter

The Curing Agent (part B) contains the followings:

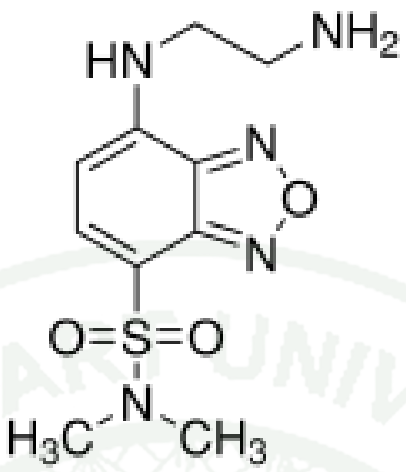
1. Dimethyl, methylhydrogen siloxane - 68037-59-2
2. Dimethyl siloxane, dimethylvinyl terminated - 68083-19-2
3. Dimethylvinylated and trimethylated silica - 68988-89-6
4. Tetramethyl tetravinyl cyclotetra siloxane - 2554-06-5
5. Ethyl benzene - 100-41-4

Maximum VOC content including water: 85 grams/liter

Source: http://drajput.com/research/sylgard184-pdms.php

Appendix E

4-(2-Aminoethylamino)-7-(N,N-dimethylsulfamoyl)benzofurazan (DBD-ED)

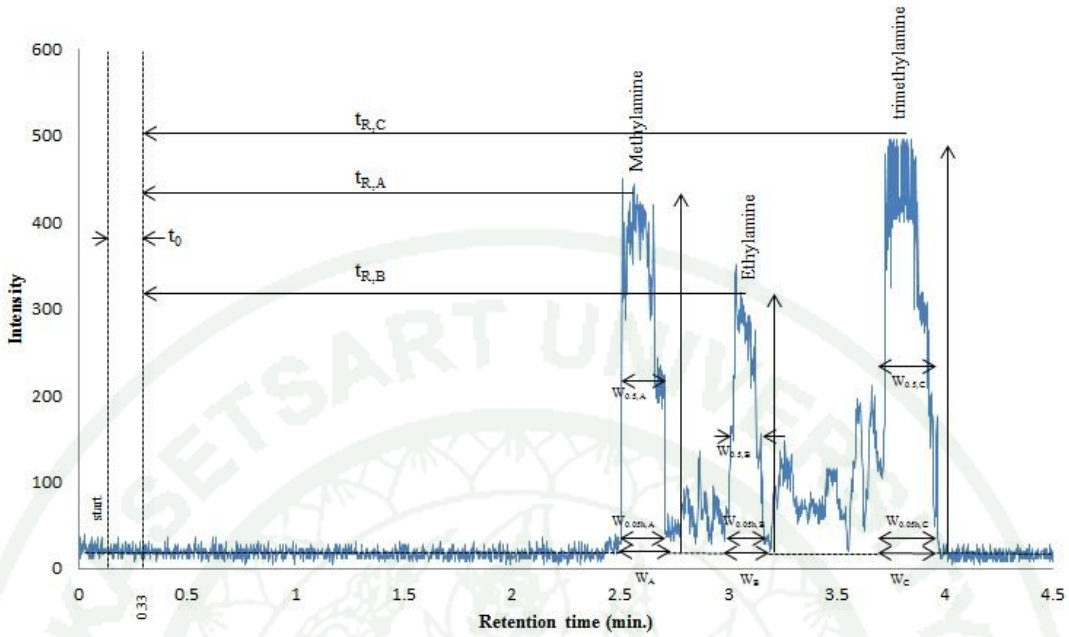


Appendix Figure E1 4-(2-Aminoethylamino)-7-(N,N-dimethylsulfamoyl) benzofurazan

4-(2-Aminoethylamino)-7-(N,N-dimethylsulfamoyl)benzofurazan (DBD-ED) is a reagent for fluorimetric detection for amine and aromatic amine. The excitation wavelength of DBD-ED was 405 nm and the emission was 518 nm.

Appendix F

Chromatographic parameter calculations



Appendix Figure F1 Separation of amine mixture on a 10 cm C_8-monolith microchannel of the microchip configuration A. Conditions: mobile phase, pure methanol; flow rate: 0.5 mL min^{-1}

$$\text{Dead time}, t_0 = \frac{\text{Injection volume}}{\text{Flow rate}}$$

$$= \frac{\text{volume in capillary} + \text{volume in microchannel}}{\text{Flow rate}}$$

$$= \frac{1.5x10^{-3} + 10^{-3}\ \text{mL}}{0.5\ \text{mL min}^{-1}} = 0.005\ \text{min}$$

$$\text{Retention factor (k')} = \frac{t_R - t_0}{t_0}$$

$$\text{Retention factor (k') of methylamine (A)} = \frac{t_{R,A} - t_0}{t_0}$$

$$k'_A = \frac{2.1784 - 0.005}{0.005} = 434.68$$

$$\text{Retention factor (k') of Ethylamine (B)} = \frac{t_{R,B} - t_0}{t_0}$$

$$k'_B = \frac{2.7015 - 0.005}{0.005} = 539.3$$

$$\text{Retention factor (k')} \text{ of Trimethylamine (C)} = \frac{t_{R,C} - t_0}{t_0}$$

$$k'_C = \frac{3.4055 - 0.005}{0.005} = 680.1$$

$$\text{Tailing factor, TF} = \frac{W_{0.05}}{2f}$$

$$TF_A = \frac{0.2748}{(2)0.0747} = 5.0067$$

$$TF_B = \frac{0.2471}{(2)0.1145} = 1.0790$$

$$TF_C = \frac{0.3193}{(2)0.0879} = 1.8162$$

$$\text{Plate number, N} = 5.54\left(\frac{t_R}{W_{0.5}}\right)^2$$

$$N_A = 5.54\left(\frac{2.1784}{0.1495}\right)^2 = 224.8986$$

$$N_B = 5.54\left(\frac{2.7015}{0.0112}\right)^2 = 600.4059$$

$$N_C = 5.54\left(\frac{3.4055}{0.2595}\right)^2 = 360.783$$

$$\text{Plate height, H} = \frac{L}{N}$$

$$H_A = \frac{10}{224.8986} = 0.0446$$

$$H_B = \frac{10}{600.4059} = 0.0167$$

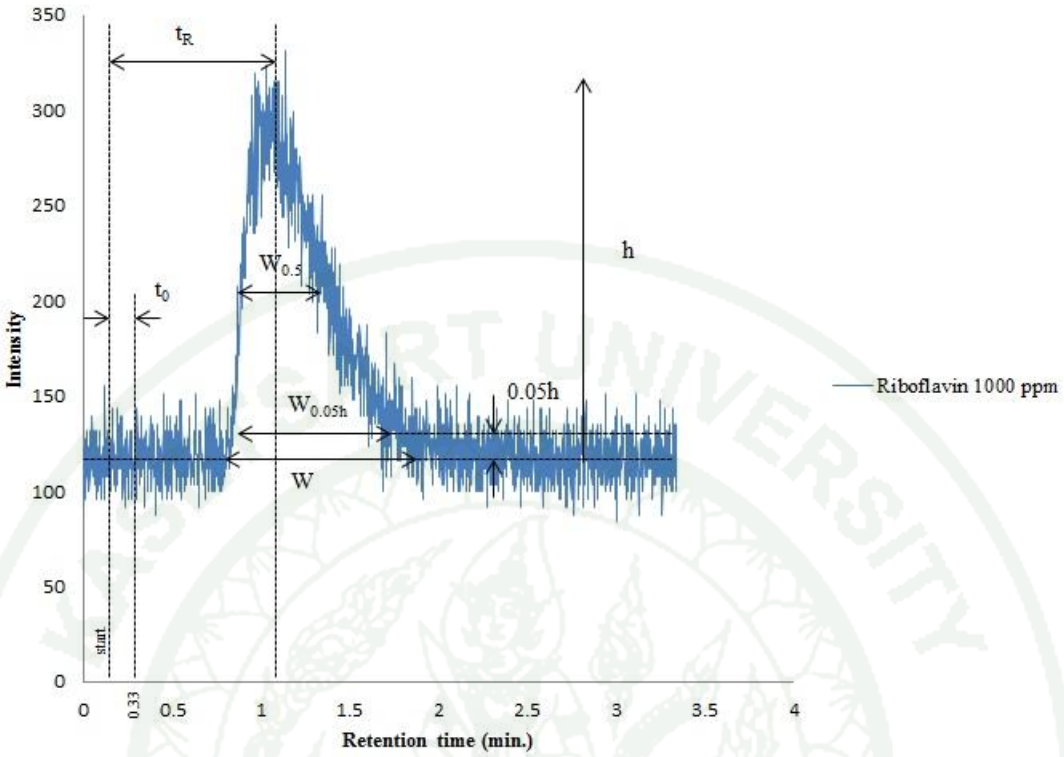
$$H_C = \frac{10}{360.783} = 0.0277$$

$$\text{Selectivity factor, } \alpha_{A-B} = \frac{k'_B}{k'_A}$$

$$\alpha_{A-B} = \frac{539.3}{434.68} = 1.2407$$

$$\text{Selectivity factor, } \alpha_{B-C} = \frac{k'_C}{k'_B}$$

$$\alpha_{B-C} = \frac{680.1}{539.3} = 1.2611$$



Appendix Figure F2 Separation of riboflavin on a 20 cm C8-monolithic microchannel of the microchip configuration B. Conditions: mobile phase, pure methanol; flow rate: 0.5 mL min^{-1}

$$\text{Dead time}, t_0 = \frac{\text{Injection volume}}{\text{Flow rate}}$$

$$= \frac{\text{volume in capillary} + \text{volume in microchannel}}{\text{Flow rate}}$$

$$= \frac{1.5x10^{-3} + 2x10^{-3}\ \text{mL}}{0.5\ \text{mL min}^{-1}} = 0.007\ \text{min}$$

$$\text{Retention factor (k')} = \frac{t_R - t_0}{t_0}$$

$$k' = \frac{0.9627 - 0.007}{0.007} = 136.5286$$

$$\text{Tailing factor, TF} = \frac{W_{0.05}}{2f}$$

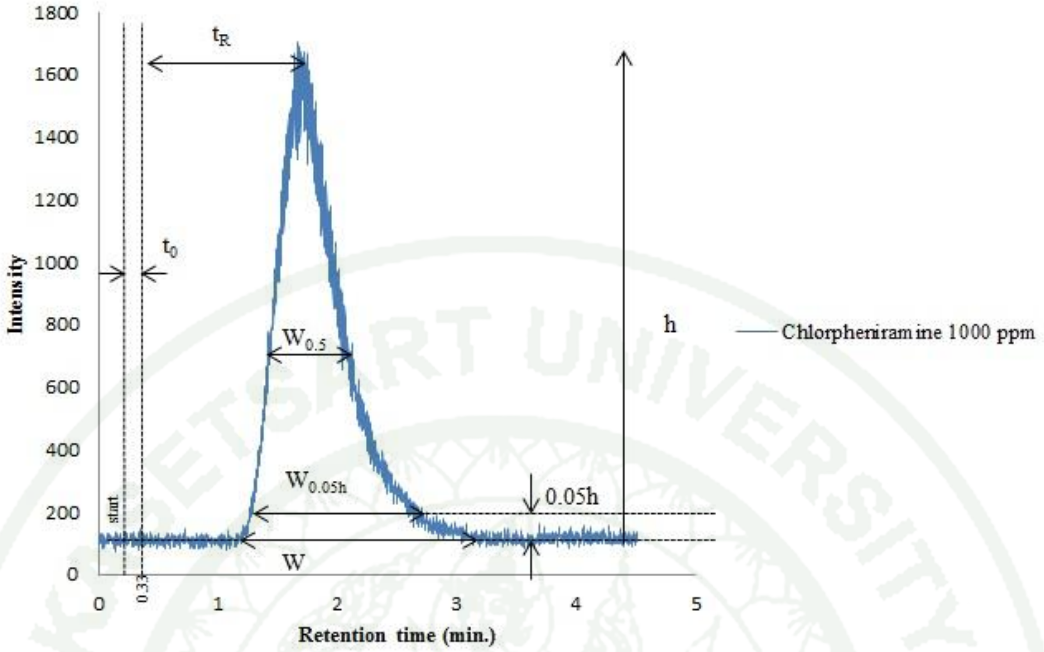
$$TF = \frac{0.9724}{(2)0.1773} = 2.7422$$

$$\text{Plate number, } N = 5.54 \left(\frac{t_R}{W_{0.5}}\right)^2$$

$$N = 5.54 \left(\frac{0.9627}{0.9558}\right)^2 = 5.6203$$

$$\text{Plate height, } H = \frac{L}{N}$$

$$H = \frac{20}{5.6203} = 3.5585$$



Appendix Figure F3 Separation of derivatised chlorpheniramine on a 20 cm C_8-monolithic microchannel of the microchip configuration B. Conditions: mobile phase, pure methanol; flow rate: 0.5 mL min^{-1}

$$\text{Dead time}, t_0 = \frac{\text{Injection volume}}{\text{Flow rate}}$$

$$= \frac{\text{volume in capillary} + \text{volume in microchannel}}{\text{Flow rate}}$$

$$= \frac{1.5x10^{-3} + 2x10^{-3}\ \text{mL}}{0.5\ \text{mL min}^{-1}} = 0.007\ \text{min}$$

$$\text{Retention factor}\ (k') = \frac{t_R - t_0}{t_0}$$

$$k' = \frac{1.6439 - 0.007}{0.007} = 1.6369$$

$$\text{Tailing factor}, TF = \frac{W_{0.05}}{2f}$$

$$TF = \frac{1.3032}{(2)0.3808} = 1.7109$$

$$\text{Plate number, N} = 5.54\left(\frac{t_R}{W_{0.5}}\right)^2$$

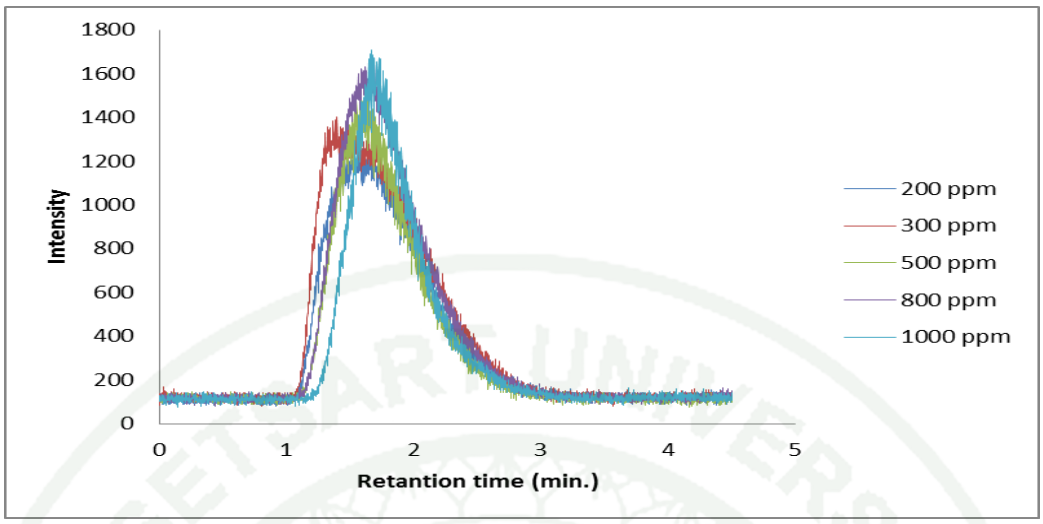
$$N = 5.54\left(\frac{1.6439}{1.79825}\right)^2 = 4.6298$$

$$\text{Plate height, H} = \frac{L}{N}$$

$$H = \frac{20}{4.6298} = 4.3198$$

Appendix G

Peak of chlorpheniramine



Appendix Figure G1 Peak of chlorpheniramine with a concentration at 200, 300, 500, 800, and 1000 ppm. The retention time was 1.4722, 1.3061, 1.5319, 1.5506, and 1.6439 min. as shown in Table 5.

The peak area of chlorpheniramine sample was 600070.1 and the value was put into y in the linear equation of y = 290.585x + 350409, then the concentration (x) was calculated as follows.

$$600070.1 = 290.585x + 350409$$

$$x = 859.181 \text{ ppm}$$

The concentration of riboflavin sample was 859.181 ppm in 1000 ml (after filtration) that was 0.8592 mg mL^{-1} and the percentage of sample in tablet can be calculated as

$$\% \text{ sample in tablet} = \frac{\text{amount of experimented chlorpheniramine}}{\text{amount of declared chlorpheniramine}} \text{ x weight of chlorpheniramine tablet}$$

$$\% \text{ sample in tablet} = \frac{0.8592}{0.05} \text{ x } 0.13$$

$$\% \text{ sample in tablet} = 2.23 \text{ mg} = 17.184 \text{ mg/g}$$

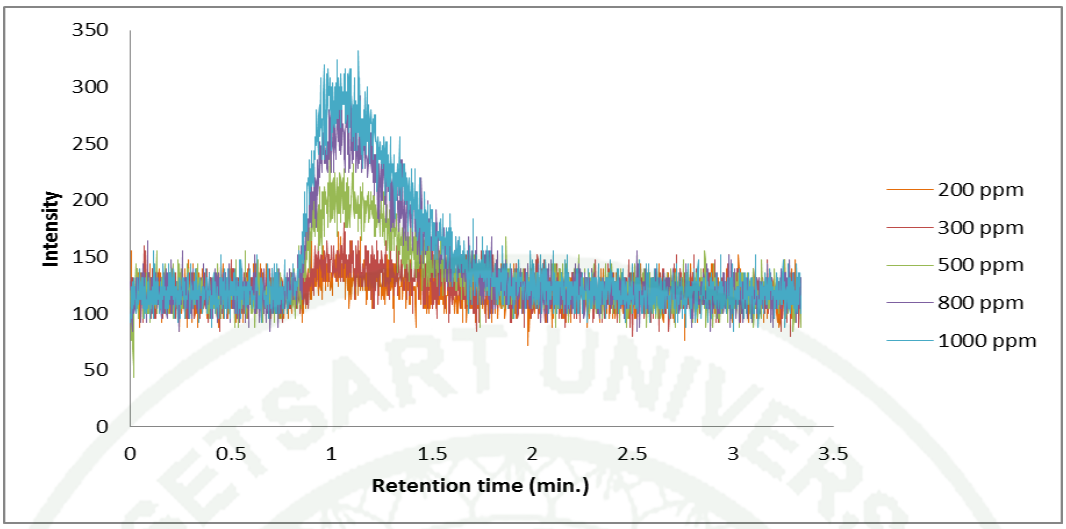
One tablet of chlorpheniramine weighed 0.135 g and was declared to have 4 mg, which was equivalent to 29.63 mg g^{-1}.

Efficiency of chlorpheniramine extraction = (17.184/29.63) x 100

= **57.99** %.

Appendix H

Peak of riboflavin



Appendix Figure H1 Peak of riboflavin with a concentration at 200, 300, 500, 800, and 1000 ppm. The retention time was 1.09987, 1.06132, 1.01645, 1.05233, and 0.962767 min. as shown in Table 3.

The peak area of riboflavin sample was 46394.5 and the value was put into the y in the linear equation of y = 57.706x – 7793.7, then the concentration (x) was calculated as follows.

$$46394.5 = 57.706x + 7793.7$$

$$x = 939.04 \text{ ppm}$$

The concentration of riboflavin sample was 939.04 ppm in 1000 mL (after filtration) that was 1.1738 mg mL^{-1} and the percentage of sample in tablet can be calculated as

$$\% \text{ sample in tablet} = \frac{\text{amount of experimented riboflavin}}{\text{amount of declared riboflavin}} \text{ x weight of riboflavin tablet}$$

$$\% \text{ sample in tablet} = \frac{1.1738}{0.05} \text{ x } 0.27$$

$$\% \text{ sample in tablet} = 6.33852 \text{ mg} = 23.476 \text{ mg/g}$$

One tablet of riboflavin weighed 0.275 g and was declared to have 50 mg, which was equivalent to 181.81 mg g^{-1}.

Efficiency of riboflavin extraction = (23.476/ 181.81) x 100

= **12.91** %

Appendix I

Peak height of riboflavin and chlorpheniramine at very low concentration for examining the standard deviation (SD)

Appendix Table I1 Peak height of riboflavin and chlorpheniramine at very low concentration determination.

Number	Riboflavin	Chlorpheniramine
1	4240.86	390007.10
2	4837.46	349455.10
3	4195.78	389913.60
4	4003.36	447239.70
5	4853.86	349546.70
6	4813.3	389947.10
7	4353.5	375022.90
8	4645.1	390003.90
9	4140.7	366830.90
10	4364.7	389968.70
11	4903.0	387845.40
12	4835.7	346905.10
13	4398.3	382775.20
14	4409.6	447239.70
15	4387.2	389971.90
16	4656.3	389952.80
17	4880.6	371742.10
18	4376.0	353279.60
19	4667.5	366782.90
20	4891.8	358330.80
Standard deviation (SD)	**287.42**	**27466.29**

PRESENTATION, PUBLICATIONS and AWARDS

PRESENTATIONS:

1. Chunhakorn, V.; Puttaraksa, N.; Singkarat. S.; Whitlow, H. J. and O. Chienthavorn. 2011. "Monolithic Materials for a Microfluidic System". **PERCH-CIC Congress VII: Chemistry, Environment and Society.** 4-7 May 2011, Jomtien Palm Beach Hotel & Resort Pattaya, Chonburi, Thailand.

2. Chunhakorn, V.; Unai, S.; Singkarat. S.; Whitlow, H. J.; Sweedler, J. H. and O. Chienthavorn. 2011. "Separation of Riboflavin in a Silica Monolithic Microfluidic System".**Separation Science Asia 2011.** 27-28 July 2011, Biopolis, Singapore.

3. Chunhakorn, V.; Unai, S.; Singkarat. S.; Whitlow, H. J.; Sweedler, J. H. and O. Chienthavorn. 2011. "Liquid Chromatography with C_8-silica Monolith in a Microchip".**The 14th Asian Chemical Congress 2011.** 5-8 September 2011, Bangkok, Thailand.

PUBLICATIONS:

1. Chunhakorn, V.; Unai, S.; Singkarat. S.; Whitlow, H. J.; Sweedler, J. H. and O. Chienthavorn. 2011. "Liquid Chromatography with C_8-silica Monolith in a Microchip".**The 14th Asian Chemical Congress 2011.** 5-8 September 2011, Bangkok, Thailand.

AWARDS:

1. **Outstanding Poster Presentation**, "Monolithic Materials for a Microfluidic System". **PERCH-CIC Congress VII: Chemistry, Environment and Society.** 4-7 May 2011, Jomtien Palm Beach Hotel & Resort Pattaya, Chonburi, Thailand.

2. **The Bangkok Bank Young Chemist Awards**, "Liquid Chromatography with C_8-silica Monolith in a Microchip".**The 14th Asian Chemical Congress 2011.** 5-8 September 2011, Bangkok, Thailand.

3. **Graduate School Honor Awards for Students**, "Monolithic Materials for a Microfluidic System". **PERCH-CIC Congress VII: Chemistry, Environment and Society.** 4-7 May 2011, Jomtien Palm Beach Hotel & Resort Pattaya, Chonburi, Thailand.

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