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THESIS

MONOLITHIC SOLID-PHASE EXTRACTION COUPLED WITH HPLC-TOF-MS DETERMINATION OF TETRACYCLINE FROM WASTE WATER

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A Thesis Submitted in Partial Fulfillment of

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A C18-silica monolith was fabricated via sol-gel method, and used as an adsorbent in extraction and preconcentration step for determination of tetracycline and its related compounds, namely tetracycline, oxytetracycline and chlortetracycline in waste water. The C18-silica monolithic adsorbent was synthesised via a sol-gel method and developed to solve shrinkage problem in a 5 mL-syringe. The synthesized C18 monolith was compared with commercial C18-packed solid adsorbent in term of extraction performance. Recoveries of tetracycline, oxytetracycline and chlortetracycline spiked in water were 76.46, 72.55 and 81.69% for the C18-silica monolithic SPE, and 92.70, 83.18 and 77.62 for C18-packed silica SPE. Analysis method provided the limit of detection for tetracycline and related compounds in a range of 0.17-0.55 mg/L and linear range between 10-60 ppm with correlation coefficient between 0.98-0.99. For the real waste water, low recoveries of the spiked analytes were obtained when using C18-monolithic SPE, while those for commercial SPE were not obtained because the polar tetracyclines did not well adsorb on the surface of C18 solid phase. For a complicated water matrix with high concentration of analytes, TOF-MS detection was proved to be more beneficial than UV detection because of very low background signal of the chromatogram.

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Student's signature Thesis Advisor's signature

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Khomsan Khaunmeung

June 2014

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LIST OF ABBREVIATIONS

C18-TEOS	=	Trimethoxy(octadecyl)silane
CTC	=	Chlortetracycline
ESI	=	Electrospray ionization
HPLC	=	High-performance liquid chromatography
HPLC-UV	=	Liquid chromatography with ultraviolet detector
HPLC-TOF-MS	=	Liquid chromatography couple with time-of-flight -mass spectrometry
LOD	=	Limit of detection
OTC	=	Oxytetracycline
ppm	=	parts per million
SIM	=	Selective ion monitoring
SPE	=	Solid-phase extraction
SEM	=	Scanning electron microscopy
TC	=	Tetracycline
TMOS	=	Tetramethoxyothosilicate

MONOLITHIC SOLID-PHASE EXTRACTION COUPLED WITH HPLC-TOF-MS FOR DETERMINATION OF TETRACYCLINE FROM WASTE WATER

INTRODUCTION

Tetracycline is an antibiotic drug group. The first drug in this group was chlortetracycline which was developed by Benjamin Minge Duggar in 1940. Chlortetracycline was derived from soil-bacteria called *Streptomyces areofaciens*. Later, oxytetracycline was discovered by AC Finlay *et al.* via the development of similar soil-bacteria, *Steptomyces rimosus*. Robert Burns Woodward determined the molecular structure of tetracycline and its synthesis was developed successfully by Lloyd. H. Conover and his team. Three drugs of tetracycline have been used as prototypes for development and following researches ever since.

Tetracycline is a pale yellow, odorless and solid soluble. It reacts rapidly to some dissolved metal ions and instable in water. It is one of an important drugs for human health and livestock that has been used a large amount in many farms each year, as part of animal feed and water. Tetracycline is one of the most popular antibiotic drugs because of its broad spectrum antibiotic, low toxicity for human and short withdraw time. The livestock excretes drugs from the body in urine and feces rapidly, which is eventually discarded to environment. Sources of drugs in the aquatic environment originate from effluents of livestock and household. An inappropriate disposal of antibiotic drugs can cause environmental pollution, as the excretion of antibiotic drugs can mix up with natural waters, either through leaching of livestock and household waste by rainwater or upon direct input into natural water. Adverse effects of antibiotic drugs on aquatic life including human are therefore of concerned. Although tetracyclines and their metabolites have short half-lives in nature, they give an acute effect to micro-organism, and destruction of the microbes severely damage the food chain in the impact area. Low concentration of tetracycline in environment may induce a development of resistance in microbial assemblages as well as pathogenic

species. Since the long-term effect has not been investigated, the monitoring of their activities in environment is then essential.

In the early 1950s, tetracyclines and their metabolites have been studied on the acute effect in several laboratories. Many researchers have reported the LD_{50}(oral) value 1500 - 6443 mg/kg of body weight (mg/kg bw) which are relatively high values [Appendix C]. These values indicate low toxicity. In the experiment, the tetracycline was mixed with the animal food and feeding to rat. The mortality rate of rats was found decreasing and not significant related to procreation of tumors. These drugs have been widely used in livestock as a growth promoter.

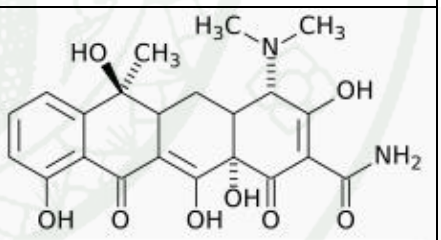
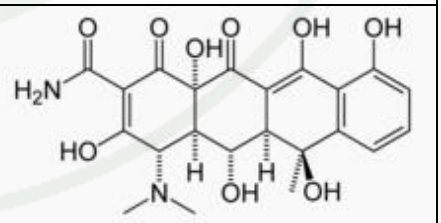
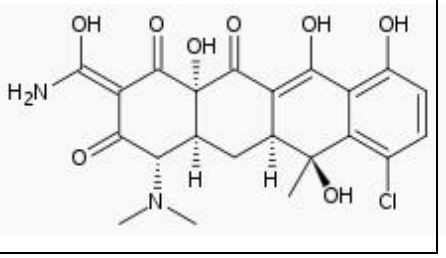
The investigations of tetracyclines and their metabolites in the environment were not popularly implemented, because of its low toxicity and rapid decomposition. Drinking water treatability database of US-EPA (United States Environmental Protection Agency) presents only a contamination index for observation. The only one of tetracycline group indicated in this index is chlortetracycline. Environmentalists put effort in determining tetracyclines and their metabolites in soil, sediment and waste water in many places in USA. However, they detected chlortetracycline only 1 sample out of 84 samples (D.W. Kolpin *et al*, 2002). The detected samples were top soil (0 to 30 cm below ground surface) with values between 4.6-7.3 ug/kg, and the tested method used high performance liquid chromatography coupled with electrospray mass spectrometry (HPLC/MS-ESI) for determination. No found analytes was in the deeper layers at the same place.

Environmentalist was attempted to analyse tetracyclines and their metabolites in the environment. The developed analysis method for extraction and analysis are divided into three major studies. Research studies were focused on contamination in soil, water and residues in the tissues of living organisms. The determination of contaminants in soil was performed by an extraction method using organic solvent and then pre-concentrationed by using solid phase extraction (SPE), which was the same protocol for determining contaminants in water. Subsequently the extract of tetracycline was analysed by using HPLC, high performance capillary electrophoresis (HPCE) or

HPLC/MS. Studies of the residues in the organisms may involve radiologic techniques using isotropic labelling, for example the study of the drug elimination from human body, C. Zhenxing and L. Rutao (2011). It was found that within 48 hours the tetracycline and its metabolites were eliminated in the urine about 70% and feces about 5-20%. The rapid excretion implied a possibility of environmental impact by the antibiotics.

It was found that bacteria in the area with consistent use of tetracycline would resist to the antibiotics and that substantial bacteria species remained in the nearby livestock waste lagoons and water sources, Z. Yingjie *et al* (2014). Therefore, the monitoring of tetracycline at low concentration in water is recommended and investigating the result in the future.

Table 1 Chemical structure of tetracycline group study

Tetracycline compound	Abbreviation	Chemical structure
Tetracycline	TC	
Oxytetracycline	OTC	
Chlortetracycline	CTC	

Monolithic material was introduced in chromatography for two decades as a stationary phase. Monolithic stationary phase have grown in interest because of several advantages such as enhanced surface area, low back pressure, and high loading capacity, superior to regular packed columns. Monolithic material can be divided into different groups on the basis of specific chemical base. There are organic based, inorganic-based and mixed mode based. The mixed mode based monolith is a combination between organic and inorganic cross-linked polymer. For example, pure silica monolithic surface exhibits inorganic functionality, however, when organic silica monomer is used in the polymeric reaction, it forms organic silica monolith, which is a mixed mode base material. In this study, C18-organic silica monolith base is of interest to be fabricated and used as an adsorbent, according to its extraction compatibility to tetracycline chemical property. The C18-silica monolith was fabricated from two alkoxysilanes, namely tetramethoxysilane (TMOS) and trimethoxy(octadecyl)silane (C18-TMOS), as precursors, reacting via hydrolysis and condensation to become C18-organic silica monolith. The fabricated formulation of C18-silica monolith was adapted from the work of Chen *et al*. (2010)

Monolithic materials can be synthesised in various containers, such as capillary, microplate and pipette tip, where the particles range from micro- to nano-size, thus when compared with an equivalent amount of conventional solid phase materials with large particle size, the monolith provides higher surface area. Its special properties offer many advantages more than a classical solid phase, such as improved performance, reduced size of material and high contained substance and etc. For example, the silica monolith can be fabricated in a cartridge form of 2-mL SPE syringe and it is connected to a SPE vacuum manifold. The silica monolith SPE was used to extract epinephrine, normetapineprine and metaephrine from urine samples (Nema *et al*., 2010)

The organic silica monolith is therefore expected for excellent adsorption of trace tetracycline and its related compounds in waste water. The organic silica monolith will be synthesised by a sol-gel method with an optimized formulation for very small size particles.

BACKGROUND

Mass spectrometry is one of analytical technique to determine ion or molecular mass of compound of interest. Three main components of a mass spectrometer are ion source, mass analyser and detector. The ion source is the first part used to ionise sample to be ion molecule. The mass analyser is a part to separate analyte by mass and charge. Finally, a detector is used to measure and display the result be mass per charge of analyte. When a mass spectrometer is utilized as a HPLC detector, one more important part named an interfacing system must be involved for the coupling between the HPLC and MS. In this study, electrospray ionization was used as both the interface and ion source of the mass spectrometer, while the time-of-flight (TOF) was used as a mass analyser.

Electrospray ionization (ESI)

The electrospray ionization is an interphase between HPLC and TOF-MS to produce ions. To describe the detail of mechanism of the ionization, the configuration of ESI is illustrated in Figure 1.

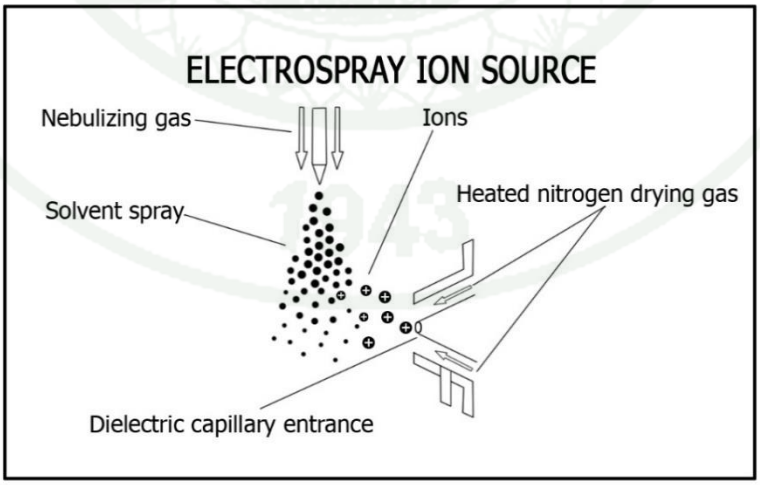


Figure 1 Configuration of electrospray ionization interface. (adapted from Particle Sciences: Drug development services, *Mass Spectrometry in Bioanalysis*, Technical brief 2009, Volume 4)

The mobile phase and analyte are passed from the capillary emitter where the nebulizing gas is flowed along. The capillary emitter provides high voltage electricity, thus when the eluent passing out of the emitter was nebulized, the droplets have surface charged. The nebulizing gas was used to evaporate solvent to reduce droplet size. The solvent continuously evaporates from a charged droplet until it becomes unstable until reaching its Rayleigh limit. At this point, on the droplet surface the electrostatic repulsion is equal to the surface tension. When the solvent evaporation continues, the repulsion becomes more powerful than the surface tension, causing an explosion of the droplet to be smaller droplets. The process continues until the droplets become molecular ions or quasi-molecular ions.

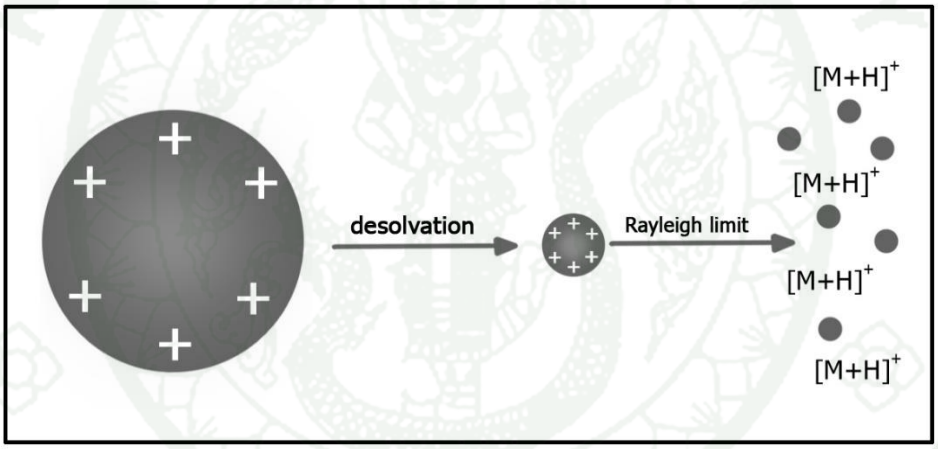


Figure 2 Step of ESI ionisation

These ions move passing through the sample cone into the mass analyzer. The sample cone was protected by heated nitrogen cone gas flowing close to the probe

Time-of-flight (TOF) mass analyser

Time-of-flight is now widely used for ESI interface in HPLC-MS application. It is an analyser to determine mass of molecules or ions by an individual time to arrive at detector. To describe the detail of TOF, the schematic diagram of TOF is given in Figure 3. The molecular ions were accelerated by an electric field with the same energy

to the flight tube. The flight tube is usually a vacuum enclosure, free of electrical field, between the ion source and the detector. Adequate pumping is required to prevent the ions from colliding with molecules on the way to the detector. The time that takes the particle to reach a detector at a known distance is measured. This time depends on the mass-to-charge ratio of the particle. A heavy molecule reaches the detector a low speed. As it is difficult to construct a long flight tube, another way to extend the focal length of the instrument is the use of a reflector. The long flight time improves the mass separation, but decreasing the signal.

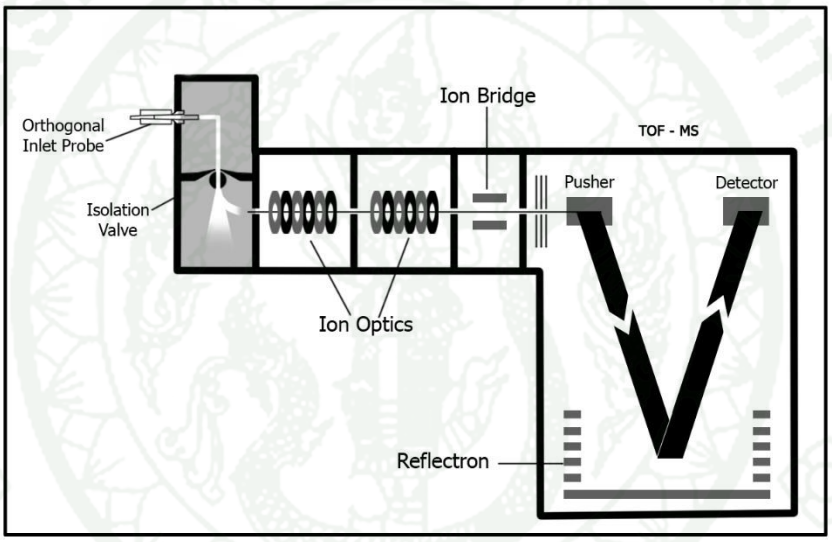


Figure 3 Schematic diagram of TOF-MS (Adapted from *Waters Micromass LCT Premier Mass Spectrometer: Operator's Guide*)

References

Particle Sciences: Drug development services, *Mass Spectrometry in Bioanalysis*, Technical brief 2009, Volume 4. Retrieved from http://www.particlesciences.com/news/technical-briefs/2009/mass-spectrometry-bioanalysis.html (access date : 26/6/2014)

Waters, *Waters Micromass LCT Premier Mass Spectrometer: Operator's Guide*, Chapter 1, Revision B, Milford, MA, USA, 2004, p.4.

OBJECTIVES

1. To develop a synthesis method of organic silica monolith SPE and characterize its morphology.

2. To study variable factors affecting the structure of monolith

3. To optimise all parameters involving the extraction and determination.

4. To preconcentrate tetracycline and its related compounds from waste water by using the monolith SPE.

5. To evaluate efficiency of the organic silica monolith SPE and compared with commercial SPE

LITERATURE REVIEW

Monolith solid phase extraction

Monolithic material was emerged in the late 1980s. It was introduced in chromatography for two decades as stationary phase. Monolithic stationary phases have grown in interest because of several advantages such as, enhanced surface area, low back pressure, high loading capacity superior to regular packed columns, and time-saving process.

Monolithics can be divided into two groups on the basis of the specific chemical base: polymer-based and silica-based materials. Monolith can be utilised as solid phase for extraction of organic species and others form several matrices. Some reviewed articles about polymer-based monolith for extraction are given as follows.

Podgornik *et al.* (2000) developed a large –volume of monolithic column with glycidyl methacrylate-based monolith. The monolith was polymerised from glycidryl methacrylate (GMA) and ethylene methacrylate (EDMA). It was suitable for ion exchange, hydrophobic interaction, reverse phase, or affinity separations. The GMA-EDMA monolith was fabricated with three sizes cylinder shape. Experiments has been analysed a heat release during the polymerisation and has been derived a mathematical model for the prediction of the maximal thickness of the monolithic annulus having a uniform structure. They were consisted three cylinder monolithic to a single unit. It was became a large one piece. There was 1 mm gap between transversal annular. The synthsised monolith was used for separation and purification of proteins and other large molecules at elevated flow rates, but still moderate pressure drops.

Tan *et al.* (2003) prepared chip-based solid-phase extraction pretreatment for direct electrospray mass spectrometry. The chip contained eight parallel channels (10 mm long, 360 mm i.d.), and the monolith in the chip was fabricated from zeonor polymer with UV-initiated polymerisation by hot embossing and thermal bonding. The monolith in the chip used for extraction was evaluated by using electrospray ionization-

mass spectrometry (ESI-MS), resulting sample capacity, recovery, reproducibility of peak area or peak height ratios, and linearity. The average sample capacity was estimated to be 0.30 µg with a relative standard deviation (RSD) of 26.5% for the eight monolithic columns on the same polymeric chip. For two chips prepared using the same monomer mixture, the difference in average sample capacity was 7.0%. The average recovery for the eight monolithic SPE columns on the same chip was 79.1% with a RSD of 7.9%. Two different types of real-world samples including human urine sample and P450 drug metabolism incubation mixture were tested.

Eeltink *et al.* (2005) prepared methacrylate ester-based monolith in fused-silica capillaries and study the morphology. It was prepared for capillary electrochromatogaphy (CEC) and HPLC analysis to test column performance. Monoliths were prepared from polymerisation mixtures consisting of butyl methacrylate (BMA), ethylene dimethacrylate (EDMA), [2-(methacryloyloxy)ethyl]-trimethylammonium chloride (META), and a pore-forming solvent composed of 10 wt % and 90 wt % of 1,4-butanediol and 1-propanol combined in various ratios. Azobisisobutyronitrile was added as an initiator. Prior to the polymerisation, surface modification of the inner wall of fused-silica capillary was performed with 3-trimethoxysilyl propyl methacrylate to enable covalent attachment of the monolith to the wall. After mixing, the polymerisation mixture was sonicated for 5 min to obtain a clear solution and then purged with helium for 10 min. The deaerated mixture was introduced into 40-cm-long pieces of fused-silica capillaries with a syringe. The morphology was verified by using a scanning electron microscope (SEM) and the monolithic performance was determined from a van Deemter equation in CEC and HPLC mode.

Drisko et al. (2011) prepared strong silica monoliths with large mesopore by using agarose gel template. The synthesis reagents consisted of agarose gels with tetraethyl orthosilicate and tetrabutyl ammonium fluoride (TBAF) catalyst. The silica monoliths were fabricated with agarose pellets insertion into the monolithic structure. After calcination, agarose pellets was removed, leaving formation of mesopores. Therefore, the mesopore size could be modified by altering the weight percent of used

agarose gel. To control the structure of the mesoporous silica monoliths, 3, 5, 7, and 8 wt % agarose gel template pellets were used. The maximum attainable agarose density was 8 wt %, while still forming a homogeneous gel. The agarose gel was made from agarose powder dispersed in water, stirred and heated to 80 ^{o}C to dissolve the powder. The clear solution was poured into test tube molds while it was hot, and allowed to set overnight. After that the gels were cut into pieces, roughly 0.25 cm^3. Over a period of 36 h, the solvent within the gels was washed ethanol. Fifty gel pellets were soaked overnight in 100 mL of solution composed of TEOS (50 mL) and 1 M TBAF in tetrahydrofuran (50 mL). The infused agarose gel pellets were then transferred to distilled water to initiate hydrolysis and condensation reactions. The silica visibly began to gel within 1 min without perceptible TEOS leakage from the agarose template. After 6 h, the pellets were removed from solution and dried, followed by calcination in air at 600 ^{o}C for 5 h.

Wang *et al*. (2012) developed SiO_2/TiO_2 composite monolithic capillary column by sol-gel method. The fabricated capillary was used to enrich phosphopeptides from the digestion mixture of phosphoproteins and bovine serum albumin (BSA), as well as human blood serum, nonfat milk, and egg white. The monolithic column was synthesised from tetramethoxysilane (TMOS) and tetrabutoxytitanium (TBOT) as a monolith base. The prepared monolith was characterised by energy dispersive X-ray spectroscopy (EDX) and X-ray diffraction (XRD). The results revealed an approximate 1/2 molar ratio of titanium to silica. The scanning electron microscopy (SEM) results demonstrated an excellent anchorage between the column and the inner capillary wall.

Yang *et al.* (2012) prepared polymeric ion exchange monolith. The functional monomer containing anion-exchange functionality, trimethyl-2-methacroyloxyethyl ammonium chloride (MATE), was utilised. The anion exchange monolithic material was prepared by direct co-polymerisation of MATE and ethylene glycol dimethacrylate (EGDMA) in a fused silica capillary. A metallic needle of the syringe was replaced by a 5 cm long prepared monolithic capillary, and the capillary was used to preconcentrate three substances, namely 2,4,6-tribromophenol (TBP), tetrabromobisphenol A (TBBPA) and 4,4_-dibrominated diphenyl ether (DBDPE) in aqueous samples. The

analysis was evaluated by HPLC. The limit of detection were 0.2, 0.15 and 0.1 ng/mL for TBP, TBBPA and DBDPE respectively. The recovery of method was 78.7-106.1% and standard deviation was 1.3-4.4%.

Zhang *et al*. (2013) developed a novel polymer-based monolith with molecular imprinting technique for selective micro solid-phase extraction of berberine in aqueous solution. Berberine is an alkaloid found in medicinal herbs such as Coptis chinensis, Phellodendron amurense, etc. The monolith support was fabricated inside a polypropylene micropipette tip by using dimethylsulfoxide as a porogen, acrylamide (AA) as a functional monomer and ethyleneglyol dimethacrylate (EGDMA) as a cross-linker for polymerisation. The analysis was evaluated by HPLC with ultraviolet (UV) detection. The results showed that the micropipette tip based (MI-m-SPE) method exhibited high clean-up efficiency, low organic solvent consumption, and good extraction efficiency.

In this study, organic silica monolithic base is of interest, according to its extraction compatability to tetracycline. The silica monolith base was synthesised to be a sorbent in SPE form. The silica monolith SPE was used to trap low-polar analyte from a sample. A number of reviewed articles about employing silica and organic silica monolith for extraction are given as follows.

Oguri *et al*. (2003) developed on-line preconcentration technique with capillary electrochromatography (CEC) to determine biogenic amines. C-18 monolithic material was fabricated in a capillary tube by thermal sol-gel reaction. The standard amine solution consisting of histamine, methylhistamine, and serotonin were preconcentrated at the inlet site of capillary column. After that, the amines were subsequently derivatised, separated, and detected by using CEC with an optimum CEC running buffer solution containing 60% acetonitrile in 5 mM *o*-phthalaldehyde/2-mercaptoethanol-10 mM borate buffer (pH 10) with a continuously applied voltage of 5 kV. The system was equipped with a fluorescence detector instead of a UV/visible detector, giving the detection sensitivity for amines up to 0.1 µM level. The sensitivity

was increased by a factor of 103 times, greater than that of CEC with normal on-column derivatisation.

Wu *et al.* (2006) developed silica monolith in glass microchip. The monolith was fabricated by using tetramethylorthosilicate via sol-gel method for micro-solid-phase extraction (μSPE). The SPE microchips were made by using standard photolithographic techniques and etched a channel of 2 cm long, 60 μm deep, and 400 μm wide at the center. The sol-gel method was prepared as follows: 2 mL TMOS was added to a solution of 0.44 g PEG (MW 10 000) in 10 mL 0.01 M acetic acid, and the mixture was kept at 0 °C for 45 min. The hydrolysed sol solution was filled into a chip sealed with a polydimethylsiloxane (PDMS) layer and placed in an oven at 40 °C for 10 h to form monolith. The monolith in the chip was used to isolate DNA from clinical samples. The monolithic microchip provided large surface area for DNA extraction with low flow back pressure. DNA extraction efficiencies for simple systems were 85%, while efficiencies for reproducible extraction of human genomic DNA from complex biological matrixes (human blood) were 70%.

Zhang *et al*. (2009) prepared a monolith silica with hydrophobic and strong cation-exchange functional groups. It was used as sorbent for micro-solid phase extraction (micro-SPE). The hybrid silica monolith functionalised with octyl and thiol groups was conveniently synthesised by hydrolysis and polycondensation of a mixture of tetraethoxysilane (TEOS), n-octyltriethoxysilane (C8-TEOS) and 3-mercaptopropyltrimethoxysilane (MPTMS) via a two-steps catalytic sol–gel process. It was then flushed with 30% w/w hydrogen peroxide for 12 h at room temperature and then washed with water to remove the excess hydrogen peroxide. The application of the hybrid monolith was demonstrated extraction performance by micro-SPE of sulfonamide residues from milk by HPLC–UV analysis.

Deng *et al.* (2012) developed aptamer modified organic-inorganic hybrid silica monolithic capillary column. It was specific monolith for biocompatibility. The hybrid silica monolith was prepared by sol−gel method with tetraethoxysilane and 3-aminopropyltriethoxysilane. The 5′-NH_2-modified aptamer for human α-thrombin was

covalently bonded on the surface of monolith. The hybrid silica monolithic capillary column was prepared as follows. The capillary (250 μm i.d.) activated by hydrochloric acid was filled with polymerisation solution consisted of 112 μL tetraethoxysilane (TEOS), 118 μL 3-aminopropyltriethoxysilane (APTES), 215 μL anhydrous ethanol, 8.0 mg hexadecyltrimethyl ammonium bromide (CTAB), and 32 μL water mix for 1 min and at temperature no higher than 25 °C to slow down the polymerisation reaction. Both ends were sealed by silicon. The capillary was put in water bath at 40 °C for 24 h, followed by rinsing with ethanol and water, respectively. TEOS and APTES (molar ratio as 1:1) as precursors, and CTAB as template in the formulation. The hybrid monolithic columns were used for continuous adsorption/desorption mode in HPLC for α-thrombin preconcentration. The results were showed advantages of good stability and high recovery for target protein.

Many articles were reported silica monolith for SPE coupled with HPLC-MS, especially chemical contaminants in blood and water resources. The preparation methods included several aspects in this study, such as load volume, sample preparation, mobile ratio and electrospray ionisation (ESI) setting conditions. Some reviewed articles were given as follows.

Jikun *et al.* (2009) reported a robust approach for fabricating cyclic olefin polymer (COP) containing in situ photopolymerised polymethacrylate monolithic stationary phases. The polymer monolithics were prepared in microfluidic chips for high-performance liquid chromatography (HPLC) separations of peptides. It was 15-cm long separation column containing a reversed-phase polymethacrylate monolith as a stationary phase, with its front end seamlessly coupled to a 5 mm long methacrylate monolith which functions as a solid-phase extraction (SPE) element for sample cleanup and enrichment. To demonstate sample clean up and enrichment, a simple protein mixture containing 0.25 μM fluorescein isothiocynate (FITC) labeled ribonuclease A and 0.5 M cytochrome C was loaded to methacrylated monolith trap column at a flow rate of 4 μL/min for 1.5 min. The HPLC condition was employed at 4 min linear acetonitrile gradient from 20% to 85% and a flow rate of 700 μL/min. The FITC-labeled ribonuclease A peak was 2 times higher while cytochrome C was 15 times high in the

HPLC separation using the trap column. The resolution of peaks was improved from 0.6 to 1.2 while peak width was decreased 40% and 50% for ribonuclease A and cytochrome C respectively.

Nema *et al*. (2010) reported the preparation method of silica-based monolith as solid phase extraction cartridge for extracting polar compounds from urine. The prepared silica monolith cartridge was housed into a 2-mL syringe and installed on a SPE vacuum manifold. The silica monolithic cartridge was demonstrated by extracting epinephrine, normetanephrine and metanephrine from urine samples. The extracted analytes, after concentration and reconstitution were then quantitatively determined by high-performance liquid chromatography coupled to electrospray ionization-mass spectrometer (HPLC/ESI/MS). The limit of detection was 3 ng/mL for metanephrine and 5 ng/mL for normetanephrine and epinephrine. The relative recoveries ranged from 60 to 67%, 55 to 59% and 99 to 105% for epinephrine, normetanephrine and metanephrine, respectively.

Analysis of tetracycline with HPLC-MS

Tetracycline was intensively used as antibiotics in livestock production and veterinary medicine in many countries. The tetracycline administered to livestock are excreted, unmetabolised, and gave a cute effect to damage microbes. Because of its high water solubility, the mobility of tetracyclines in water resources is very widespread. Therefore, continual and rapid monitoring is very important. Analysis of tetracycline have been developed continuously. An initial analysis was developed from micro assay test. Recently, the analysis method has been improved by using liquid chromatography, and HPLC has become a standard method to assay tetracycline and its related compounds in clinical and pharmaceutical industries. Some reviewed articles are given as follows.

Moats (2000) developed a determination of tetracycline antibiotics in tissues which improved stability of these compounds in sample extracts. About 15 g of tissue sample was cut and weighed accurately into a 300-mL blender jar. Deionised water (3

mL/g) was added and the mixture was blended for 2 min or until no visible pieces of tissue remained. For spiked samples for recovery experiments, an appropriate volume of standard solution was added directly to the tissue in the homogeniser before adding water and blending. A 4-mL aliquot of homogenate was mixed with 16 mL of acetonitrile, and then with 1.0 mL of 0.1 M H_3-PO_4. The liquid mixture was filtered and collected for chromatographic analysis. The HPLC mobile phase was a mixture of 4 mM oxalic acid, 4 mM sodium oxalate, and 4 mM sodium decane sulfonate in water-acetonitrile of which ratios between aqueous and organic solvent were 70:30 for oxytetracycline and tetracycline, and 66:34 for chlortetracycline. The separation was carried out by using a C18 column with UV detector at 370 nm. Recoveries were ranged between 90-100% with limits of quantitation of 0.05-0.1 ppm. The procedure was evaluated for beef and pork muscle, liver and kidney.

Fabio *et al*. (2012) reported a method for nonaqueous extraction and high-performance liquid chromatography for detection of tetracyclines and macrolides. The preparation process for SPE was described as follows. A 5 gram of sieved feed samples was doubly extracted with methanolic solution containing citric acid and nitric acid. After homogenisation and centrifugation, the extract was loaded onto HLB columns that were preconditioned with methanol, diluted HCl, and water. Thereafter, the SPE columns were washed with 20 mM oxalic acid buffer (pH 4.0) and water. The analytes were eluted with methanol, and applied to HPLC with a mobile phase containing acetonitrile (A) and 20 mM trifluoroacetic acid in water (B) at a flow rate of 0.7 mL min^{-1}. The gradient elution program was operated as follows: 0−10 min: 15% A, 85% B; 10−15 min: 60% A, 40% B. The UV detector was set at 355 and 280 nm, respectively. The method was detecting from 0.2 to 24.0 μg kg^{-1} of tetracycline, oxytetracycline, chlortetracycline. The limit of detection were 0.2, 0.5, 0.2 and limit of quantitation were 0.6, 1.5, 0.6 for OTC, TC and CTC respectively. The average recoveries were 62.3, 74.4 and 94.5 for OTC, TC and CTC respectively. A polar analyte could be lost during the SPE process.

The monitoring of organic chemical compounds in environment is rather difficult because of complex matrix and low concentration of analytes in nature. Mass spectroscope is a high resolution and high performance detector to determine analytes

in complicate matrix. Some review articles of liquid chromatography coupled with mass spectrometer in tetracycline determination are given as follows.

Alfredsson *et al*. (2005) demonstrated a dipstick for screening test of tetracycline, oxytetracycline and chlortetracycline and it was confirmed by LC-MS/MS. The dipstick was a competitive receptor-based assay that could be applied for analysis of tetracyclines contamination in eggs and honey. The mass spectrometer was operated in a positive electrospray ionisation (ESI) mode. Desolvation temperature was 400 ◦C and the block temperature was 135 ◦C, while the cone voltage and hexapole 1 were set at 30 and 10 V, respectively. Desolvation gas flow was 680 L/h and cone gas flow was 205 L/h. The chromatography was performed on a 50 mm× 2.1 mm C18 column with 4 µm particle size. A gradient eluent containing 100% methanol (A) and 0.2% formic acid containing 0.1 mM oxalic acid (B) was applied, starting at 10% A and ending at 75% A after 2.6 min. The runtime was 7 min and flow rate was 0.3 mL/min. A summary of the results of the screening showed no false screening tests in the analysis of OTC in honey. From the diluted samples of the screening samples for LC-MS/MS, it was found the recoveries of 67.5%, 60.4% and 71.4% for OTC, CTC and TC respectively.

Wendy *et al*. (2005) reported a determination of tetracycline residues in shrimp and whole milk using liquid chromatography with ultraviolet detection. The residue confirmation was also performed by using mass spectrometry. The chromatographic conditions was optimised on a 150 mm polar end-capped C8 column for the milk analysis and on a 4.6 x 250 mm C8 column with 5 µm particle for the shrimp analysis with an isocratic mobile phase consisting of 75% organic acid (0.01 M oxalic acid for milk samples and 0.1% formic acid for shrimp samples), 18% acetonitrile, and 7% methanol and the flow rate was 1.0 mL/min. For the LC-MS/MS conditions, the source voltage was 5 kV, and the capillary temperature was 270 ◦C. The methods were validated over the concentration range of 50–400 ng/g, with overall average recoveries from 77 to 93% and RSD values of less than 10%

Ye *et al*. (2007) reported trace analysis of trimethoprim and sulfonamide, macrolide, quinolone, and tetracycline antibiotics in chlorinated drinking water using

liquid chromatography electrospray tandem mass spectrometry. A quadrupole mass spectrometer was coupled with a dual off-axis ESI interface. All analytes were ionised in a positive ion mode. The electrospray settings were optimised by infusing a 1 mg/L solution of all analytes in (1:1) water-acetonitrile into the ESI source at a flow rate of 10 µL/min. The LC mobile phases was 0.1% formic acid in (50%:50%) water/acetonitrile at 0.2 mL/min. The nebuliser needle voltage and shield voltage were set at 5000 and 600 V, respectively. The optimal ion-transfer voltage was 60 V. Nitrogen was used as drying gas at a flow rate of 4 L/min at 300 °C and was also used as the nebuliser gas at a flow rate of 1 L/min. The ESI chamber temperature was 50 °C. MS/MS breakdown experiments were conducted by direct infusion of analytes as described previously for ESI optimisation, using [M + H]+ as precursor ion and argon as collision gas in collision induced dissociation in the second quadrupole (Q2). While the collision energy for Q2 varied from 0 to 50 eV, quadrupole 3 (Q3) was scanned to identify the major product ions of each precursor ion. The limit of detection were 1.0, 0.5, 2.0 and limit of quantitation were 4.4, 3.2, 6.5 ng/L for OTC, TC and CTC respectively. Analytes recoveries from SPE obtained by drinking extraction were 105, 106, 107 for OTC, TC and CTC respectively

The contamination of antibiotics in waste water is of concern and must be determined because of their toxicities. SPE has been used to preconcentrate organic analytes prior to determination by LC-MS for improving analyte signals. The review article is given as follows.

Batt *et al.* (2005) reported simultaneous analysis of multiple classes of antibiotics by ion trap LC/MS/MS for assessing surface water and groundwater contamination. Preliminary experiments were conducted to assess the methanol extraction efficiency of two SPE cartridges, namely, the 500-mg Oasis HLB cartridges and 1-g C18 Sep-Pak cartridges. The samples were passed through the cartridges at a rate of 3 mL/min. The analytes were eluted twice using methanol with 10 mM oxalic acid (method I), methanol with 1% TFA (method II), acetonitrile (methods III and VI), acetonitrile with 1% ammonia (method IV), or acetonitrile with 1% TFA (method V). The volume of each eluate was reduced and adjusted to 1 mL for LC/MS analysis. The

LC column was a 100 x 2.1 mm x 3-μm BetaBasic-18 C18 column equipped with a 10 x 2.1 mm x 3-μm Uniphase guard cartridge. The limit of detection were found at 0.060, 0.043, 0.059 µg/L, while the limit of quantitation were 0.2, 0.2, 0.1 µg/L for OTC,TC and CTC respectively. The SPE recoveries were 108±5 %, 113±3 %, 119±6 % for OTC, TC and CTC respectively.

MATERIALS AND METHODS

Materials

1. Chemicals and equipments

Monolith silica was synthesised from silica reagent, porogen and catalyst via a sol-gel method. Synthesis reagents were analytical grade or HPLC grade. The reagents included tetramethoxyothosilicate (TMOS, ≥ 98% purity) (Merck, Darmstadt, Germany), trimethoxy (octadecyl)silane (C-18-TMOS, 90% purity) (Aldrich, Milwaukee, IL, USA), *N*-dodecylamine of 98% purity (Fluka, St. Louis, Mo, USA) and methanol of HPLC grade (RCI Labscan, Bangkok, Thailand), hydrochloric acid of analytical grade (Carlo Erba, Strada Rivoltana, Milano, Italy), while water was purified by using a Cascada RO lab water purification system (Pall Life Science, Ann Arbor, MI, USA).

The monolith mixture was prepared in a 50-mL centrifuge tube obtained from Vivantis (Oceanside, CA, USA). The 3 and 5-mL syringes were purchased from Nipro (Osaka, Japan) and the 500 mg/ 6 mL Strata-X® C18-E solid phase extraction cartridge was purchased from Phenomenex (Torrance, CA, USA).

The synthesized silica monolith was sieved through 500 micron aluminium sieve. (Retsch, Haan, Germany).

The main analyte and related compounds, namely tetracycline (99.5% purity) was obtained from Calbiochem (China), oxytetracycline (≥95% purity) and chlortetracycline (≥97% purity) were standard grades obtained from Fluka (St. Louis, MO, USA)

2. Apparatus

A FED240 oven (Binder, Tuttlingen, Germany) and an incubator (MMM Group, Brno, Czech republic) were used to control temperature for polymerization of monolith. The synthesised C18-monolith was characterised by using a scanning electron microscope (Quanta 450, FEI, Hillsboro, Oregon, USA) and determined carbon loading by elemental analyser (LECO CHNS-932, St. Joseph, Michigan, USA). The solid phase extraction assembly (SPE) 12-position vacuum manifold set obtained from Phenomenex (Torrance, CA, USA) was connected to either a fabricated C-18 silica monolithic cartridge or a commercial C-18 silica cartridge used as sample and/or analyte extractants. A high performance liquid chromatography (HPLC) with a ultraviolet (UV) detector couple with time-of-flight (TOF) mass spectrometer (MS) from Waters (Milford, MA, USA) consist of water 2695 separation module, Waters 2485 UV visible detector, Waters micromass LCT premier mass spectrometer and a computerized system with MassLynx v. 4.0 was used to qualitatively and quantitatively determined tetracycline and its related compounds from waste water.

Methods

1. Reagent and sample preparation

1.1 0.5 M hydrochloric acid

A 5-mL of 37% HCl was diluted in a 100-mL volumetric flask with deionised water.

1.2 Tetracycline, oxytetracyclin and chlortetracycline solution at a concentrate range of 10, 20, 40, 50, 60, 80,100 and 200 ppm in 30% methanol.

A 200 ppm standard stock solutions of tetracycline, oxytetracycline and chlortetracycline were prepared by weighing 20 mg each in a 100-mL volumetric flask, and mixing with 30% methanol. The mixture was sonicated in an ultrasonic bath until chemical pellets were completely dissolved, and then adjusted to the volume with the

solvent. The diluted solution was prepared by diluting the 200 ppm stock solution with 30% methanol.

1.3 2.5 ppm spiked sample

The spiked sample solution was prepared by diluting 50 mL of 50 ppm standard stock solution into a 1000-mL volumetric flask. Adjusted to the volume with deionised water and stirred homogeneously.

1.4 Buffer solution pH 6.5

The buffer solution consisted of 0.075 M sodium acetate, 0.035 M calcium chloride and 0.025 M ethylenediaminetetraacetic acid (EDTA). It was prepared by weighing 6.15g of sodium acetate, 4.52g of calcium chloride and 7.31g of EDTA and dissolving the salts with DI water in 1000-mL volumetric flask. The buffer solution was adjusted to pH 6.5 by sodium hydroxide.

2. Preparation of silica monolith SPE device [see Appendix B]

2.1 Preparation of C-18 silica monolithic clusters

To obtain monolith cluster, a 50 mL polypropylene centrifuge tube was washed with 3 x 50 mL distilled water to remove any possible contaminants. The tube was then dried at 60 ^{o}C for 3 hours. The formulation mixture consisted of 4,000 µL methanol, 200 µL 0.5 M HCl, 700 µL water, 800 µL C18-TEOS and 3200 µL TEOS. The solution was mixed thoroughly in a plastic centrifuge tube using a vortex mixer. After sealing the tube, the mixture was hydrolysed at 60 ^{o}C for 4 h, and then cooled down to room temperature. After that 100 µL *n*-dodecylamine was added into the solution to solidify the monolith. The tube was uncovered and dried at 40 ^{o}C for 24 h. Organic monolithic solid was formed inside the tube; however, shrinkage of the monolith occurred, leaving the gap between the monolith and the tube wall. The monolith was ground and sieved through 500 micron aluminium sieve to obtain coarse monolithic clusters.

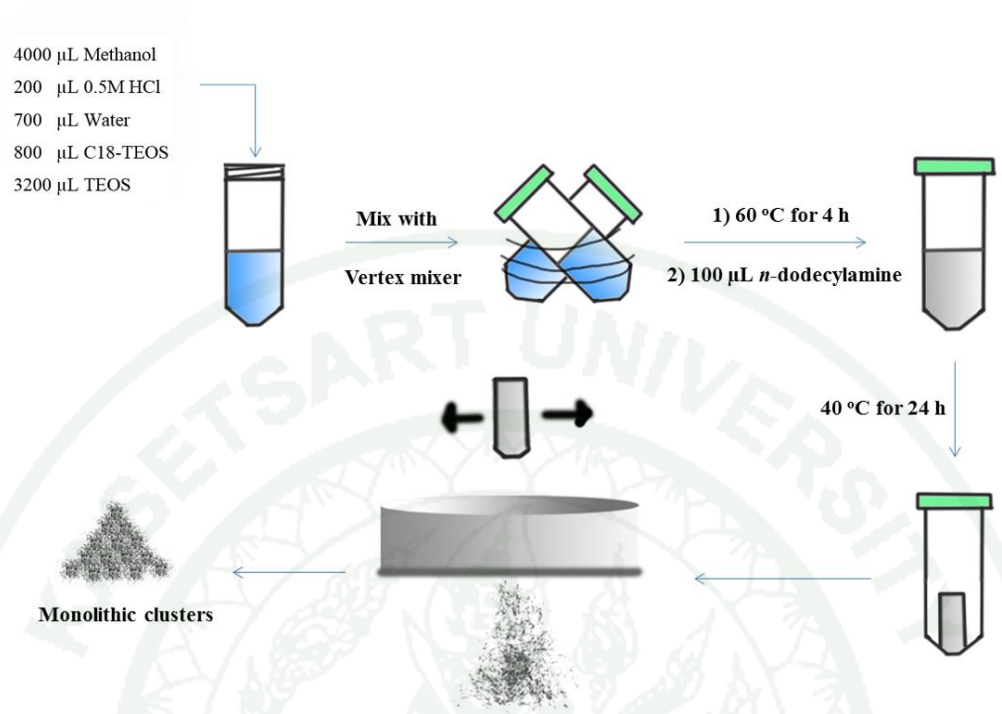


Figure 4 Preparation of monolithic cluster via a sol-gel method

2.2 Fabrication of monolithic silica solid phase for extraction

A 5 mL polypropylene syringe was cleaned with distilled water, and dried at 60 °C for 3 hours. Weighed 500 mg of the synthesised organic monolithic cluster into the polypropylene syringe where glass wool was put at the bottom of the syringe to protect a removal of monolithic cluster. The filled syringe was equipped with a SPE assembly.

A 400 µL methanol, 20 µL 0.5 M HCl, 70 µL water, 20 µL C18-TEOS and 180 µL TEOS were mixed in a 1.5 mL Eppendorf vial. The vial was sealed and hydrolysed at 60 °C for 4 h. The hydrolysed liquid mixture was immediately poured into the cluster-filled syringe, leaving the liquid on the cluster surface. A 10 µL *n*-dodecylamine was added into the coated cluster.

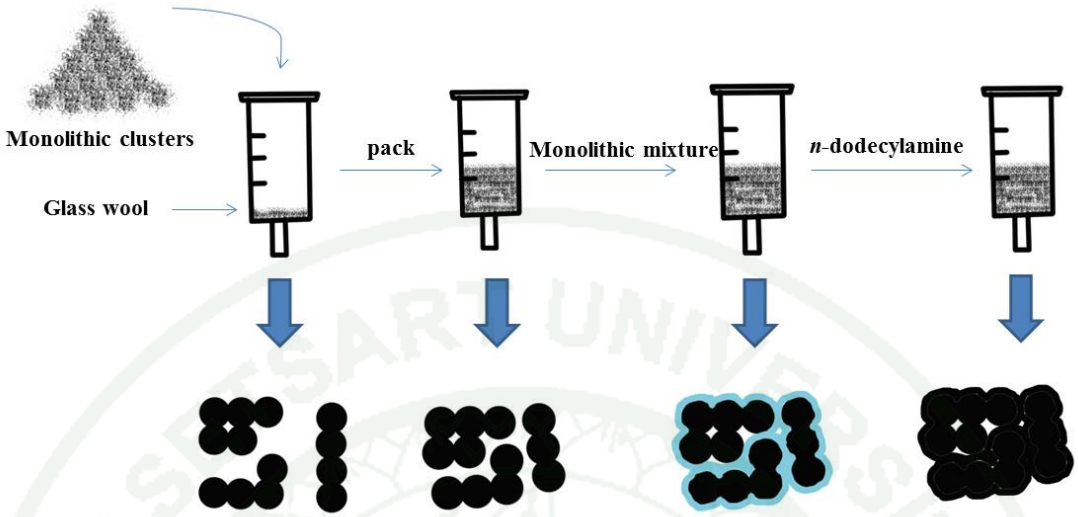


Figure 5 Fabrication of C-18 monolith SPE.

3. Scanning Electron Microscope (SEM)

Fabricated monolith was stuck on an aluminum stud cell by using a double-sides carbon tape, and then sputtered with gold nanoparticle for 5 minutes to avoid charging effects before the SEM observation by using a SC 7620 sputter (Quorum Technologies, Laughton, UK). The morphology of the newly synthesized C18 organic silica monolith was investigated by using Quanta 450 scanning electron microscope (FEI, Hillsboro, Oregon, USA). The scanning electron micrograph was operated at 15 kV.

3. Solid phase extraction

The fabricated C18-monolithic SPE cartridge was activated by rinsing with 5.0 mL methanol, followed by 5.0 mL water. A 100 mL of 2.5 ppm of spiked analyte in water was directly loaded onto the monolithic SPE cartridge fitted with the SPE assembly under applied vacuum. The adsorbed analytes were eluted with 5.0 mL of methanol, and collected in a 5 mL collection vial. The eluent was adjusted to the volume of 5.00 mL using methanol.

4. Analytical method of tetracycline and related compounds in HPLC system

4.1 Specificity and HPLC-UV and HPLC-TOF-MS conditions

Specificity of HPLC was obtained by setting chromatographic conditions, such as mobile phase composition, column length, oven temperature and detector wavelength. In this study, some optimum setting values were obtained from the balance between both optimised HPLC system and HPLC-TOF-MS system. The combination between retention time in HPLC and mass to charge (m/z) ratio in MS revealed specific conditions for particular analyte in waste water. A mixture solution for testing HPLC system consisted of 50 ppm each of tetracycline, oxytetracycline and chlortetracycline in deionised water.

For the liquid chromatographic system, the injection volume was 20 µL, sample temperature was 25^{o}C and the column oven temperature was 30^{o}C.

When the TOF-MS was the main detector, the 50 mm x 4.6 mm x 2.6 µm Kinetex column was operated at the flow rate of 0.3 mL/min of (70:30) 0.3% formic acid-methanol in an isocratic mode. The capillary cone and sample cone voltage were optimised at 3500 and 100 V. The desolvation temperature was 220 ^{o}C and desolvation gas flow was 250 L/hr. The ionization was operated in a positive mode, except for the determination of real waste water sample, both positive and negative ionization were performed.

When the UV detector was the main detector, the separation was carried on the 150 mm x 4.6 mm x 5 um Inersil ODS-4 column with a flow rate of 0.8 mL/min of a mobile phase mixture of buffer pH 6.5 and DI water. The gradient elution was operated as follows: 70% buffer pH 6.5-water, gradient to 60% between 0-6 min, then gradient to 50% between 6-10 min, isocratic between 10-20 min, then further gradient to 30% between 20-22 min. then isocratic between 22-27 min. The UV adsorption wavelength was 360 nm.

4.2 Calibration of standard and sample

A calibration curve of tetracycline, oxytetracycline and chlortetracycline were prepared at a concentration range between 10 - 100 ppm.

4.3 Repeatability

The precision under the same operating conditions was verified by triple injections of each concentration of analyte.

4.4 Limit of detection (LOD) of tetracycline and related compounds

Limit of detection is the lowest concentration of tetracycline, or its related compounds, in the sample which was obtained from a concentration that produced a signal-to-noise (S/N) ratio of 3:1 –the concentration of which the peak height was higher than 2 or 3 times of baseline noise level.

4.5 Relative recoveries and reproducibility

Reproducibility of C-18 SPE cartridge was carried out by determining a difference of two batch recovery. Relative recovery of tetracycline was demonstrated by testing 3 pieces of synthesized C-18 silica monolith cartridges.

4.6 Comparison of efficiency between C-18 silica monolith SPE and commercial C-18 silica SPE

A set of experiments of examining relative recoveries of both synthesized monolith and commercial cartridge were carried out by determining the tetracycline and its related compounds at a concentration of 50 ppm.

5. Analysis of tetracycline and related compounds spiked in waste water with HPLC-TOF-MS

5.1 Comparison of waste water background with 50 ppm each of analytes between HPLC-UV and HPLC-TOF-MS

A waste water sample with 50 ppm analytes were injected into HPLC-UV and HPLC-TOF-MS system with the optimised conditions and the two chromatograms were compared.

5.2 Analysis of tetracycline and related compounds in waste water with HPLC-TOF-MS

Since the waste water sample was very concentrated, it was then diluted at 1:1 ratio. A 1000 mL of the diluted waste water was added 50 mg of each TC, OTC and TCT. A C18 monolith cartridge was conditioned with 5 mL methanol, followed by rinsing with 5 mL water. A 100 mL of the spiked water sample was loaded onto the preconditioned C18 monolith cartridge, which was then eluted with 5 mL methanol. The volume methanol extract was readjusted to 5.00 mL with methanol, and 20 µL of the extract solution was applied into the HPLC-TOF-MS for analysis. The flow chart of the procedure was given in Appendix B.

The calibration curves of tetracycline, oxytetracycline and chlortetracycline were prepared at concentration ranges of 10 – 60 ppm. After the mass analysis, the areas of each extracted compounds from chromatograms were averaged and the concentration of analyte extract solution was calculated based on the calibration curve.

RESULTS AND DISCUSSION

Fabrication of C-18 silica monolithic SPE

In this study, C18-silica monolith was synthesized in a syringe cartridge for solid phase extraction assembly (SPE). The syringe has a large diameter compare to those of another monolith containers, such as capillary tube and pipette tip. The shrinkage was found after the fabrication. This agreed with those previously, Podgornik *et al.* (2000). that shrinkage of (organic) silica monolith occurred for large and high volume container more that small size container. The monolithic technique and/or formulation was then developed to solve such problem.

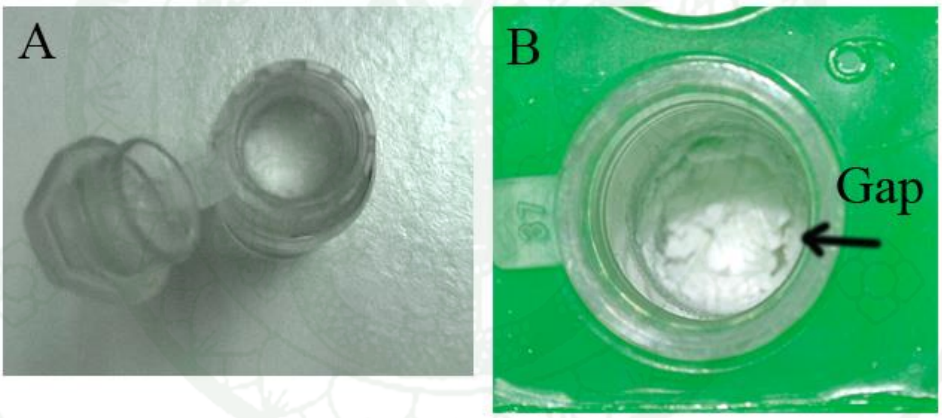


Figure 6 Shrinkage of monolith after 1 day in an appendorf vial; A, monolith initial day, B, After 1 day the monolith was shrunk, producing a gap between the wall and the monolith.

In the synthesis step of silica monolithic material, formulation of C-18 silica monolith formation consists mainly of silica mixture, porogens and co-solvent. The ratio of silica mixture and porogens determines the flow through pores and the polar distinction determines the size of particles and methanol acts as a co-solvent for assisting compatability. Therefore, optimisation of ratio of each ingredient in the mixture ratio is critical. The organic silica cluster was subjected to scanning electron

microscopy (SEM), and the SEM image confirmed particles in cluster, as shown in Figure 9.

Technique of a sol-gel method is mixing the mixture with two different phases: polar and non-polar phase. The mixture of two phases gave emulsion. During the reaction the non-polar phase became harden, releasing polar phase out of the system. The solid material of non-polar phase was then porous. In the experiment the factors that must be considered were the substances that used into the two parts. In the polar phase, methanol, hydrochloric acid, and deionised water were chosen, and non-polar phase were TMOS and C18-TMOS.

The first step of preparation was performed by adding methanol, HCl and deionised water into the centrifuge tube and mixing well. After that, C18-TEOS and TEOS were poured into the centrifuge and mixed vigorously. C18-TEOS and TEOS have the same TEOS base, but the distinct point is the bonding or non-bonding with C18. Therefore, the ratio between two types of TEOS controlled the amount of carbon loading value of C18 silica monolith.

The most important part is adjusting the ratio between the amount of polar phase and non-polar phase. The volume of methanol acts as a co-solvent to reduce the difference in polarity between the two phases. Added methanol should have a suitable volume depending on the two phase ratio and it gave various effects to physical characteristic of the synthesised monolith. If the methanol is added lower than the suitable volume, the difference in polarity between two phases remains high. During the process of mixing, the two-phase substances gave emulsion that resembled oil droplet suspending in water. The high polarity distinction made high separation between two phases, and the non-polar droplets eventually combined together, resulting larger droplets. In the worst case that occurred, the two phases separated completely. This made the synthesis failed immediately.

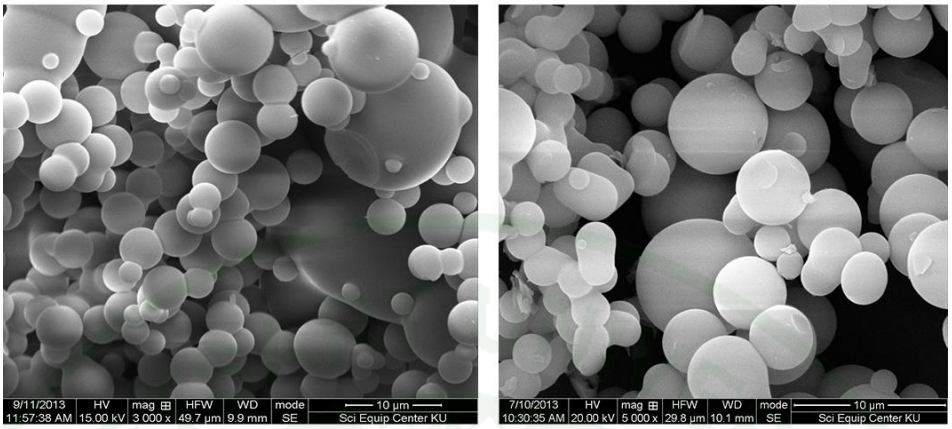


Figure 7 Scanning electron micrographs of silica monolith with the volume ratio of (4:3) TEOS base/methanol. The methanol volume was lower than the suitable volume

In contrast, if the volume of methanol was higher than the suitable volume, the difference in polarity between the two phases was low. Repulsive force between the phases was not high enough to maintain the droplets to be in spherical shape. The formed skeleton became rough monolith without significant spherical particles, or rough ball, depending on the volume of methanol. Furthermore, if a further increased volume of methanol was added, a spread sheet of monolith that resembled a leaf or several overlapping layers of leaves occurred. In the worst case, the non-polar phase highly distributed all over the liquid mixture, and it became virtually homogeneous. As a result the formed monolith was virtually non-porous, similar to that occurred when adding low amount of methanol. To differentiate between the monolith occurred from too high or too low volume of methanol, the visual appearance of the surface of the monolith must be studied by SEM. The high methanol volume gave rough and spiny monolith, but the low volume methanol resulted smoother surface.

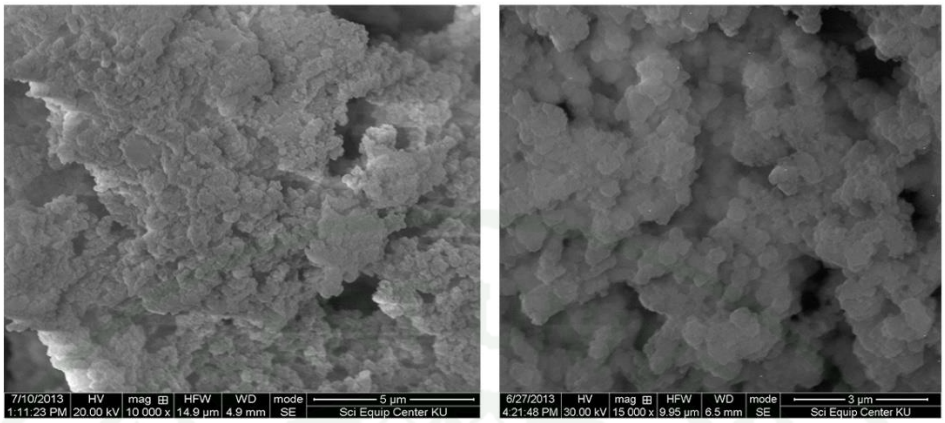


Figure 8 Scanning electron micrographs of silica monolithic with the volume ratio of (2:4) TEOS base/methanol. The methanol volume was higher than the suitable volume

Generally the suitable volume ratio in this research was 1:1. However, the mixing force and temperature were also important factors for fabricating the monolith.

After mixing all ingredients in a centrifuge tube, the tube was baked at a temperature of 60^{o}C for 4 hours. In this step TMOS underwent hydrolysis and condensation reaction using HCl and heat, resulting dispersing clouds of colloid in the mixture. The reaction that occurs highly depended on the volume of methanol that caused TMOS distribution in the solution and temperature of the oven. High temperature increased the kinetic energy of TMOS, giving more collision of TMOS with water to cause hydrolysis and condensation. However, the temperature should not exceed 70 ^{o}C, otherwise the bonding between C18 and TMOS of C18-TMOS was disrupted. To avoid the damage to the C18-TMOS that caused by a thermal fluctuation of the operated oven, the temperature was set at 60^{o}C.

Amount of colloids was very important to the monolith formation, because they were the precursor of the monolith structure. The more colloids occurred, the better particle size distribution of the monolith was. However, one should be aware of the high density of colloids occurred for the long chain organic silica, such as C18, monolith synthesis of which reaction gave a rapid skeletal formation. The organic carbon chain

is an electron donating group that may support the elimination of water and methyl group. The rate of reaction was then increased. When the high density colloids occurred in the reaction and n-dodecyl amine was added as a catalyst. If the reaction occured too quickly, when dropping n-dodecyl amine, the surface of the solution became covered with dispersing solid, thus resulting the following drop of n-dodecyl amine not be able to disperse to the bottom of the solution. As a result, it gave a layer of solid on top of liquid mixture. This may affect many physical characteristics of the resulting material, such as mixed clear and opaque particles, non-macroporous monolith, etc.

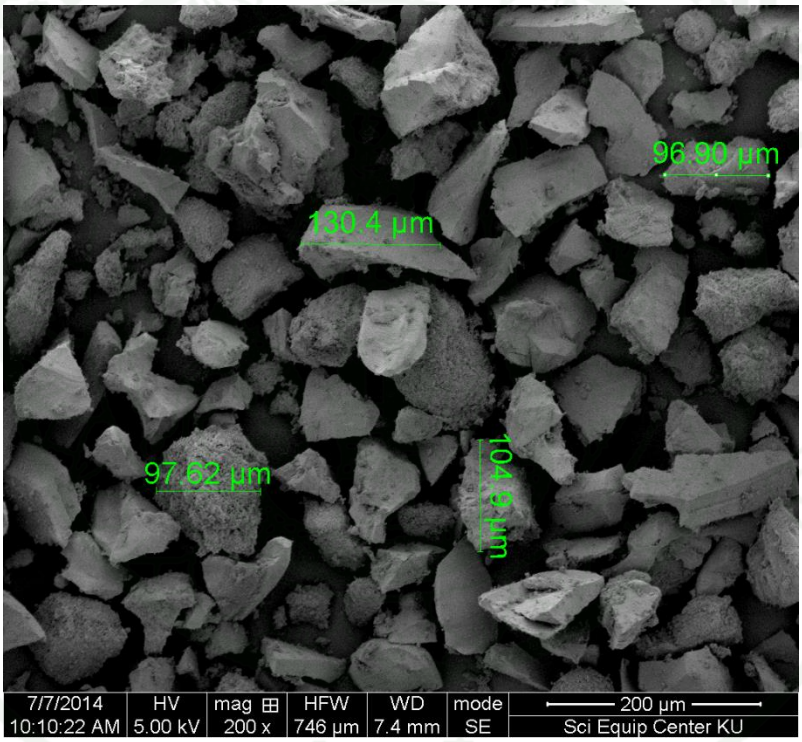


Figure 9 Scanning electron micrograph of organic silica monolithic clusters. The size of cluster is between 90-150 micron.

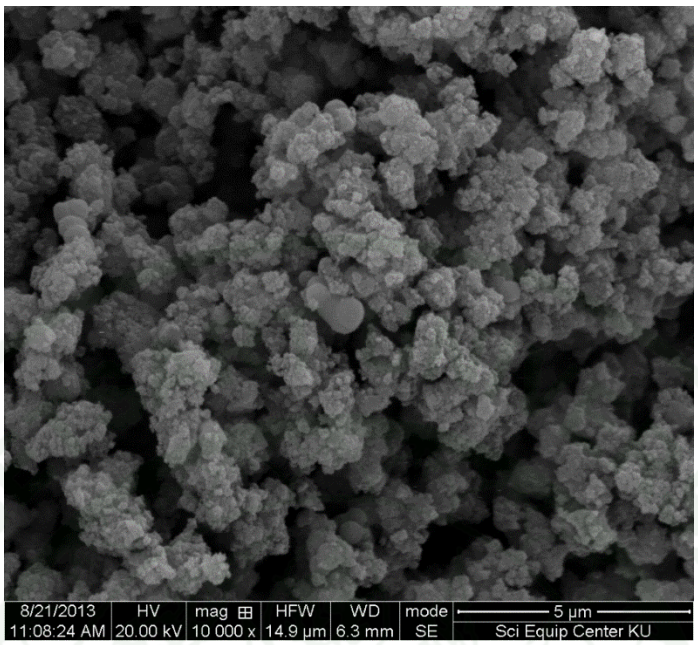


Figure 10 Scanning electron micrographs of silica monolithic clusters with (1:1) TEOS base/methanol

After obtaining the monolith, the monolith was sieved through a sieve to obtain coarse monolithic clusters. In practice, the clusters indicated the macroporous size of C18-silica monolith for SPE. The cluster size is therefore important for a consideration in a ratio of polar to non-polar phase for reformation of the cluster to be a monolith.

C18-silica monolith clusters were subsequently refabricated to monolith by a sol-gel method. The monolithic clusters was added with synthesised liquid mixture. After synthesis the liquid mixture became solid that connected each cluster together. The ratio of the syntheised liquid mixture to clusters depended on the size of clusters. If the exceeding volume of the liquid mixture was used, the newly formed monolith was clogged on the solid silica monolith. On the other hand, with the deceeding volume of the liquid mixture, the clusters could not be connected, causing cracking.

Figure 11 C-18 silica monolith left three months after fabrication. No shrinkage and no gap between the wall and the monolith inside.

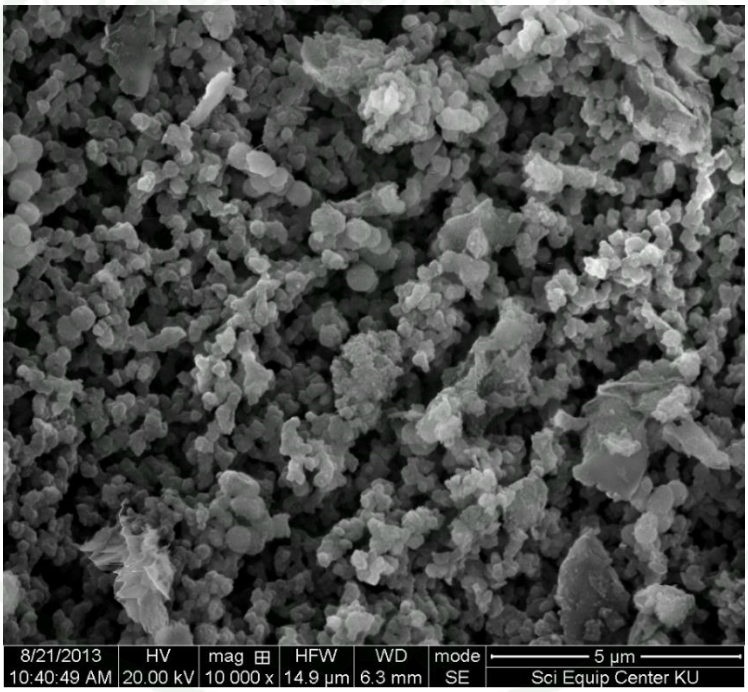


Figure 12 Scanning electron micrographs of silica monolithic particles after refabrication process

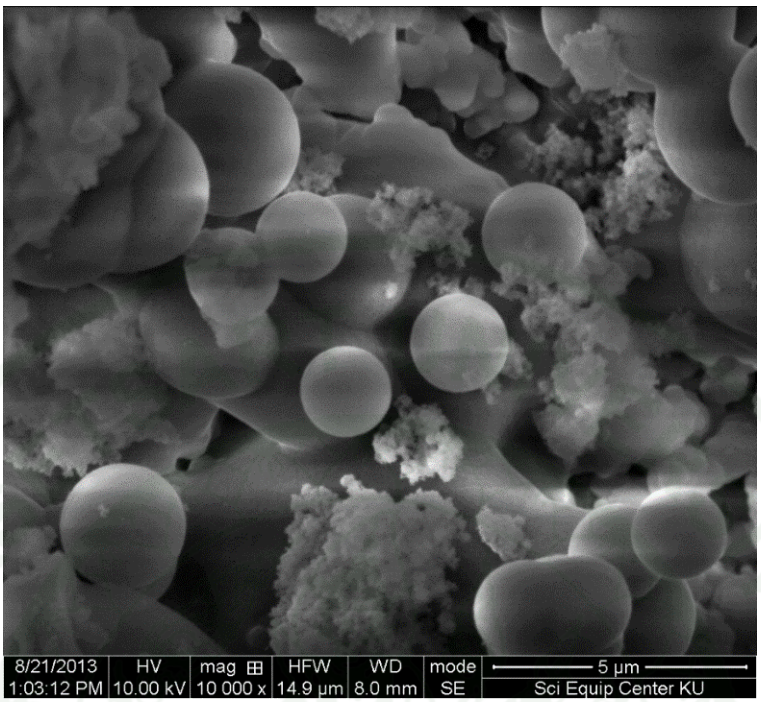


Figure 13 SEM micrograph of C18-silica monolith with an exceed volume of secondary silica mixture

The resulting final monolith was a well-fabricated monolith with well-defined particles and narrow particle size distribution. The new technique definitely solved the shrinkage problem of synthesising organic silica monolith. The advantage of the method was a capability of defining particle size of monolith before refabrication process. Another advantage of the technique is to adapt it for regenerating a collapsing C18-column in a following study.

Analytical method

HPLC-TOF-MS was expected to gain much benefits when it was integrated to the use of monolithic adsorbent for the tetracycline determination in waste water. As a sequence of setting the method, initially the experiment should be then set up for the tetracycline separation by using the monolithic adsorbent, then determination by using the HPLC-TOF-MS. After that the determination was compared with the HPLC-UV detector at 360 nm, as it is more typical and available in most laboratories. However

because the HPLC-TOF-MS is a new high performance instrument, conditions for the separation of three selected tetracycline and conditions for the mass detection must therefore be optimized, as well as those of HPLC-UV.

1. High Performance Liquid Chromatogaphy-time-of-flight-mass spectrometry (HPLC-TOF-MS)

The HPLC-TOF-MS was considered to gain good and acceptable results in the separation of the analyte mass from complicated matrix. All conditions of the separation by using HPLC-TOF-MS were studied and optimised.

1.1 Optimisation of HPLC-TOF-MS conditions

For an initial study, factors affecting sensitivity of TOF-MS must be optimised. As the HPLC-TOF-MS coupled with an electrospray ionization (ESI) interface, different type of mobile phase produced different sensitivity of analyte signal. In this work the mobile phase mixture containing formic acid and methanol were selected as they were volatile in the ionization source. Formic acid donated protons to analytes to become a preformed state, which improved the protonation of the analytes. Methanol served as an organic solvent that reduced surface tension of the mobile phase, thus supporting the fine spraying of the nebulizer to produce very small droplets. The capillary functioned as a counter electrode, adding the charge onto the liquid droplets. The smaller droplet, the higher charge density of the droplet was. From the result, the ratio of 70:30 of 0.3% formic acid and methanol was the most suitable for separating the analytes with HPLC column prior to the mass spectrometer.

ESI is a device that employed pressurized gas to spray the solution to form charged aerosol, which was evaporated, giving the ion analytes passing to the multi-channel plate analyzer by electromotive force. To maximize the sensitivity of the analyte signal, an adjustment of ESI setting must be optimized.

1. Aluminium capillary which functioned as a transfer pipe for solution in the nebulizer needed to be adjusted. It must be protruding from the nebulizing gas tube and the length must be related to the gas flow rate. This adjustment was to optimise the radial distribution of aerosols. The most appropriate extended length of the capillary was 2 mm from the nebulizing gas tube and the gas flow rate was 250 L/hr.

Figure 14 Distribution characteristics of aerosols with different extended length of the aluminium capillary (adapted from แมสสเปกโทรเมตรี, ศุภศร วนิชเวชา รุ่งเรือง และ ณรงค์ ประไพรักษ์สิทธิ์. สำนักพิมพ์แห่งจุฬาลงกรณ์มหาวิทยาลัย. 2553, หน้า 107)

2. Care must be taken to avoid contamination at the aluminium capillary tip. When the tip was deposited with contaminants, the voltage dropped. The analytes in the droplet cannot be protonates with a regular voltage, causing a reduction of analyte signals or disappeared signal. Contamination can occur at the ion guide, which acted as a repeller to push and accelerate the analyte ion to the flight tube of TOF analyzer. Contaminantion at the ion guide led to an error of the result.

3. The angle between nebulizer and skimmer must be adjusted to obtain the highest sensitivity of a particular analyte. When the outlet of the capillary was projected directly to the skimmer, some unionized analytes may deposit on it. This caused a reduction of sample cone voltage, lessening the number of analyte ions entering the skimmer, thus decreasing the sensitivity. On the other hand, moving the outlet of capillary away from the skimmer also reduced the number of analyte ions entering the skimmer. By tuning the angle, the most optimized position was chosen from the highest signal of analyte.

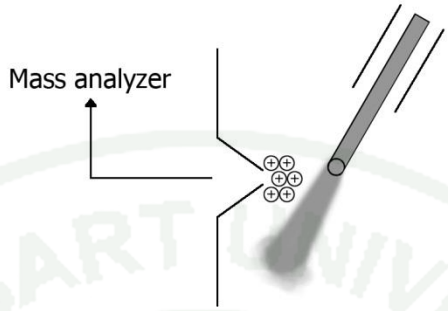


Figure15 Degree adjustment of nebulizer (adapted from แมสสเปกโทรเมตรี, ศุภศร วนิชเวชารุ่งเรือง และ ณรงค์ ประไพรักษ์สิทธิ์. สำนักพิมพ์แห่งจุฬาลงกรณ์มหาวิทยาลัยม. 2553, หน้า 108)

To optimize the other conditions, 20 µL of a mixture of 50 ppm each of tetracycline, oxytetracycline and chlortetracycline standard was injected to the HPLC-TOF-MS. The system was coupled with 50 mm x 4.6 mm x 2.6 um Kinetex C18 column and the mobile phase was (70:30) 0.3% formic acid-methanol. Because there were numerous chromatograms during the change of overall conditions, therefore only the most optimised parameters of both HPLC-TOF-MS that provided the best separation are shown in Table 2. The chromatogram of the separation of analytes was shown in Figure 15.

Table 2 Optimised conditions of separation of standard tetracycline by using HPLC-TOF-MS:

Parameters	Value
Injection volume	20 µL
Sample temperature	25 ºC
Column oven temperature	30 ºC
Column	50 mm x 4.6 mm x 2.6 µm Kinetex
Flow rate	0.3 mL/min
Mobile phase (isocratic)	(30:70) methanol-0.3% formic acid
Capillary voltage	3500 V
Sample cone voltage	100 V
Desolvation temperature	220 ºC
Desolvation gas flows	250 L/hr.

1.2 Identification

Each separated peaks in the chromatogram was analysed by the time-of-flight mass spectrometer. It was confirmed by comparing molecular weight with a reference standard of each compound as shown in Figure 16.

With the 0.3 mL/min of (70:30) 0.3% formic acid-methanol mobile phase in the isocratic mode, the retention time of tetracycline, oxytetracycline and chlortetracycline were 16.72, 18.06 and 26.00 min, respectively. From the TOF mass identification, the mass ion of tetracycline, oxytetracycline and chlortetracycline were 444, 460 and 478, respectively. The mass spectrum of three tetracyclines were given in Appendix L.

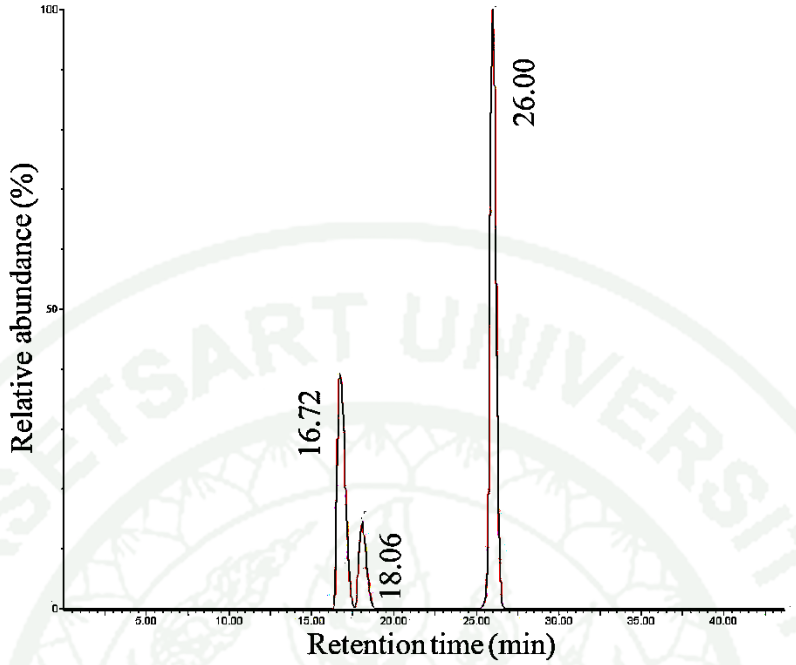


Figure 16 Chromatogram obtained from HPLC-TOF-MS of the separation of tetracycline compounds.

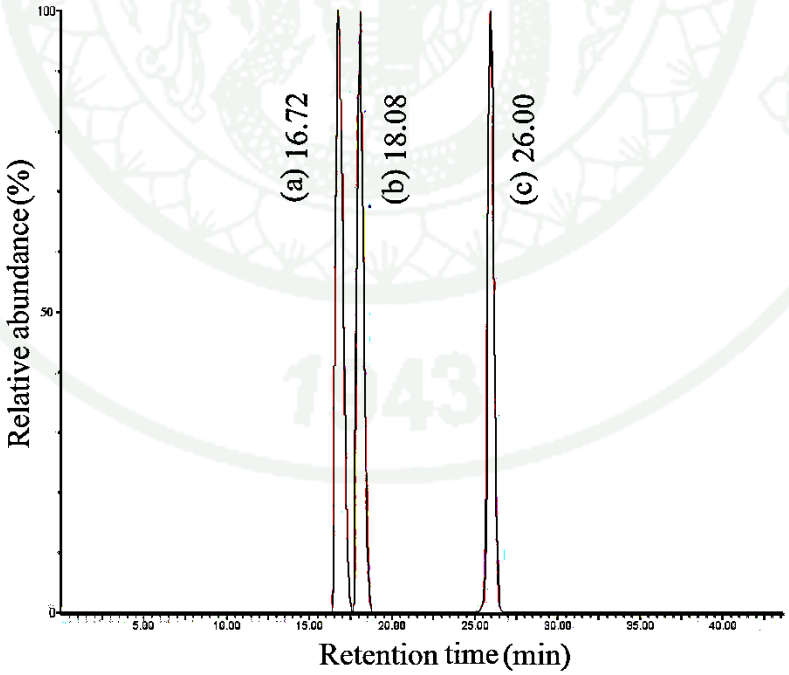


Figure 17 Ion selective chromatogram of (a) tetracycline, (b) oxytetracycline and (c) chlortetracycline.

Since the UV detector was connected between the analytical column and the TOF-MS detector, with the mobile phase system and the UV wavelength of 360 nm, the chromatogram obtained from UV detector was compared with that from the mass detector, as shown in Figure 17 below.

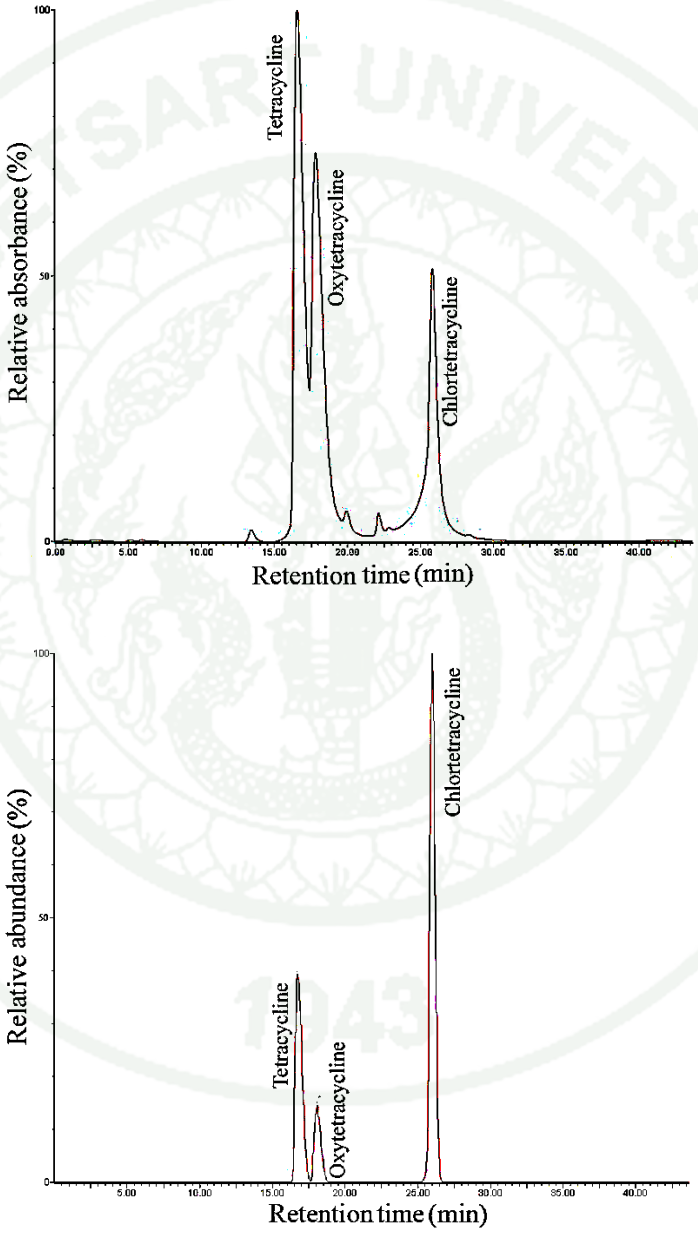


Figure 18 UV chromatogram and mass chromatogram of tetracycline, oxytetracycline and chlortetracycline when using (30:70) methanol-0.3% formic acid as a mobile phase.

From the chromatogram of UV detector, the peaks of tetracycline and oxytetracycline were partially overlapped, although the signals obtained by the UV detector were higher than those detected by the TOF detector. Owing to the low signals, the overlapping peaks became separated. Meanwhile the signal of chlortetracycline was higher than that by using the UV detector, and the tailing effect of the peak was reduced.

In the ion selective mode of the mass detector, the analyte mass with high resolution can be locked for high precision of mass analysis to show each analyte peak in three chromatograms, this is very beneficial when a complicated matrix was analysed and the separation was difficult. In such the cases, the result of both high resolution mass ion and retention time can be used to verify the analytes. Waste water sample is an example of such matrix, as it includes many chemical compounds which is generally difficult to analyze with only one method. HPLC-TOF-MS is therefore suitable for analysis tetracycline and its related compounds in waste water rather than the HPLC-UV.

1.3 Efficiency of the HPLC-TOF-MS instrument

To ensure the quality of the analytical procedure and the reliability of the result, performance of the HPLC-TOF-MS instrument was evaluated by using standards, spiked samples and blank sample.

1.3.1 Linearity

In order to test linearity of the calibration curve, various concentration of spiked standard tetracycline solution in water ranging from 10 to 200 ppm were analysed. A 20 µL of each standard solution was directly injected into a HPLC-TOF-MS under the optimal conditions. The calibration graphs were constructed by plotting the peak area against the concentration of each tetracycline, as shown in Figure 18. It was found that the concentration range of all compounds that gave linear relationship was below 60 ppm for oxytetracycline and chlortetracycline, while that for tetracycline was below 100 ppm. The higher concentration gave reduced signal than expected. This

may be postulated that at low concentration all analyte molecules from the nebulizer passing through the skimmer, but at the high concentration the increased number of molecules were partially blocked by the skimmer.

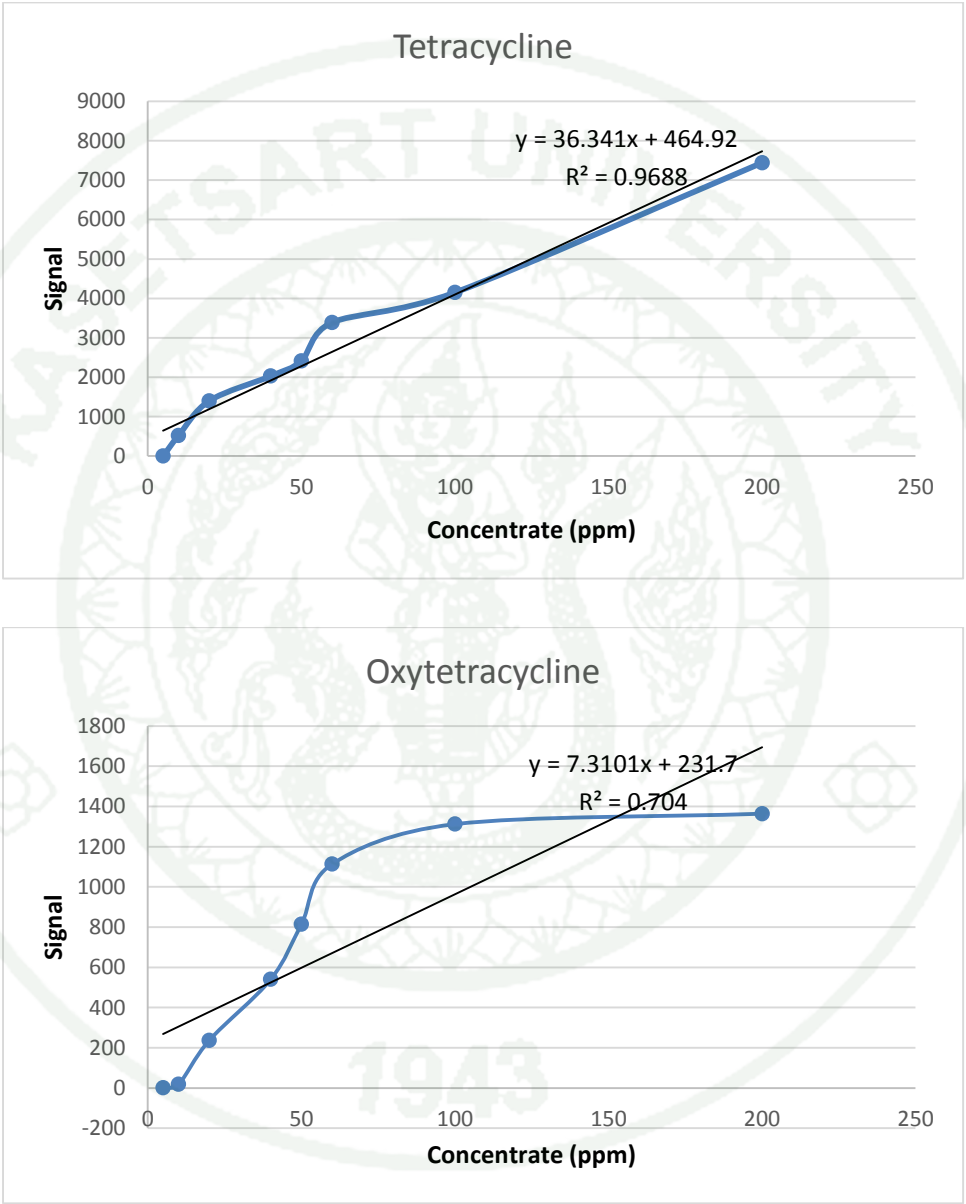


Figure 19 Calibration curves of tetracycline and its related compounds in the concentration range of 10 – 200 µg/L

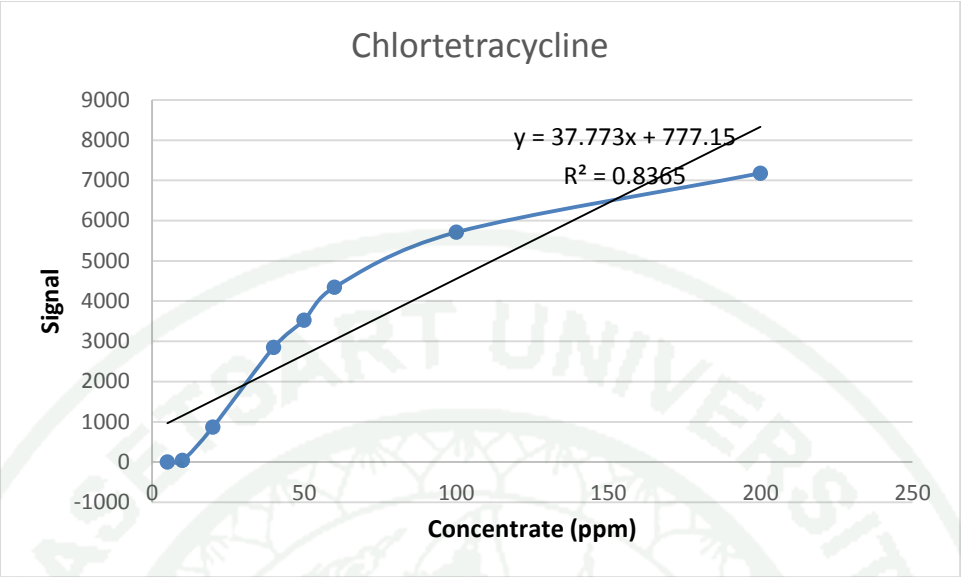


Figure 19 Calibration curves of tetracycline and its related compounds in the concentration range of 10 – 200 µg/L (continue)

From the configuration setting of ESI mentioned above, the ESI has several limitation which affect the limit of detection of HPLC-TOF-MS. At a low concentration, analyte ions diffused away from the skimmer could be lost from the nebulizing process, thus producing lower signal than expected. In contrast, for the high concentration and/or high gas flow rate, the analytes were not totally protonated. The analyte molocules did not enter the skimmer, leading to the reduced signals, as seen in the calibration graph of tetracycline, oxytetracycline and chlortetracycline above.

Slope, intercept and least square of correlation coefficient (r^2) obtained from calibration curve of tetracycline, oxytetracycline and chlortetracycline in the concentration range between 20 - 80 ppm are summarized in Table 3.

Table 3 Statistic values from each regression line of selected tetracyclines determined by using HPLC-TOF-MS (10-100 ppm)

Compounds	Regression equation	r^2
Tetracycline	y =38.107x – 425.88	0.9797
Oxytetracycline	y = 20.935x – 209.26	0.9821
Chlortetracycline	y = 87.273x – 812.84	0.9969

1.3.2 Detection limit of HPLC-TOF-MS

Method sensitivity was evaluated by measuring the limit of detection (LOD) of tetracycline and its related compounds, and calculated according to Miller & Miller (2005). A definition of limit of detection (LOD) in analytical chemistry is an analyte concentration giving a signal equal to the blank signal, y_B, plus three standard deviations of blank, s_B as presented in equation.

$$y - y_B = 3s_B$$

To estimate a standard deviation of blank (s_B), the experiment was set up by an injection of a series of the selected analyte at a concentration range of 10-200 ppm to the HPLC-TOF-MS under the optimal conditions. By determining 10 replicates of the blank sample, average peak area, standard deviation (SD) and three times of standard deviation (3SD) of each compounds were calculated and given in Table 4.

Table 4 Lowest concentration sample average peak area, standard deviation (SD), three times of standard deviation (3SD) of each compound (see Appendix D)

Compounds	Average peak area	SD	3SD
Tetracycline	504.90	50.58	151.73
Oxytetracycline	15.70	4.47	13.42
Chlortetracycline	43.90	10.60	31.79

The limit of detection can be obtained from a calibration graph plotted between the concentration of each tetracycline (x-axis) and its signal (y-axis), where the slope (b) and intercept (a) were given, according to the linear equation, $y = a + bx$. To examine the true detection limit, the 3SD was substituted in the equation, $y - y_B = 3SD$, while y is the true detection limit and y_B is the average peak area of blank sample. When y was replaced in the linear equation, then x was calculated as the true detection limit which was equal to 0.17, 0.55 and 0.50 ppm for tetracycline, oxytetracycline, and chlortetracycline , respectively, as shown in Table 5. The result shows that the limit of detection of the three tetracyclines was in a range of 0.17 to 0.55 ppm. It should be noticed that the TOF-MS is not a very sensitive detector when the trace analysis is concerned, although when the mass resolution is taken into account, the TOF-MS is a high resolution mass analyser. As a result, in this work to obtain the low detection limit the UV detection was therefore considered as a more optimised detector.

Table 5 Detection limit of each tetraclycline determined by using HPLC-TOF-MS detection. (see Appendix F)

Compounds	Detection limit (ppm)
Tetracycline	0.17
Oxytetracycline	0.55
Chlortetracycline	0.50

When using the HPLC-TOF-MS, salts in buffer solution were more problematic. At the electrospray (ESI) interface, organic solvents and any volatile components in the mobile phase that passed through was evaporated and ionization occurred. As the buffer solution may consist of non-volatile salts, when the solvent in the mobile phase was evaporated and those salts passing from the nebulizer deposited on the skimmer, and blocked the passage through the ionization chamber of ESI. Non-volatile salt was then not allowed in the mobile phase of the HPLC coupled with TOF-MS. However, since an absolute separation of tetracycline and oxytetracyline was required for higher sensitivity, a buffer system with non –volatile salt then was tested in the mobile phase of HPLC-UV detection.

2. High Performance Liquid Chromatography-UV Detection (HPLC-UV)

Since the sensitivity of the HPLC-TOF-MS was lower than expected, the detection was then examined by using a UV detector with the highest absorption wavelength of tetracycline, 360 nm. Unfortunately the UV detector in this work was a single wavelength, only one absorption wavelength was then operated.

2.1 Optimisation of HPLC-UV conditions

To optimise 20 µL of a mixture of 50 ppm each of tetracycline, oxytetracycline and chlortetracycline standard was injected to the HPLC-UV coupled with a 150 mm x 4.6 mm x 5 um Inersil ODS-4 and the mobile phase was a mixture of methanol and

buffer pH 6.5. Some of the optimised parameters of both HPLC-UV are shown in Table 6.

Table 6 Optimised conditions of separation of standard tetracycline by using HPLC-UV:

Parameters	Value
Injection volume	20 µL
Sample temperature	25 ºC
Column oven temperature	30 ºC
Column	150 mm x 4.6 mm x 5 µm Inersil ODS-4
Mobile phase	DI water : Buffer pH 6.5
Gradient mobile phase ratio	0 min (30 : 70) 6 min (40 : 60) 10 min (50 : 50) 20 min (50 : 50) 22 min (70 : 30) 27 min (70 : 30)
Flow rate	0.8 mL/min
Adsorption wavelength	360 nm

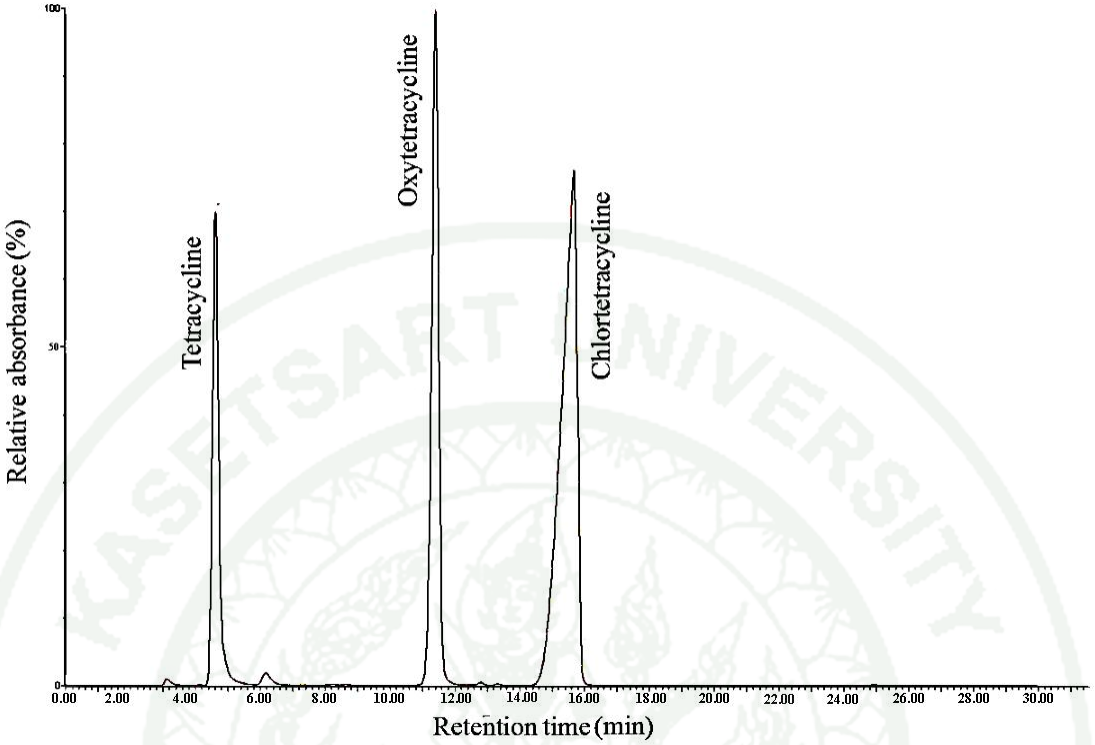


Figure 20 Chromatogram of the separation of tetracyclines obtained from HPLC with the UV detection at 360 nm.

With the mobile phase of deionised water and buffer pH 6.5 with gradient elution, the retention time of tetracycline, oxytetracycline and chlortetracycline obtained were 4.61, 11.40 and 15.67, respectively. From Table 2 and 6, when compared the optimised conditions of both instruments the difference in conditions was the mobile phase and the flow rate. The mobile phase condition of HPLC-UV was shown good separation but it could not be used in HPLC-TOF-MS system, because of the aggregation of salt at the skimmer. Tetracycline and oxytetracycline peaks that were overlapped when using the organic solvent and DI water were separated by using the buffer at pH 6.5.

2.2 Linearity

Linearity was evaluated by plotting signal as a function of analytes concentration. The linearity data must be subjected to a statistical transformation prior to the calculation with regression analysis and r^2 from equation can be estimated the

degree of linearity. In order to test linearity of the calibration curve, concentration of standard tetracycline, oxytetracycline, and chlortetracycline ranging from 20 to 100 ppm were analysed. A 20 µL of each standard solution was injected into a HPLC under the optimal conditions. The calibration graphs were constructed by plotting the peak area against the concentration of each analyte, showing a good linearity with correlation coefficients greater than 0.99 for all of studied compounds.

Table 7 Regression equation and r^2 of each tetracycline compound at the concentration between 10 to 80 ppm for tetracycline and oxytetracycline, 20 to 80 ppm for chlortetracycline

Compounds	Regression equation	r^2
Tetracycline	y = 3303.1x – 1482.3	0.9982
Oxytetracycline	y = 4730.4x – 19497	0.9972
Chlortetracycline	y = 1833.2x – 22220	0.9993

2.3 Accuracy

Accuracy was calculated by injecting three replicate of three concentration levels covering the linear range, 20 ppm to 100 ppm. The selected concentrations were 20 ppm, 50 ppm and 80 ppm.

Table 8 Accuracy test of three tetracyclines at the concentration of 20, 50 and 80 ppm (see Appendix H)

Compounds	% RSD		
	20 ppm	50 ppm	80 ppm
Tetracycline	2.37	0.04	0.39
Oxytetracyckine	0.23	0.15	0.28
Chlortetracycline	1.75	0.50	0.14

It was found that the accuracy of the overall concentration in the range was very good, below 2.4% RSD. The accuracy at the low concentration was higher than that of higher concentration, which is considered to be typical.

2.4 Detection limit of HPLC-UV

In a similar way to the detection limit determined by using the HPLC-TOF-MS, the experiment was set up by an extraction of the lowest concentration of tetracycline and the other selected anlytes spiked in water, and the extracts were subjected to HPLC-UV under the optimal conditions. By determining 10 replicates of the blank sample, average peak area, standard deviation (SD) and three times of standard deviation (3SD) of each compounds were calculated and given in Table 9.

Table 9 Average peak area, standard deviation (SD), three times of standard deviation (3SD) of the tetracycline extract of the lowest concentration of spiked sample

Compounds	Average peak area	SD	3SD
Tetracycline	1726.0	163.31	489.93
Oxytetracycline	1626.6	176.59	529.78
Chlortetracycline	1114.7	329.54	988.61

The true limits of detection were then calculated in a similar way to those of HPLC-TOF-MS, by using the equation $y - y_B = 3SD$, while y is the true detection limit and y_B is the average peak area of blank sample. When y was replaced in the linear equation, then x was calculated as the true detection limit which was equal to 70.6, 69.3 and 68.2 µg/L for tetracycline, oxytetracycline and chlortetracycline respectively, as shown in Table 10. The detail of calculation of the LOD was also given in Appendix I. Since the 100 mL water sample was extracted to give the final liquid extract of 5 mL, the preconcentration factor was then equal 20. When compared between HPLC-TOF-MS and HPLC-UV determination, it was found LODs examined by HPLC-UV were

lower than those by HPLC-TOF-MS. This confirmed that the HPLC-UV was more suitable and reliable than HPLC-TOF-MS for validating the true efficiency of C18 monolithic SPE.

Table 10 Detection limit of each tetracycline compound by using HPLC-UV determination (preconcentration factor= 20) [Appendix J]

Compounds	Detection limits (µg/L)
Tetracycline	70.6
Oxytetracycline	69.3
Chlortetracycline	68.2

3. Coupling between the monolith SPE with HPLC-UV

In this experiment the monolith SPE was employed for the extraction of tetracycline in waste water. Since the synthesized monolithic adsorbent affected the efficiency of extraction, it was therefore investigated for the repeatability when the variation of within-batch synthesis was concerned, and for reproducibility when the between-batch synthesis was involved. The experiment was carried out by an extraction of 50 ppm spiked each tetracycline in 100 mL water. After passing the spiked sample through the monolith SPE, and preconcentrating to 5 mL methanol, the 20 uL extract was subjected to the HPLC-UV. The result was compared with that of the same concentration that did not subject to the monolith SPE.

3.1 Repeatability

To study the repeatability 50 ppm tetracycline spiked water sample was extracted by using the monolith SPE that was synthesized from the same batch.

Table 11 Standard deviation of peak area between SPE sample in the same batch production (n=3)

Compounds	Variation between samples (%)
Tetracycline	3.32
Oxytetracycline	2.76
Chlortetracycline	6.07

2.4.3 Reproducibility

Typical variation to be studied is batch to batch production of C18 SPE monolith adsorbent. To study the reproducibility 50 ppm tetracycline spiked water sample was extracted by using the monolith SPE that was synthesized from the different batch.

Table 12 Standard deviation of average peak area between batch production (n=2)

Compounds	Between-batch variation (%)
Tetracycline	6.18
Oxytetracycline	11.16
Chlortetracycline	10.22

7. Recovery

Recovery was used to evaluate extraction efficiency of the method. In this experiment the efficiency of C18-monolithic SPE was then compared with a commercial C18-SPE. Because of the lower sensitivity of the TOF-MS than UV detector, the determination was then carried out by the HPLC-UV. The recovery of the experiment was carried out by adding 2.5 ppm of each standard analyte spiked in 100mL DI water and the extract was subjected to the chromatographic determination.

Table 13 % Recovery of tetracycline and its related compounds by using C18-monolithic SPE compared with a commercial C18-SPE [Appendix L]

Compounds	% Recovery		
	Tetracycline	Oxytetracycline	Chlortetracycline
C18-Monolithic SPE	76.46	72.55	81.69
C18-Commercial SPE	92.70	83.18	77.62

The recoveries of tetracycline and oxytetracycline when using C18 monolithic SPE were lower than that of commercial SPE, while that of chlortetracycline was greater than that of commercial SPE. The low recoveries of the analytes when using the monolith SPE was hypothesized that tetracycline and oxytetracycline were very polar compounds, therefore they had low interaction with C18 non-polar stationary phase.

Since the interaction of tetracyclines with the surface of the C18 adsorbent affecte the recoveries, the carbon loading of the adsorbents must then be considered. The carbon loading of synthesized monolithic cluster, synthesized C18-monolith adsorbent, and commercial C18 adsorbent were therefore analysed by using a CHNS analyser and the results were compared as shown in Table 14.

Table 14 Comparison of carbon loading of three solid adsorbents

Solid adsorbents	**Carbon loading (%)**
Synthesized monolithic cluster	31.28
Synthesized C18-monolith adsorbent	22.85
Commercial C18 adsorbent	17.03

The carbon loading is the value of the C18-organics chain bonded with silica It also represented the polarity of stationary phase. The higher carbon loading, the low polarity of the stationary phase is. The synthesized monolithic cluster has a higher carbon loading than the synthesized C18-monolith adsorbent because of lower ratio of

C18-TEOS to TEOS in the second fabrication formulation. The surface of synthesized monolithic cluster was then less polar than that of synthesized C18-monolith adsorbent.

The synthesised C18-monolith had a carbon loading of 22.85%, higher than that of commercial SPE (17.03 %), it was therefore less polar and reduced the adsorption to the tetracyclines. Its recoveries of tetracyclines were then lower than those of commercial SPE.

8. Spiked waste water samples

In this study, the selected tetracyclines were spiked at 50 mg/L each in waste water from the swine farm located in Pratumthani, Thailand. The waste water samples were subjected to either the monolith SPE, and eluted with 5 mL methanol. The liquid extract was applied to the chromatographic system, and the chromatograms obtained by using UV and TOF-MS detectors are shown in Figure 21. Although the mobile phase of HPLC-UV was shown a better separation of each analyte than that of HPLC-TOF-MS, the buffer in mobile phase interfered the ESI interface.

It was noticed that the background of chromatogram obtained from HPLC-UV was high, while that obtained from HPLC-TOF-MS in the range of m/z 400-500 gave virtually no signal of contaminant. This confirmed that the HPLC-TOF-MS was suitable to analyse tetracycline and its related compounds in waste water more than HPLC-UV.

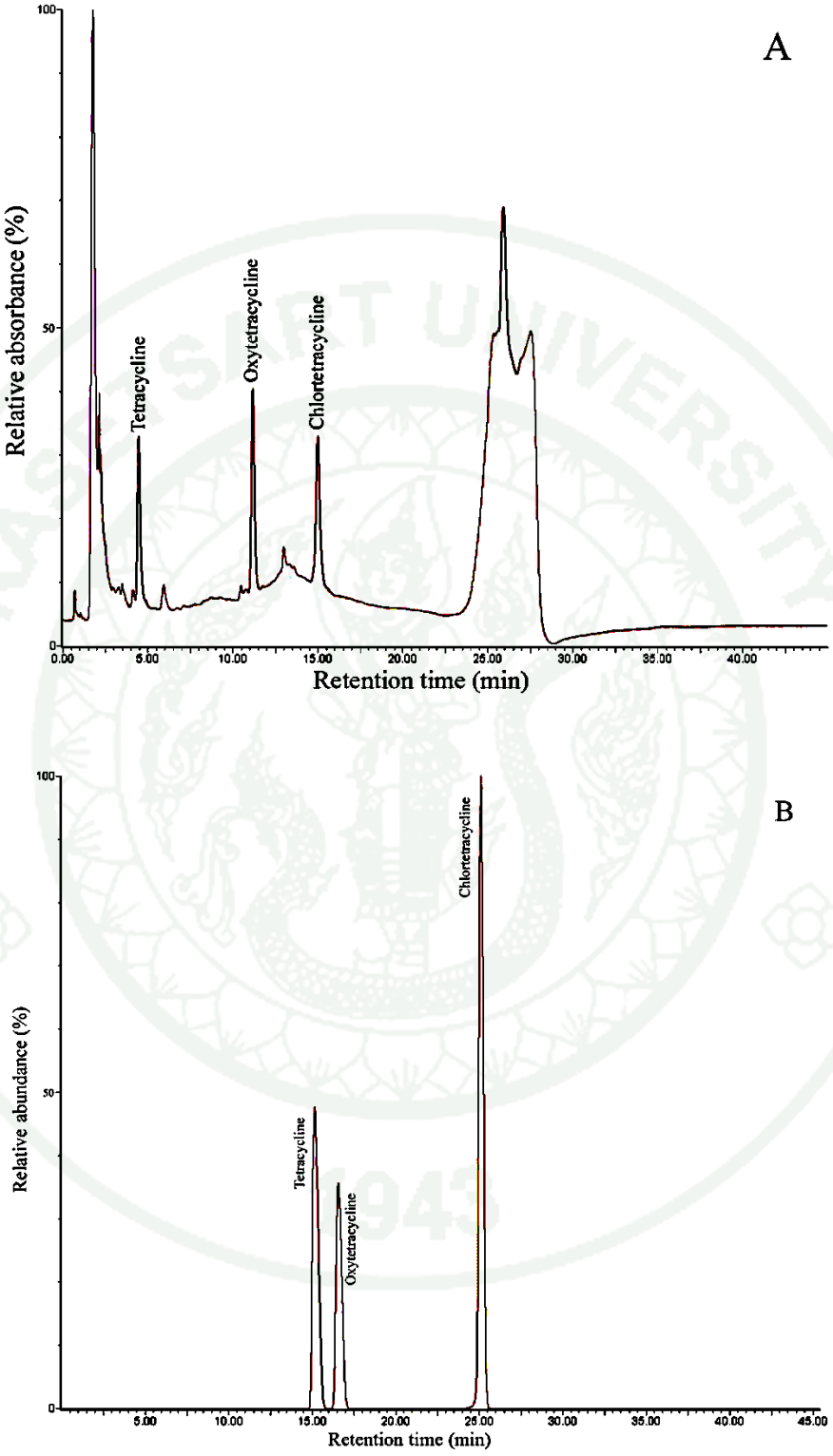


Figure 21 Chromatogram of tetracycline and its related compounds at 50 mg/L in waste water, using (A) UV detector and (B) TOF-MS detector.

9. Real waste water samples

The waste water sample was collected from swine farm located at Pratumthani, Thailand. It was preconcentrated by using a commercial SPE cartridge and the extract was directly injected into the TOF-MS. In the mass spectrum with negative ion mode matrix compounds were found in the mass range of 60-320 and for the higher mass no peak was detected. The extract was subsequently injected into the HPLC-TOF-MS under optimized conditions in a positive ion mode. The chromatogram showed no peak of those analysts and contaminants were detected in the mass range of 400-500. The result form TIC proved no analytes in the waste water sample.

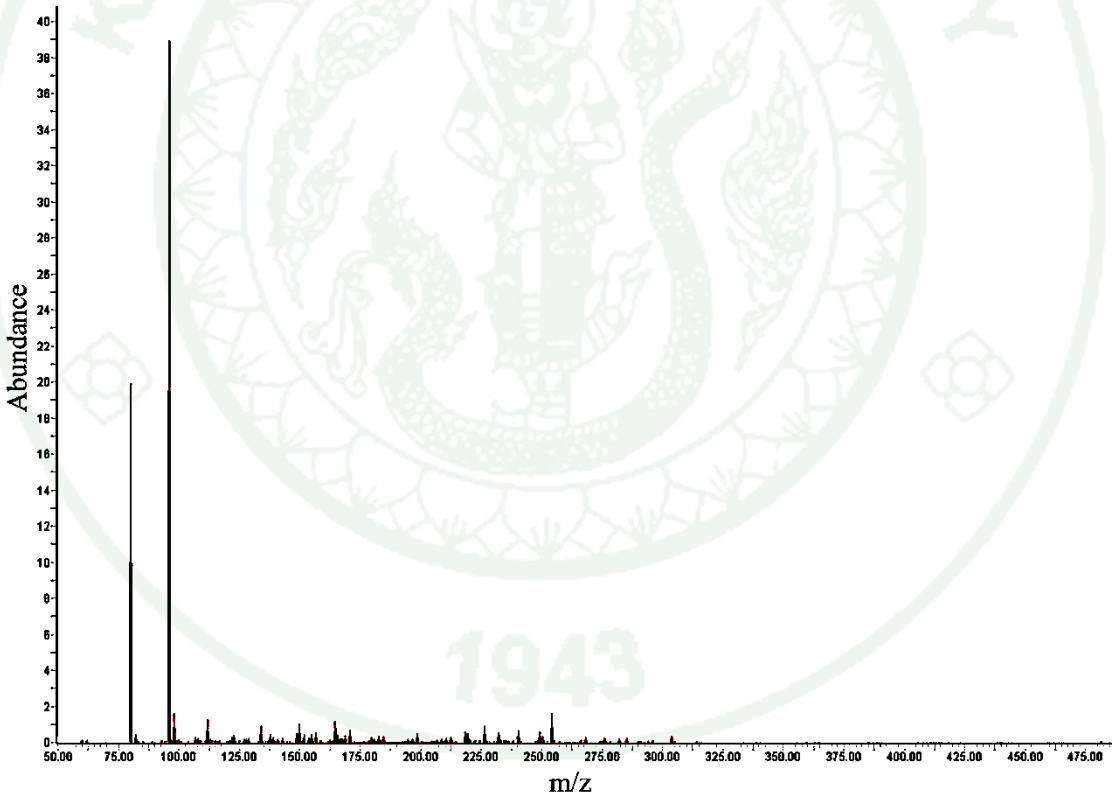


Figure 22 Mass spectrum of the extract of waste water with negative ion mode

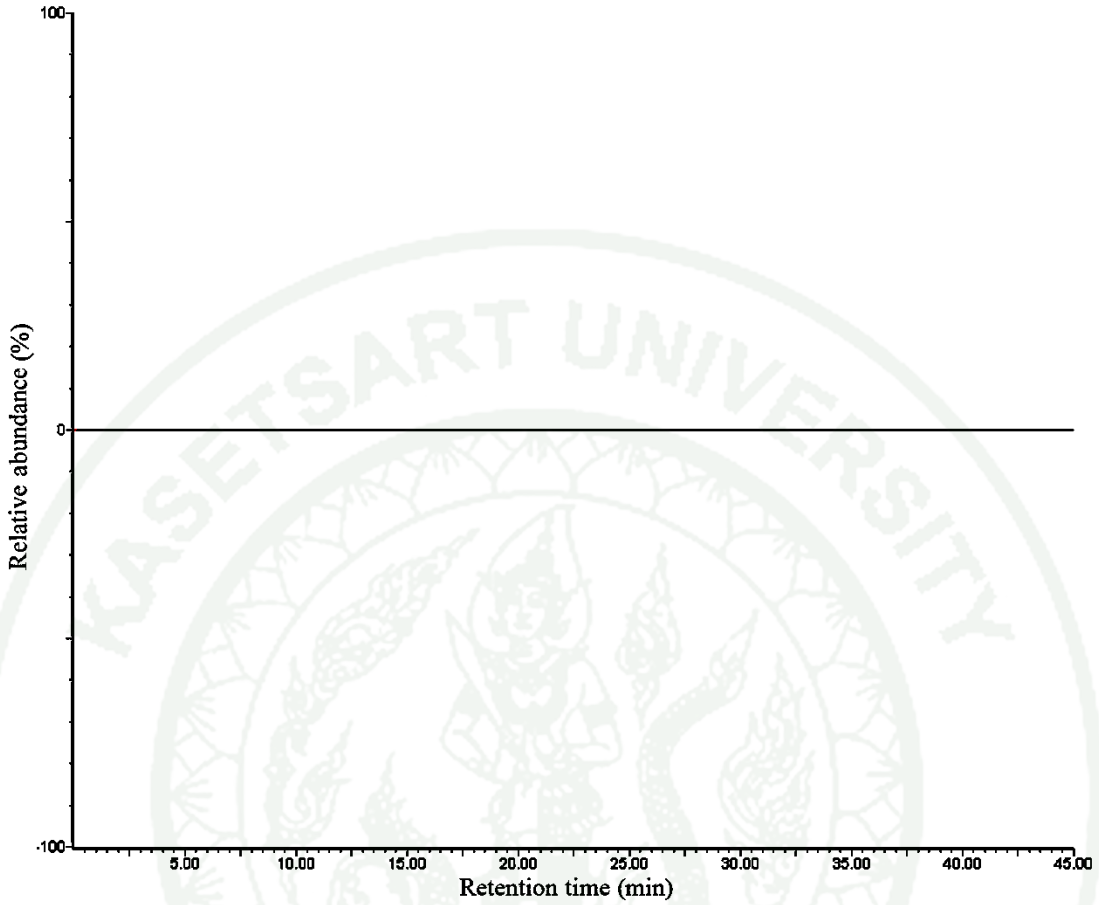


Figure 23 Total ion chromatogram in a mass range of 400-500 of the extract of waste water sample .

10. Comparison with other methods

A comparison of the proposed method to the other extraction and chromatographic methods was performed for water and liquid samples, in term of sample mass, sample preparation, analytical method, detection limit and % recovery, as shown in Table 6.

Antibiotics drugs contaminated in the environment occurred in many circumstances, but only some reported the contamination in water and liquid sample. The solid phase extraction (SPE) is the most popular method in many references. For aqueous matrix the most used solid phase adsorbent was hydrophilic lipophilic balanced or HLB- solid phase. HLB-SPE was mostly used in many works because of its wide

spectrum of retention and more reproducibility than C18 solid phase. However, the analysis method of HPLC typically utilises C18 column to separate tetracyclines, therefore the proposed research focused on using monolith as the adsorbent was then possible. The HPLC with UV detector and mass detector was used for analysis tetracycline and related compounds. Since tetracycline and its related compounds give fluorescence, a fluorescence detector has also been utilised in a number of applications, but none of them was carried out for water matrix. The detection limit of the propose method was in the same order of concentration magnitude of those reported for reference 1, 2, and 3, while the recovery was in the same range of those reported for reference 1 and 3, but higher than that of reference 4, and lower than that of reference 2. The relative recovery results in this work were not good as expected, because the high carbon loading of C18-silica monolith SPE was not suitable for highly polar analytes. Compared exactly between the same matrix of the proposed method, reference 2 and 4, the sample volume needed for one analysis was reduced five times. Although the MS/MS detector is more sensitive than UV detector, it has high cost and is more complicated, thus not available in most laboratories.

Table 15 Comparison between methods for determination of tetracycline and related compounds in water and liquid samples

References	Sample matrix	Amount of sample	Preparation method	Analytical method	Compounds	Detection limit	% Recovery
Proposed method	waste water	100 mL	Extracted with C-18 silica monolith SPE	HPLC-UV at 370 nm HPLC-TOF-MS	TC, OTC, CTC	0.11-0.71 mg/L 68.2-70.6 µg/L	68.2-92.7
1 Fletouris et al. (1990)	milk	5 g	Extracted with acetonitrile and used tetrabutyl ammonium reagent for ion pair	HPLC-UV at 355 nm	TC, OTC	-	72.7-85.1
2 Batt *et al.* (2005)	waste water	500 mL	Extracted and preconcentrated by using HLB SPE, eluted with methanol.	HPLC-MS/MS	TC, OTC, CTC and 9 antibiotics	0.03-0.19 Mg/L	108-119 for TC, OTC, CTC
3 Wendy *et al*. (2005)	shrimp and whole milk	2 g shrimp 10 mL milk	Extracted with 10 mL succinic acid, 5 mL 0.05M oxaric and 4 mL water Cleaned up with HLB SPE	HPLC-UV 370 nm and HPLC-MS/MS	TC, OTC, CTC	50-400 µg/kg	Shrimp 76.8-93.2 Milk 79.8-87.7
4 Ye et al. (2007)	drinking water	500 mL	Preconcentrated with HLB, eluted with methanol and 0.1%formic in methanol	HPLC-MS/MS	tetracycline, sulfonamines, macrolide, andquinolone	0.5-2.0 ng/L	54-76 for TC, OTC and CTC

CONCLUSION

In this work, a study in fabrication organic monolithic SPE for extracteing tetracycline and related compounds from waste water, namely tetracycline, oxytetracycline and chlortetracycline was performed. The HPLC-UV and HPLC-TOF-MS was employed for validating organic monolithic SPE efficiency and quantitatively analysed tetracycline groups in waste water.

The organic silica monolithic was synthesized in a 5-mL syringe via a sol-gel method. The monolith synthesis involved with two steps. In the first step, the organic monolith was synthesized in a centrifuge tube and it was sieved to obtain coarse monolithic clusters. In the second step, the second monolith formulation solution was poured into the cluster-filled syringe, leaving the liquid on the cluster surface for re-mastering to a single piece. Morphological verification by SEM proved the monolithic structure with less than 1 μm particles and no gap between clusters. The fabricated monolithic SPE was used to extract and pre-concentrate tetracycline and its related compounds from waste water. While loading the water sample, the monolithic SPE produced lower back pressure than the commercial SPE and the TOF results showed no signal of analytes in spiked water sample.

The validation method was determined for HPLC-UV and HPLC-TOF-MS for tetracycline determination from waste water. HPLC-UV provided a good performance for validation method when the tetracycline spiked DI water was involved. However for the real waste water samples, contaminant peaks that could not be separated was shown on the chromatogram with UV detection signal. Because TOF-MS is a high resolution mass spectrometer, it can separate each signal by molecular mass completely. The LOD when using TOF mass spectrometer was 0.17, 0.55 and 0.50 mg/L for tetracycline, oxytetracycline and chlortetracycline respectively.

In this research a novel method to fabricate organic monolithtic silica in a container was achieved, with no shrinkage and gap between the monolith and the wall

of container. However, when compared with commercial pack SPE, the recoveries of tetracycline and chlortetracycline were not satisfactory, but the recovery of chlortetracycline was better than that obtained from commercial SPE.

The C18-monolithic SPE had a carbon loading value higher than commercial C18 SPE. The higher carbon loading, the low polarity of stationary phase is. Therefore low polarity of C18-monolithic SPE reduces the adsorption of tetracycline to be lower than commercial SPE.

The advantage of this work was the re-mastering technique for synthesizing monolith in a large size container, such as a typical SPE cartridge without shrinkage. This technique supported the measurement of the cluster and particles size of monolith before re-mastering to obtain one single piece monolith with no gap between the first fabricated monolith and the second one. Therefore, it was feasible to verify quality of C18 silica monolith as a product in manufacturing process.

Future work.

The research presented the novel process to fabricated monolithic without shrinkage, that could be further developed in a number of

1. Repaired old organic silica HPLC or collapsed column

When a C18 organic silica HPLC column has been used for a long time, the efficiency of column usually dropped, because of some reasons, such as the organic chains was collapsed, and the high and unstable pressure caused broken particles. Contamination of highly adsorbed compounds at the beginning of the column that caused the dropping efficiency may need to be replaced with new C18-particle adsorbent. The proposed fabrication process may be introduced to repair the HPLC column performance. From the research, the procedure of re-mastering the cluster to SPE by loading the second formulation liquid mixture to coat the organic silica particles in the old column. The column will be improved the performance with the new surface. However the performance of the repaired column must be investigated and compared with those new commercial columns.

2. Low cost SPE kit

As the back pressure of the fabricated organic SPE monolith in the cartridge was very low, compared with the commercial pack organic silica SPE, which needed a vacuum chamber with a pressure of at least 15-20 psi, when the water sample was poured onto the organic silica monolith, it flowed through the cartridge in the atmospheric pressure without a vacuum pump. It is therefore supporting a production of low cost SPE kit that can be used without a pump. Although the novel fabrication process can solve the shrinkage and applied for a development in any containers, the homogeneity of clusters size and formula of secondary mixture must be developed to improve for higher quality of the monolith product.

LITERATURE CITED

Alfredsson, G., C. Branzell, K. Granelli and A. Lundstrom. 2005. Simple and rapid screening and confirmation of tetracycline in honey and egg by a dipstick test and LC-MS/MS. **Anal. Chim. Acta** 529: 47-51

Batt, Angela L. and Diana s. Aga. 2005. Simultaneous analysis of multiple classes of antibiotics by ion trap LC/MS/MS for Assessing surface water and ground water contamination. **Anal. Chem.** 77: 2940-2947.

Charles, W.K., A.E. Christina, L.H. Mark, L.K. Patricia, J.H. Kenneth and W.G. Devid. 2008. Indirect evidence of transposon-mediated selection of antibiotic resistance genes in aquatic systems at low-level oxytetracycline exposures. **Environ. Sci. Technol.** 42: 5348-5353

Casella, I.G. and P. Fabio. 2009. Determination of tetracycline residues by liquid chromatography coupled with electrochemical detection and solid phase extraction. **J. Agric. Food Chem.** 57: 8735-8741.

Chen, G. and L. Liu. 2004. Hyphenation of sorbent extraction and solid-matrix time-resolved luminescence using tetracycline in milk as a model analyte. **J. Agric. Food Chem.** 52:7199-7205.

Chen, X., L. Zhao, X. Tian, S. Lian, Z. Huang and X. Chen. 2014. A novel electrochemiluminescence of tetracyclines sensor based on a Uu(bpy)3^{2+}-doped silica nano particles/Nafion film modified electrode. **Talanta.** 129: 26-31

David W.G, O.-R. Susana, W.K. Charles, L. Lazaro, W. David and B. Emma. 2011. Antibiotic resistance gene abundances associated with waste discharges to the almendares river near Havana, Cuba. **Envirron. Sci. Technol.** 45: 418-424

Deng, N., Z. Liang, Y. Liang, Z. Sui, L. Zhang, Q. Wu, K. Yang, L. Zhang and Y. Zhanf. 2012. Aptamer modified organic-Inorganic hybrid silica monolithic capillary column for highly selective recognition of thombin. **Anal. Chem.** 84: 10186-10190

Eelting, S., J.M. Manuel-Martinez, G.P. Rozing, P.J. Schoenmakers and W.T Kok. 2005. Tailoring the morphology of methacrylate ester-based monoliths for optimum efficiency in liquid chromatography. **Anal Chem.** 77: 7342-7347

Fabio G.-C, S. Jorge, G. Fernando and R. Rodriguez. 2012. A novel green chemistry method for nanoqueous extraction and high-performance liquid chromatography detection of first-, second-, and Thired-generation tetracycline, 4-epitetracycline, and tyrosin in animal feeds. **J. Agric. Food Chem.** 50: 7121-7128

Gerd H., S. Silke, H. Heinrich and N. Heinz. 2002. Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. **Anal. Chem.** 74: 1509-1518

Jikun L.,C. Chien-Fu, T. Chia-Wen, C. Chien-Cheng, C. Chin-Chou and L.D. Don. 2009. Polymer microchips integrating solid-phase extraction and high-performance liquid chromatography using reversed-phase polymethacrylate monoliths. **Anal. Chem.** 81: 2545-2554

Khayoon W.S., S. Bahruddin, S. Baharuddin, H.A. Normaliza and A.L. Aishah. 2014. Micro-solid phase extraction with liquid chromatography-tandem mass spectrometry for the determination of aflatoxins in coffee and malt beverage. Food Chemistry 147: 287-294

Lopez M.I., S.P. Jeffery, I.B. Smith and C. Pak-Sin. 2008. Multiclass determination and confirmation of antibiotic residues in honey using LC-MS/MS. **J. Agric. Food. Chem.** 56: 1553-1559

Miaomiao L., Z. Yu, Y. Min, T. Zhe, R. Liren and Z. Shujun. 2012. Abundance and distribution of tetracycline resistance genes and mobile elements in an oxytetracycline production wastewater treatment system. **Environ Sci. Technol.** 46:7551-7557

Michele E.L., M. Michael and E.M. Thurman. 2001. Analysis of trace levels of sulfonamine and tetracycline antimicrobials in groundwater and surface water using solid-phase extraction and liquid chromatography/mass spectrometry. **Anal. Chem.** 73: 4640-4646

Oguri S.,H. Tanakaki, M. Hamaya, M. Kato and T. Oka. 2003. On-line preconcentration prior to on-column derivatization monolith octadecasiloxane capillary electrochromatography for the determination of biogenic amines**. Anal Chem.** 75: 5240-5245

Podgornik A., M. Barut and A, Strancar. 2000. Construction of large-volume monolithic columns. **Anal. Chem.** 72: 5693-5699

Tan A., S. Benetton and J.D. Henion. 2003. Chip-based solid-phase extraction pretreatment for direct electrospray mass spectrometry analysis using an array of monolithic columns in a polymeric substrate. **Anal. Chem.** 75: 5504-5511

Wang S.T., M.Y. Wang, X. Su, B.-F Yuan and Y.-Q. Feng. 2012. Facile preparation of SiO_2/TiO_2 composite monolithic capillary column and its application in enrichment of phosphopeptides. **Anal. Chem.** 84:7763-7770

William A. Moats. 2000. Determination of tetracycline antibiotics in beef and pork tissues using ion-paired liquid chromatography. **J. Agric. Food. Chem.** 48: 2244-2248

Wendy C.A., J.E. Roybal, S.A Gonzales, S.B. Turnipseed, A.P. Pfenning and L.R. Kuck. 2005. Determination of tetracycline residues in shrimp and whole milk using liquid chromatography with ultraviolet detection and residue confirmation by mass spectrometry. **Anal. Chim. Acta**. 529: 145-150

Wu Q., J.M. Bienvenue, B.J. Hasson, Y.C. Kwok, B.C. Giordano, P.M. Norris. J.P. Landers and JP. Ferrance. 2006. Micro-ship based macroporous silica sol-gel monolith for efficient isolation of DNA from clinical samples. **Anal. Chem.** 78: 5704-5710

Yang T.-T, L.-F. Zhou, J.-Q Qiao, H.-Z. Lian, X. Ge and H.-Y Chen. 2013. Preparation of poly(trimethyl-2-methacroyloxythylammonium chloride-co-ethylene glycol dimethacrylate) monolith and its application in solid phase microextraction of brominated flame retardants**. J. Chromatogr. A.** 1291: 1-9

Zhang W., S.M. Belinda, W.K. Charles and W.G. David. 2009. Accumulation of tetracycline resistance genes in aquatic biofilms due to periodic waste loading from swine lagoons. **Environ. Sci. Technol.** 43: 7643-7650

APPENDICES

Appendix A

Proceedings

Khaunmeung, K., Srisuksawad, K., Chienthavorn, O., Novel Solid-Phase Microextraction Coupled with LC-MS Determination of Tetracycline from Waste Water, *Proceedings Pure and Applied Chemistry International Conference 2014*, 8-10 Jan 2014.

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NOVEL SOLID-PHASE MICROEXTRACTION COUPLED WITH LC-MS DETERMINATION OF TETRACYCLINE FROM WASTE WATER

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Abstract: In this study, C-18 silica monolithic material was fabricated via a sol-gel method, and used as an adsorbent in extraction and preconcentration step of tetracycline and its related compounds from waste water samples. The monolithic structure was verified by using a scanning electron microscope (SEM). Three drug compounds in tetracycline group, namely oxytetracycline, chlortetracycline and tetracycline was extracted and preconcentrated using the developed solid sorbent prior to high performance liquid chromatography coupled with time-of-flight mass spectrometry (LC-TOF-MS). With very high active surface of the synthesized monolith the mass required for adsorption is lowered, thus reducing the consumption of organic eluent and time required for preconcentration step. The preconcentration factor of tetracycline from water of 50 was obtained.

1. Introduction

Tetracycline is a broad spectrum antibiotic drug that has been used extensively because it gives an effect to bacteria, low toxicity for human. It is also compounded into a part of animal feed and water for prevention and treatment of micro-organism. The livestock can consequently excrete the drugs from the body, leaving them in urine and feces, which are eventually discarded to environment, especially in the water resources nearby farm of which environment supports the contaminants to easily widespread.

In the past, the impact of tetracycline in environment was not of interest, because the tetracycline and its metabolites have short half-lives in nature. Nevertheless, tetracycline gives an acute effect to micro-organism. Destruction of microbes severely damages the food chain in the impact area. Since the long-term effect has not been investigated, the monitoring must then be performed. An extremely low concentration of analytes in water resources makes conventional analysis difficult. Presently many analytical methods have been developed for quantification and qualification of those antibiotics of trace level in water. Liquid chromatography coupled with mass-spectroscopy (LC-MS) is a method for the antibiotic determination after extraction and preconcentration. In this research monolithic solid phase extraction (SPE) is introduced as a novel type of solid phase extraction, supporting the adsorption and preconcentration of ultra-trace chemicals in aqueous solution.

Monolithic material was introduced in chromatography for two decades as a stationary phase. Monolithic stationary phases have grown in interest because of several advantages such as, enhanced surface area, low back pressure, high loading capacity superior to regular packed columns and time-saving process. Monolithic material can be divided into different groups on the basis of specific chemical base. There are organic-based, inorganic-based, and mixed mode based. The mixed mode base monolith is a combination between organic and inorganic cross-liked polymer. For example, pure silica monolithic surface exhibits inorganic functionality, however, when organic silica monomer is used in the polymeric reaction, it forms organic silica monolith, which is a mixed mode based material. In this study, C18-organic silica monolith base is of interest to be fabricated and used as an adsorbent, according to its extraction compatibility to tetracycline chemical property.

Monolithic solid phase can be synthesised in a syringe tube where the particles range are in micro- or nano-size, thus comparing with an equivalent amount of the conventional solid phase material of which particle size is larger, the monolith provides higher surface area. Its special properties offer many advantages over a classical solid phase, such as improved performance, reduced size of material, high contained substance, and etc. The new material is therefore expected for excellent adsorption of trace tetracycline and its metabolites in waste water. The organic silica monolith is synthesised by a sol-gel method with an optimised formulation for very small size particles.

2. Materials and Methods

2.1 Chemicals and reagent

Tetramethoxyothosilicate (TMOS) was purchased from Merck (Hohenbrunn, Germany). Trimethoxy(octadecyl)silane (C-18-TMOS) was bought from Aldrich (St. Louis, USA). *N*-Dodecylamine of 98% purity was obtained from Fluka (USA). Methanol of HPLC grade was purchased from RCI-Labscan (Bangkok, Thailand), while water was purified by a water purification system (Cascada RO Lab water system, Pall Co., NY, USA).

2.2 Instruments and analytical conditions

For detection a liquid chromatograph (Waters 2695, MA, USA) was coupled with time-of-flight mass spectrometer LC-TOF-MS system (Waters Micromass LCT Premier, MA, USA) operated by using a MassLynx 4.0 software (Milford, Massachusetts, USA). The separation was carried out on a 50 mm x 4.6 mm i.d. C18 column (Phenomenex, CA, USA). with 2.6 mm particle size under an isocratic condition of a mobile phase consisting of 70% water and 30% methanol by volume and the flow rate of 0.2 mL.min^{-1}. The injection volume was 20 µL. The mass spectrometer was operated in a positive electrospray ionization (ESI) mode with a capillary voltage of 3500 V and sample cone voltage of 70 V. Desolvation temperature was set at 150 ^{o}C and the source temperature was 100 ^{o}C.

2.3 Preparation of silica monolith SPE device

2.3.1 Preparation of C-18 silica monolithic clusters

To obtain monolith cluster a 50 mL polypropylene centrifuge tube was washed with 3 x 50 ml distilled water to remove any possible particulates. The tube was then dried at 60 ^{o}C for 3 hours. The formulation mixture was a solution of 4,000 µL methanol, 200 µL 0.5 M HCl, 700 µL water, 800 µL C18-TEOS and 3200 µL TEOS were mixed thoroughly in a plastic centrifuge tube using a vortex mixer. After sealing the tube, the mixture was hydrolysed at 60 ^{o}C for 4 h, and then cooled down to room temperature. After that 100 µL *n*-dodecylamine was added into the solution to form while solid. The tube was uncovered and dried at 40 ^{o}C for 24 h. Organic monolithic solid was formed inside the tube, however, shrinkage of the monolith occurred, leaving the gap between the monolith and the tube wall. The monolith was grinded to obtain coarse monolithic clusters.

2.3.2 Fabrication of monolithic silica solid phase for extraction

A 3 ml polypropylene syringe was cleaned with distilled water, and dried at 60 ^{o}C for 3 hours. Weigh 300 mg of the synthesised organic monolithic cluster into the syringe. In the syringe glass wool was put at the bottom to protect a removal of monolithic cluster. The filled syringe was equipped with a SPE assembly.

A 400 µL methanol, 20 µL 0.5 M HCl, 70 µL water, 40 µL C18-TEOS and 160 µL TEOS were mixed in a 1.5 mL Eppendorf vial. The vial was sealed and the hydrolysis occurred at 60 ^{o}C for 4 h. The hydrolysed liquid mixture was suddenly poured onto the cluster-filled syringe, leaving the liquid on the cluster surface. A 10 µL *n*-dodecylamine was added into the coated cluster to form "doubly fabricated monolith", which is used as monolithic solid phase for the following experiment. The fabricated monolith in syringe was rinsed with 10 ml of methanol and dried at 35 ^{o}C for 72 h in a incubator.

2.4 Solid phase extraction

The fabricated monolithic SPE cartridge was activated by using 3 ml methanol, followed by 4.5 ml water. After that 100 ml water sample was directly loaded onto the monolithic SPE cartridge under applied vacuum at a flow rate of 2 ml min^{-1}. A 100 ml 10 ppm of each spiked analyte in water was poured onto the cartridge fitted with the SPE assembly. The adsorbed analytes were eluted with 2 ml of methanol, and collected in a 1 dram vial. The eluent was adjusted the volume to 2 ml using methanol.

3. Results and Discussion

3.1. Monolithic silica SPE characteristics

Since the morphology of monolithic material depends much on formulation and reaction conditions, in this work the formulation of fabrication of C18-silica monolith was adapted from the work of Chen *et al*. (2010) and all conditions were reoptimised. The most optimal formulation was previously described in the section 2.3. Figure 1 shows the characteristic feature of the doubly fabricated C18-silica monolith under a scanning electron microscope (SEM). The well-defined monolithic structure with uniformly interconnected particles of less than 1 µm was obtained. No gap between clusters were found.

In a typical monolith formation, volatile porogens are continuously leaving the skeleton, resulting cavities which haul the monolithic structure to crack and/or shrink. However, in our method silica monolith cluster are well-packed and connected each other, so the effect of loss porogens was reduced. The particle size of silica monolithic clusters was verified by SEM before filling in a container, such as dropper, syringe pipette tip and etc. Therefore, size of particle can also be controlled for high reproducibility.

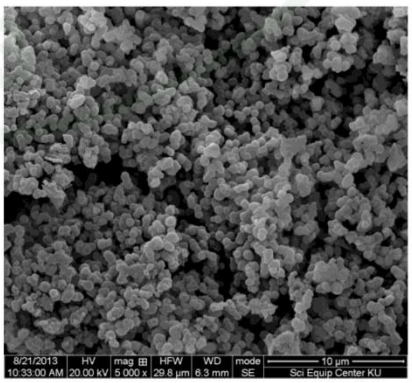

Figure 1. SEM images of C18-silica monolith in the syringe.

3.2 Separation of tetracycline and its related compound mixture by LC-TOF-MS

A mixture of tetracycline, oxytetracycline and chlortetracycline was identified by different molecular mass and retention time under the LC-TOF-MS conditions previously described. The chromatograms of each compound are shown in Figure 2. Although it was observed that the tetracycline and oxytetracycline peaks were overlapped, the separation and identification of each component could be achived by the exact molecular mass difference, supported by the time-of-flight MS.

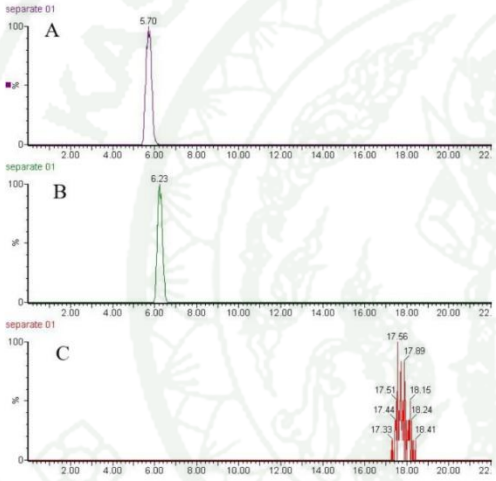

Figure 2. LC-MS chromatogram of tetracycline (a), oxycycline (b), and chlortetracycline (c) extracted from water sample.

3.3 Relative recoveries

The relative recoveries were performed from a 1 ppm spiked analyte in water sample. It was found that all analytes gave relative recoveries of 62.0, 55.2, 27.6 for tetracycline, oxytetracycline and chlorcycline, respectively. The low relative recoveries occurred because *n*-dodecylamine residues reduced dissociation of tetracycline and its metabolites, resulting the preconcentration factor of tetracycline from water of only 50.

4. Conclusions

In this work, a doubly fabricated organic silica monolith was synthesised. Our novel fabrication method overcomes the problems of sol-gel methods, such as shrinkage, reproducibility of particle size.

Although the relative recoveries of tetracycline and its related compounds were rather low, the good aspects of the method were proven. When considering the high matrix effect of complicated water sample, the HPLC-TOF-MS could be a good solution, owing to the high mass resolution.

Acknowledgements

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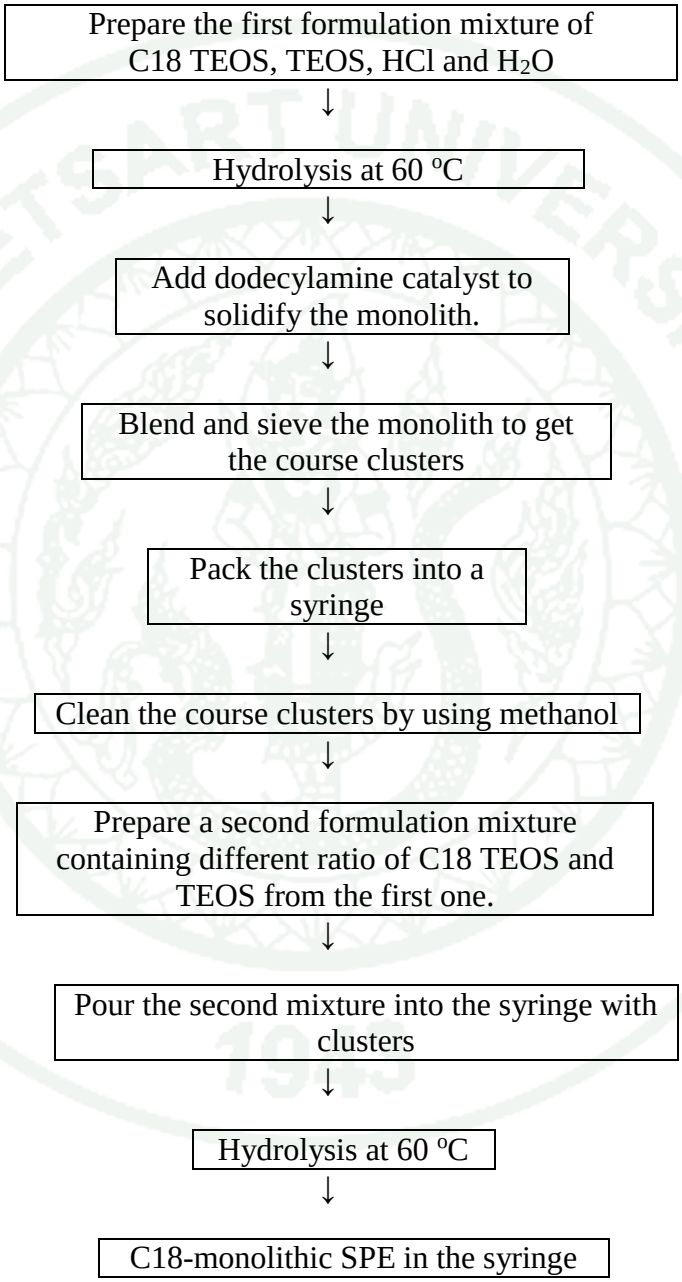
References

[1] M.L. Chen, M.M. Zheng and Y.Q. Feng, *J. Chromatogr. A*. 1217 (2010) 3547-3556.
[2] D. Peronia, D. Vanhouttea, F. Vilaplanaa, P. Schoenmakersa, S. Koningb,S and H.G. Janssena, *Anal. Chimi. Acta.* 720 (2012) 63-70.
[3] W. Zhang and Z. Chen, *Talanta* 103 (2013) 103-109.
[4] M. Zheng, G.D. Ruan, Y. and Feng,Y, *J. Chromatogr. A* 1216 (2009) 7739-7746.
[5] T.T. Yang, L.Zhou, J. Qiao, H. Lian, X. Ge and H.Chen, *J. Chromatogr. A* 1291 (2013) 1-9
[6] T. Nema, E.Chan and P.C. Ho, *Talanta*. 82 (2010) 488-49

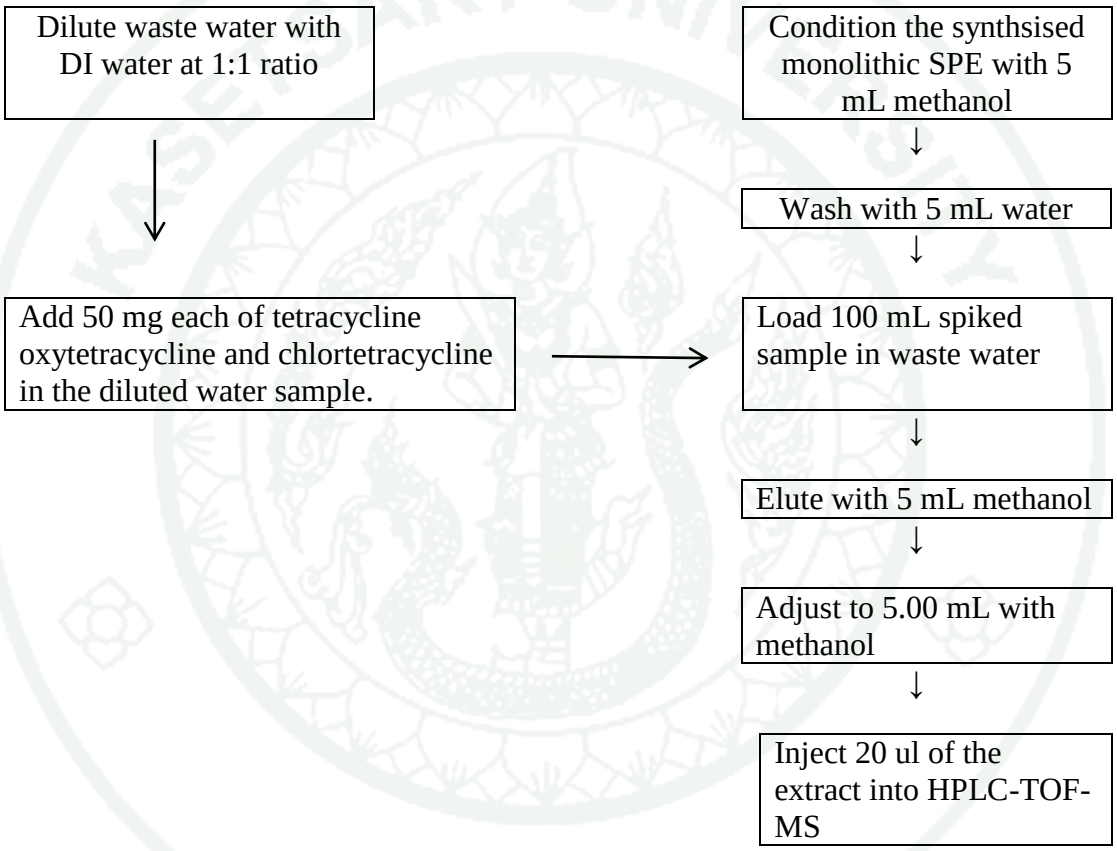
Appendix B

Summary of procedure of fabrication of C18-monolith SPE and tetracycline determination in waste water by using C18-monolith SPE

Flowchart of procedure of C18 monolithic SPE fabrication



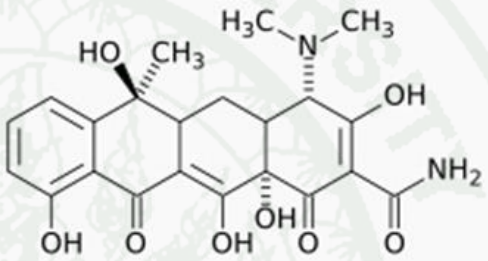
Flowchart of procedure of a determination of spiked tetracycline in waste water by using monolith SPE



Appendix C

Chemical information of tetracycline

Appendix Table C1 Chemical information of tetracycline (TC)

CAS Number	: 60-54-8
IUPAC name	: (4*S*,6*S*,12a*S*)-4-(dimethylamino)-1,4,4a,5,5a,6,11, 12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxonaphthacene-2-carboxamide
Molecular Formula	: $C_{22}H_{24}N_2O_8$
Structural Formula	: 
Molecular Weight (g/mol)	: 444.435
Solubility	: Soluble in water (1.7 mg/mL) and methanol (> 20 mg/mL) at 25 °C
LD50 (rat)	: 6443 mg/Kg

References :

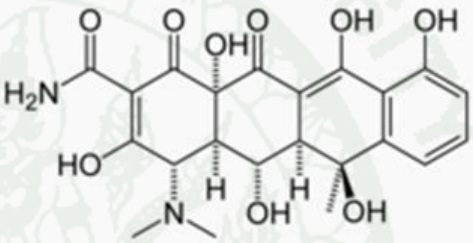
http://en.wikipedia.org/wiki/Tetracycline (access date : 26/6/2014)

http://www.scbt.com/datasheet-205858-tetracycline.html (access date : 26/6/2014)

Appendix D

Chemical information of oxytetracycline

Appendix Table D1 Chemical information of oxytetracycline (OTC)

CAS Number	: 79-57-2
IUPAC name	: (4*S*,4a*R*,5*S*,5a*R*,6*S*,12a*S*) -4-(dimethylamino)-3,5,6,10,11,12a-hexahydroxy -6-methyl-1,12-dioxo-1,4,4a,5,5a,6,12,12a-octahydrotetracene -2-carboxamide
Molecular Formula	: $C_{22}H_{24}N_2O_9$
Structural Formula	: 
Molecular Weight (g/mol)	: 460.434
Solubility	: water (<1 mg/mL) and ethanol (10 mg/mL) at 25 °C
LD50 (rat)	: 6696 mg/Kg

References :

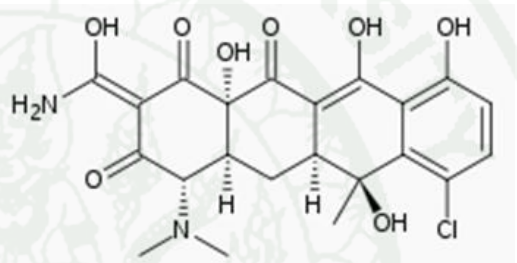
http://en.wikipedia.org/wiki/Oxytetracycline (access date : 26/6/2014)

http://www.scbt.com/datasheet-205784-oxytetracycline.html (access date : 26/6/2014)

Appendix E

Chemical information of chlortetracycline

Appendix Table E1 Chemical information of chlortetracycline (CTC)

CAS Number	: 57-62-5
IUPAC name	: (4*S*,4a*S*,5a*S*,6*S*,12a*S*,*Z*)-2-[amino(hydroxy) methylene]-7-chloro-4-(dimethylamino)-6,10,11,12a-tetrahydroxy-6-methyl-4a,5,5a,6-tetrahydrotetracene-1,3,12(2*H*,4*H*,12a*H*)-trione
Molecular Formula	: $C_{22}H_{23}ClN_2O_8$
Structural Formula	: 
Molecular Weight (g/mol)	: 478.88
Solubility	: water (< 0.5 mg/mL) at 25 °C
LD_{50} (rat)	: 1500 mg/Kg

Tetracycline and related compounds are broad spectrum antibiotics. They were indicated used against many bacteria infections. They work by interfering with the ability of bacteria to produce essential proteins. Without these proteins, the bacteria cannot grow, multiply and increase in numbers

References :

http://en.wikipedia.org/wiki/Chlortetracycline (access date : 26/6/2014)

http://iaspub.epa.gov/tdb/pages/contaminant/contaminantOverview.do?contaminantId=10240 (access date : 26/6/2014)

Appendix F

(Related to Table 4)

Data obtained for a determination of detection limit of each tetracycline by using HPLC-TOF-MS determination

Appendix Table F1 Lowest concentration sample peak area of tetracycline and related compounds obtained from mass spectrometric detector.

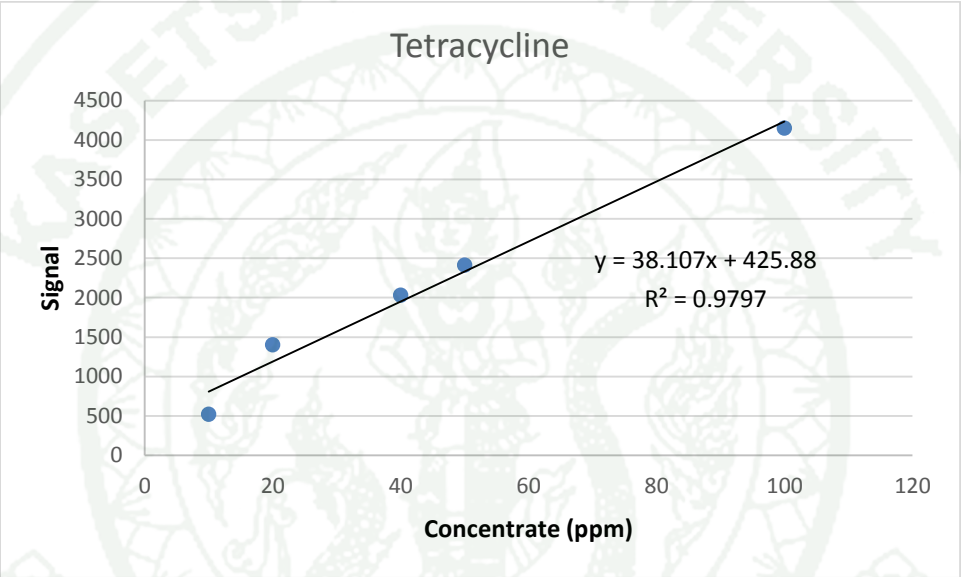
Replicate	Peak area		
	Tetracycline	Oxytetracycline	Chlortetracycline
1	482	18	46
2	576	17	59
3	463	26	55
4	555	11	43
5	551	15	42
6	448	14	51
7	443	11	31
8	445	17	54
9	538	10	29
10	548	18	29

Appendix G

(Related to Table 5)

Example for calculation of the detection limit of method of HPLC-TOF-MS

A calibration curve of tetracycline was prepared from peak area of tetracycline injected in HPLC-TOF-MS under the optimal condition. In this sample, the concentration of tetracycline linearity ranging from 10-100 mg/L was analysed and the peak area of tetracycline was plotted against the concentration as shown in Appendix Figure E1.



Appendix Figure G1 Calibration curve of tetracycline

The average peak area, stand deviation (SD) and three times of standard deviation (3SD) of tetracycline and related compound were determined by 10 injections of blank sample. In this sample, the average peak area, SD and 3SD of tetracycline were 504.90, 50.58 and 151.73 respectively.

The detection limit was calculated from the equation, $y-y_B = 3SD$, while y is the true detection limit and y_B is the average peak area of blank sample

$$y = y_B + 3SD$$

$$y = 504.90 + 151.73$$

$$y = 555.48$$

When y was replaced in the linear equation from Appendix Figure E1, then x was calculated as the true detection limit as follows:

$$555.58 = 38.107x + 425.88$$

$$x = (555.48 + 425.88)/38.107$$

$$x = 3.40 \text{ mg/L}$$

and the detection limit in the unit of mg/kg can be calculated as

$$\text{Concentration (mg/L)} = \left(\frac{\text{obtained concentration from curve (mg/L)}}{\text{preconcentrationfactor}}\right)$$

$$= 3.40 \text{ mg/L} / 20$$

$$= 0.17 \text{ mg/L}$$

Therefore, the detection limit of tetracycline was 0.17 mg/L.

Appendix H

(Related to Table 8)

Data of peak area of tetracycline and related compounds for the determination of accuracy by using HPLC-UV

Appendix H1 Peak area of accuracy test of three tetracyclines at the concentration of 20, 50 and 80 ppm determined by using HPLC-UV detection

Compounds	Peak area			Average	% RSD
	1	2	3		
Tetracycline					
20 ppm	66260	69313	66941	67505	2.37
50 ppm	159182	159149	159064	159132	0.04
80 ppm	265784	267245	266936	266655	0.39
Oxytetracycline					
20 ppm	63954	63934	64203	64030	0.23
50 ppm	218350	218852	218300	218501	0.15
80 ppm	358850	360521	360668	360013	0.28
Chlortetracycline					
20 ppm	13801	13519	13550	13623	1.75
50 ppm	71235	71418	71128	71260	0.50
80 ppm	123366	123419	123493	123426	0.14

Appendix I

Example for calculation of the accuracy test of three tetracyclines at the concentration of 20, 50 and 80 ppm determined by using HPLC-UV detection

A accuracy of tetracycline was prepared from relative standard deviation of replication in the same concentration. In this sample, The concentration of tetracycline were 20, 50 and 80 ppm of each three replicate. The relative standard deviation of replication was calculated as below ;

$$s = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}$$

% RSD = (s / average peak area) x 100

The peak area of tetracycline of 20 ppm were 66260, 69313 and 66941 respectively.

S = SQRT $((66260-67504.67)^2 + (69313-67504.67)^2 + (66941-67504.67)^2 / 3-1)$

= SQRT ((1549203.41 + 3270057.39+317723.87) / 2)

= 1602.652

%RSD = (1602.652/67504.67) x 100

= 2.37

Appendix J

(Related to Table 10)

Lowest concentration sample peak area of tetracycline and related compounds for examining the standard deviation (SD) for HPLC-UV

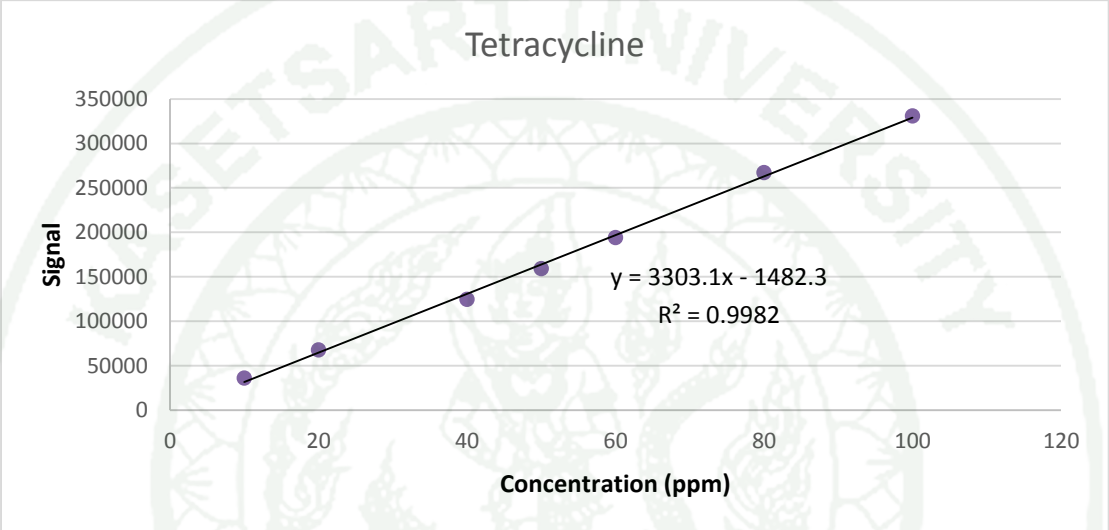
Appendix Table J1 Lowest concentration sample peak area of tetracycline and related compounds obtained from ultraviolet detector.

Replicate	Peak area		
	Tetracycline	Oxytetracycline	Chlortetracycline
1	1663	1905	1180
2	1591	1822	1204
3	1530	1534	457
4	1478	1631	1547
5	1753	1842	1643
6	1993	1519	878
7	1874	1483	842
8	1636	1719	1266
9	1887	1369	973
10	1855	1442	1157

Appendix K

Example for calculation of the detection limit of method of HPLC-UV

A calibration curve of tetracycline was prepared from peak area of tetracycline injected in HPLC-UV under the optimal condition. In this sample, the concentration of tetracycline linearity ranging from 10-100 mg/L was analysed and the peak area of tetracycline was plotted against the concentration as shown in Appendix Figure F1.



Appendix Figure K1 calibration curve of tetracycline

The average peak area, stand deviation (SD) and three times of standard deviation (3SD) of tetracycline and related compound were determined by 10 injections of the lowest concentration sample. In this sample, the average peak area, SD and 3SD of tetracycline were 1726, 163.31 and 489.93 respectively.

The detection limit was calculated from the equation, $y-y_B = 3SD$, while y is the true detection limit and y_B is the average peak area of blank sample

$$y = y_B + 3SD$$

$$y = 1726.0 + 163.31$$

$$y = 2215.93$$

When y was replaced in the linear equation from Appendix Figure E1, then x was calculated as the true detection limit as follows:

$$2215.93 = 3303.1x + 1482.3$$

$$x = (2215.93 + 1482.3)/3303.1$$

$$x = 1.41 \text{ mg/L}$$

and the detection limit in the unit of mg/L can be calculated as

$$\text{Concentration (mg/L)} = \frac{(\text{obtained concentration from curve (mg/L)}}{\text{preconcentration factor)}}$$

$$= 1.41 \text{ mg/L} / 20$$

$$= 0.0706 \text{ mg/L}$$

Therefore, the detection limit of tetracycline was 0.0706 mg/L or 70.6 µg/L.

Appendix L

(Related to Table 13)

Sample peak area of tetracycline and related compounds for recovery test and regression equation with HPLC-UV

Appendix Table L1 Sample peak area of tetracycline and related compounds obtained from HPLC-UV detection for recovery test.

Compounds	Peak area		
	Tetracycline	Oxytetracycline	Chlortetracycline
C18 monolith SPE 01	82500	91494	62788
C18 monolith SPE 02	79304	88235	63120
C18 monolith SPE 03	76054	85528	63575
Commercial SPE 01	98987	104389	62788
Commercial SPE 01	97913	102788	63120
Commercial SPE 01	98641	101470	63575

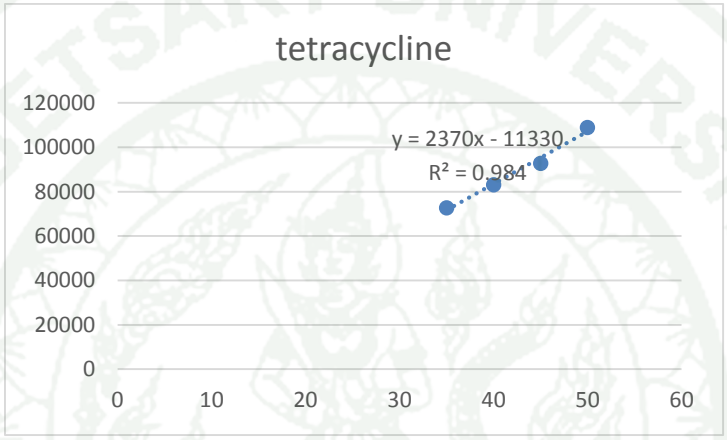
Appendix Table L2 Linear equation for the recovery test

Compounds	Regression equation	r^2
Tetracycline	y = 2370x – 11330	0.9840
Oxytetracycline	y = 2720.5x – 10264	0.9972
Chlortetracycline	y = 1850x – 22220	0.9943

Appendix M

Example for calculation of recovery.

A calibration curve of tetracycline was prepared from peak area of tetracycline injected to the HPLC-UV detector under the optimal conditions. In this sample, the concentration of tetracycline linearity ranging from 35-50 mg/L was analysed and the peak area of tetracycline was plotted against the concentration as shown in Appendix Figure K1.



Appendix Figure M1 Calibration curve of tetracycline

The standard solution of tetracycline at concentration of 50 mg/L was spiked into DI water sample (n=3) followed by extraction procedure in Appendix B. Three extracted spike sample were injected into the HPLC-UV. The average of peak area was calculated, resulting 79286 and the value was put into the peak area (y) in the linear equation y = 2370x – 11330, then the concentration (x) was calculated as follows:

$$79286 = 2370x – 11330$$

$$x = 38.23 \text{ mg/L}$$

The tetracycline concentration of experiment and theoretical spiked sample in the unit of µg/L were 35.29 and 50.00, respectively. % recovery can be calculated as

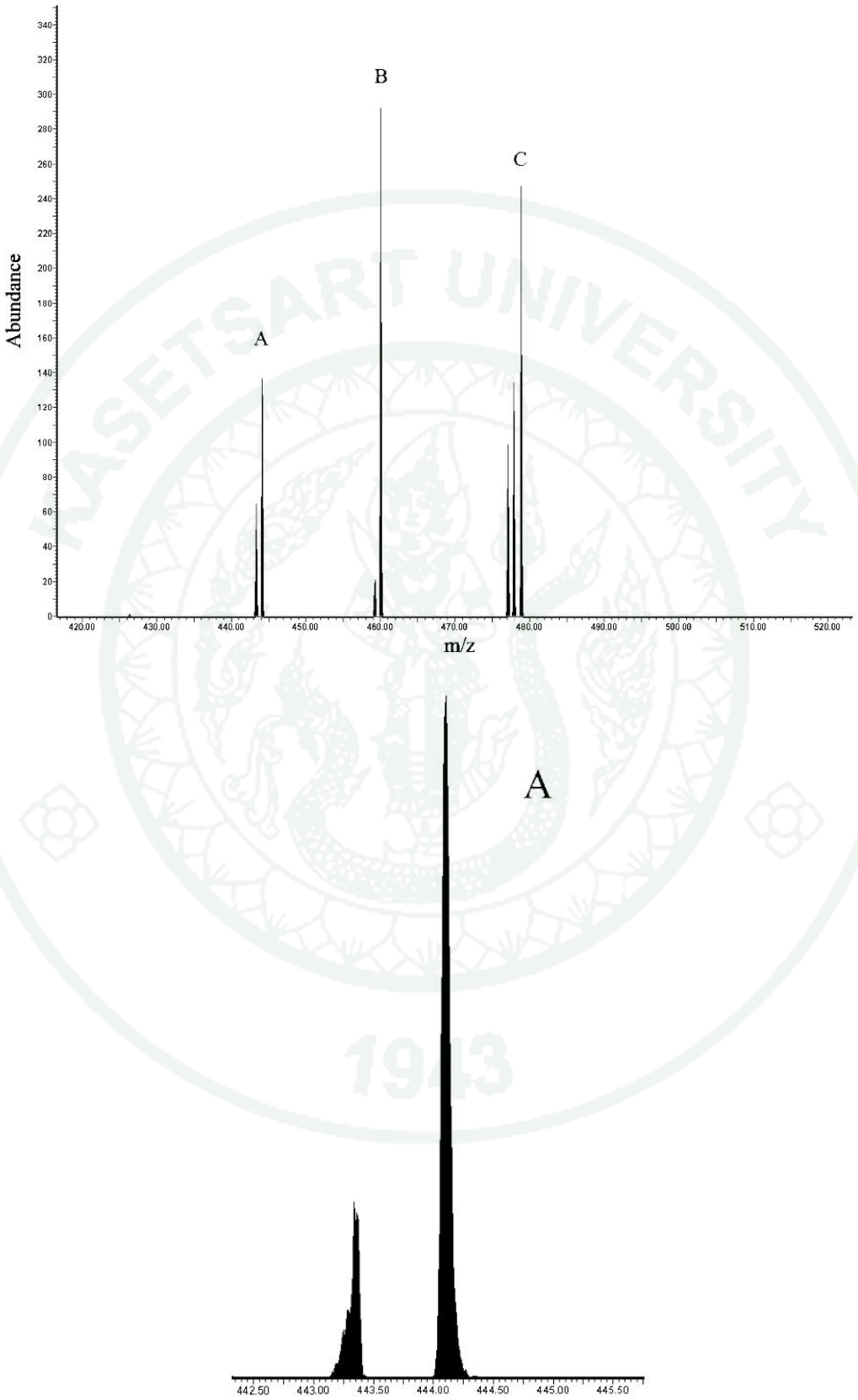
% Recovery = (concentration of experimental spiked sample / concentration of theoretical spiked sample) x 100

= (38.23/50.00) x 100

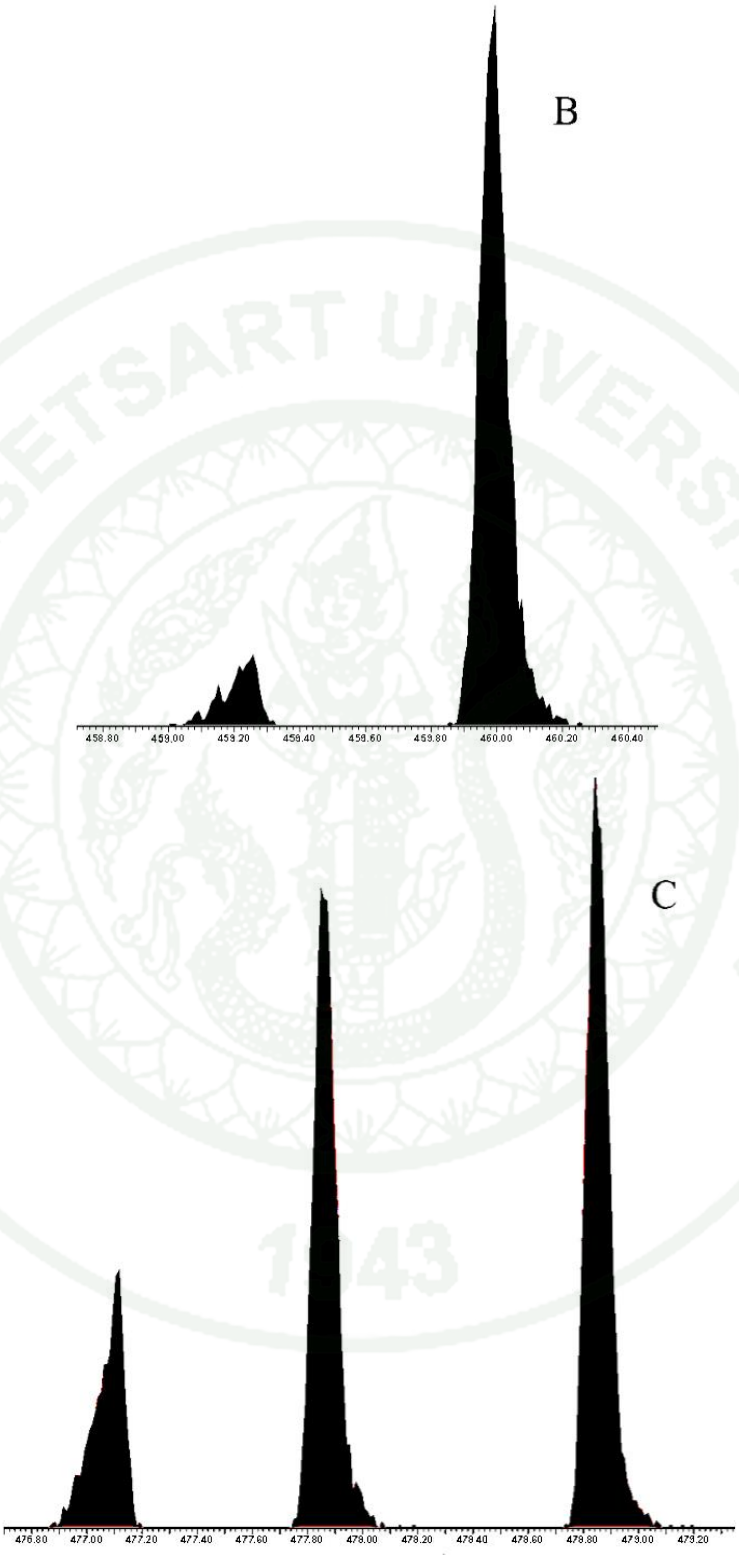
= 76.46

Appendix N

Mass Spectrum of tetracycline, oxytetracycline and chlortetracycline.



Appendix Figure N1 Mass Spectrum of tetracycline(A), oxytetracycline(B) and chlortetracycline(C)



Appendix Figure N1 Mass Spectrum of tetracycline(A), oxytetracycline(B) and chlortetracycline(C) (Continued)

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