8-20-2014 12:00 AM

Solid-phase Extraction as Sample Preparation for Bioassay-based Micropollutant Quantification

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A thesis submitted in partial fulfillment of the requirements for the Master of Engineering Science degree in Chemical and Biochemical Engineering

SOLID-PHASE EXTRACTION AS SAMPLE PREPARATION FOR BIOASSAY-BASED MICROPOLLUTANT QUANTIFICATION

by

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Master in Engineering Science

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Abstract

Solid phase extraction (SPE) using chemically bonded silica particles or small particles of an organic polymer resin, is being studied extensively for extraction of polar or non-polar compounds from various water matrices. This study focused on the evaluation of the performance of three commercial cartridges belonging to three different groups: reversed-phase, mixed-mode anion exchanger and mixed-mode cation exchanger. In the first stage of research, the performance of three cartridges was compared by extracting four antibiotics with different physic-chemical properties from water samples. The results obtained from column sorption experiments were plotted into breakthrough curves and batch equilibrium experiments results were fitted into Langmuir and Freundlich isotherms. Based on the parameters obtained from these plots, Oasis MCX was determined to be the best cartridges of the three for various analyte extractions. The recovery efficiency of each cartridge was studied by eluting the sorbent with acetone. The recovery of LC-18 sorbent was between 72% ~ 104% depending on the compounds, while both Oasis MAX and MCX cartridge can achieve approximately 100% recoveries.

In the second stage of the study two bioassays and HPLC analysis were used to evaluate the influence of different background water matrices on the performance of the SPE sorbents to extract known amount of estradiol from surface water and wastewater samples. Finally the quality of surface water and wastewater was examined in Ames assay and YES assay with samples pre-concentrated by Oasis MCX cartridge. No mutagenicity (determined by the Ames assay) and estrogenicity (determined by YES assay) were found in the raw water samples and SPE treated samples. With the assistance of bioassays and HPLC analysis, it was demonstrated that surface water has a minor influence on the recovery of Oasis MCX sorbent. However, the recovery of MCX sorbent decreased to 84.65% when wastewater was used as the background matrix. The work determined that Oasis MCX was the ideal sorbent for sample extraction in different water matrices.

Keywords

Solid phase extraction, estradiol, antibiotics, Ames test, YES assay, HPLC analysis

Acknowledgement

I would like to extend my deepest gratitude to my supervisors, Dr. Mita Ray and Dr. Lars Rehmann, for their support and guidance. Thank you for keeping my research interesting, diversified and challenging. I am also grateful for your endless patience, generosity and support. I could not have asked for better mentors.

To Erin Johnson, thank you for everything you have taught me about HPLC and answering all of my questions, even if I had already asked them before. Without your help I could have never completed my thesis.

Many thanks go to Sura Ali for teaching me how to conduct both bioassays. Your experience helped me save a lot of time in my research.

I would like to thank all my colleagues in the lab, especially Tulip Chakraborty, Jun-woo Kim and Kai Gao, for making the experience of working in the lab thoroughly enjoyable.

Finally, my most heartfelt thanks go to my dear parents for inspiring me to follow my dreams, and for their encouragement during the hard time.

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List of Abbreviations

%E = the percent of analytes being extracted from one phase into another

µg = microgram

µg L^{-1} = microgram per liter

µm = micrometer

Å = porosity

a = baseline response

A_0 = initial UV absorbance

A_{sp} = peak areas of spiked E2 standards

A_{nsp} = peak area of non-spiked sample

b = maximum response

C18 = silica based chromatography column coated with a C18 polymer

CH = cyclohexane

C8 = silica based chromatography column coated with a C8 polymer

C2 = silica based chromatography column coated with a C2 polymer

$C_{theoretical}$ = theoretical concentration of E2

C_{E2} = concentration of E2

C* = aqueous-phase concentration at equilibrium

C_0 = the initial concentration of the micropollutant in solution

CMC = critical micellar concentration

CNT = carbon nanotubes

CE = capillary electrophoresis

E2 = 17β-estradiol

EEQ = equivalent estradiol concentration

EC50 = half-maximal effect concentration

EDCs = endocrine disrupting compound

g mol^{-1} = gram per mole

g L^{-1} = gram per liter

GC = gas chromatography

GC-MS = gas chromatography-mass spectrometry

hER = human estrogen receptor

HPLC = high performance liquid chromatography

K_D = distribution coefficient

K_{OW} = octanol-water partition coefficient

LC-18 = silica based chromatography column coated with a LC-18 polymer

LLE = liquid-liquid extraction

LD = liquid desorption

LCM = lincomycin

m = hill slop

mg mL^{-1} = milligram per milliliter

mg L^{-1} = milligram per lite

m^2 g^{-1} = square meter per gram

M_E = amount of analytes eluted from the SPE devices

M_L = amount of analytes adsorbed onto the SPE devices

MAX = mixed-mode anion exchange sorbent

MCX = mixed-mode cation exchange sorbent

MIP = molecularly-imprinted polymer

MNZ = metronidazole

MWCNT = multi-walled carbon nanotube

ng L^{-1} = nanogram per liter

N = theoretical plates number

NF = electrospun polymer nanofibers

OFL = ofloxacin

ppt = one part per trillion

ppb = parts-per-billion

pKa = acid dissociation constant

PDMS = polydimethylsiloxane polymer

PH = phenyl

PhC = Pharmaceutical compounds

q = adsorptive capacity

R = absolute recovery

R^2 = regression coefficient

SBSE = stir-bar sorptive extraction

SMX = sulfamethoxazole

SPE = solid phase extraction

SPME = solid-phase microextraction

TD = thermal desorption

V_B = breakthrough volume

V_R = chromatographic elution volume

V_C = sample volume when the concentration of the analyte at the outlet equals to C_0

V = volume of the solution

VOC = volatile organic compound

W = absorption weight

YES test = yeast estrogen screen test

Chapter 1

1. Introduction

1.1 Background

Water, as a natural resource, is valuable throughout the world, especially in the regions experiencing significant industrialization and urbanization due to population expansion. Deforestation and man-made pollution are inflicting tremendous pressure on the depletion of freshwater resources. World Health Organization (WHO, 2004) reported a nearly 2 million death rate caused by waterborne diarrheal diseases each year. 88% of these deaths are a result of drinking unsafe water, inadequate sanitation, and poor hygiene. To use the freshwater sustainably, a "radical rethink" of policies to manage competing claims has been suggested (Reuters, 2012). A long-lasting sustainability of safe water supply is regulated by stringent protection and management of water sources and an efficient reclamation of used water from different effluents. However, various organic compounds such as pharmaceuticals and personal care products (PPCPs), which can include prescription drugs and nutraceuticals, fragrances and sunscreen products, etc. were reported to be found in numerous wastewater effluents and aquatic systems. Other than PPCPs, endocrine-disrupting chemicals (EDCs) were also reported and found to affect the aquatic habitat (Caliman & Gavrilescu, 2009; Onesios et al., 2009; Li et al., 2010). At present, there are no legal regulations established for the discharge of these persistent, omnipresent and biologically active substances into surface water bodies (Verlicchi et al., 2012; Fürhacker, 2008; Salgot et al., 2006; Ternes et al., 2007). The concentrations of PPCPs and EDCs in raw wastewater are generally in the range of 10^{-3} to 10^{-6} mgL^{-1} (Chen et al., 2007; Verlicchi et al., 2012). Moreover, these substances have very different physical and chemical properties such as polarity, solubility, adsorbability, absorbability, and biodegradability (Ziylan & Ince, 2011; Le-Minh et al., 2010) which have a great influence on their behavior during the treatment and their removal efficiencies in treatment plants. Although the concentration levels of PPCPs and EDCs do not have

an acute toxicity to human health and the environment, long-term exposure to these substances might adversely impact aquatic and terrestrial ecosystems and human health (Environment Canada, 2009). For instance, investigations have shown an epidemiologic link between genotoxic substances in drinking water intake and an increasing trend in certain cancers (Koivusalo et al., 1997). Ethynylestradiol (EE2), the main components in oral contraceptive pills for birth control and hormone therapy, has been shown to result in the induction of female-specific proteins in male fish (Tyler & Routledge, 1998), reduced sperm counts (Haubruge et al., 2000; Woods & Kumar, 2011), feminize wild fish populations, (Papoulias et al., 2000; Larsson et al., 2000) and prevalence of intersexuality. So, it is vital to detect and monitor the appearance and concentrations of these micropollutants in various effluents and aquatic environments.

Because of the intricacy of ecosystems and the difficulties of the potential impacts of the anthropogenic pollutants to be quantified, various bioassays have been developed over the years to address different aspects of environmental pollution. Bioassays use simple biological systems to simulate the immediate effect of a compound or mixtures of compounds on living organisms (Murphy et al., 2009). It relies on detecting the response of organisms exposed to micropollutants relative to a control (Rizzo, 2011). In contrast to chemical analysis, the results of bioassays reflect biological responses instead of just chemical concentrations. However, different compounds have different levels at which acute toxicity occurs, similarly each bioassay only responds to a given concentration of the contaminant. Therefore, current bioassays need to be modified to detect low concentrations of target compounds or their mixtures in aqueous streams.

Sample preparation, the step taken prior to a bioassay, makes the analytes at micro to nano-concentration more suitable for detection. Sample preparation would impact nearly all the later steps in the bioassays and is hence very critical for unequivocal identification, confirmation and quantification of analytes (Chen et al.,

2008). A proper sample preparation method would assist the detection and reduce the time and cost of the bioassays.

Considerable pre-concentration technologies have been used for bioassays such as solid phase extraction, continuous liquid-liquid extraction (Lippincott et al., 1989), supercritical fluid extraction (Wolfe et al., 1994) and hollow fiber-liquid phase micro-extraction (Kim et al., 2012). Solid phase extraction (SPE) is the most conventional and frequently used technique for isolation, concentration, clean-up and medium exchange for trace organics (Kim et al., 2012). Compared with other extraction techniques, SPE has the advantages of simplicity, rapidity and high recovery. It also requires low consumption of organic solvents, which reduces the cost of the extraction. Furthermore, SPE may be successfully used in combination with some analytical methods such as Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS) for a variety of compounds (Caliman & Gavrilescu, 2009).

Various sampling formats and sorbents have been developed and modified over time to facilitate the suitable processing of different samples and to extend the scopes of the technique. In the early 1980s, disposable cartridges packed with silica-based chemically bonded sorbents started to be used in the laboratory (Poole, 2003). SPE cartridges are devices that sorbents with different nominal particle sizes and different properties are packed between porous plastic frits in short columns (generally an open syringe barrel). Nowadays, numerous commercial SPE cartridges are available in the market. However, the data on the sorption properties of different types of popular commercial SPE columns are very limited. In addition, the sorption isotherms have been restricted to a relatively high concentration range of the analytes (Foo & Hameed, 2010). Isotherm fitting needs to be better examined and statistically tested at low concentrations. Finally, an optimized SPE procedure is always required for different environmental samples being tested in different bioassays.

Objectives of the Present Study

Based on the above, further research and development are required in both solid phase extraction optimization and application of the SPE procedures in water quality evaluation. The objective of this work was to address both issues, specifically to (i) determine adsorption parameters for selected micropollutants on various commercial cartridges and determine relationship with common physico-chemical properties such as acid dissociation constant (pKa), octanol-water coefficient, and solubility, (ii) optimize the sample concentration procedures for the selected SPE cartridges, and (iii) apply the optimized SPE procedures in two different bioassays, the Ames Test and the yeast estrogen screen (YES) test, to determine the effect of environmental matrices on SPE extraction.

Overview of Dissertation

This thesis is divided into the following chapters:
Chapter 1 provides the background and the objectives of the research.

Chapter 2 presents a literature review of the relevant theories for the stages in the research project.

Chapter 3 describes the first stage of the research, in which the properties of three different cartridges were evaluated by using four antibiotics as the model compounds in both column and batch sorption experiments.

Chapter 4 discusses the second stage of the research where the toxicity using two bioassays is compared for the environmental water samples after being extracted by the optimized SPE cartridge and procedure.

Chapter 5 reports the conclusions and recommendations for future work.

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Chapter 2

2 Literature Review

2.1 Background

The widespread occurrence of organic micropollutants such as pharmaceutical compounds (PhCs) and personal care products, flame retardants, pesticides, and endocrine disrupting compounds (EDCs) in receiving aquatic environments and wastewater plants have provoked increasing concern all over the world. A study conducted in Europe stated that in 264 municipal wastewater treatment plants (WWTPS) around the world, 118 pharmaceutical compounds belonging to 17 different classes were found in the effluents (Verlicchi et al., 2012). The majority of those organic compounds have not been proved to be mutagenic or carcinogenic. However, 34% of 71 compounds detected in drinking water were reported to be mutagens (Ellis et al., 1982). Although the direct effects of these suspected mutagenic micropollutants on human health and aquatic habitats are not yet fully understood, the pernicious effects of the EDCs and suspected mutagenic compounds have already been demonstrated (Sumpter, 2005). For example, chloroform was found at 366 μgL^{-1} and Dieldrin was found 8 μgL^{-1} in drinking water, which have 1.7×10^{-6} and 2.6×10^{-4} lifetime cancer risk per μgL^{-1} (Claxon, et al., 2008).

Bioassays, as one of the most precise and available tools, are used to monitor the quality of the wastewater treatment by using genetically modified bacteria or yeast strains to detect the mutagenicity or estrogenicity of the environmental samples downstream of the treatment processes. On the other hand, improving the techniques to detect micropollutants at very low concentrations and developing the methodology to evaluate the toxicity of the contaminants should be fed back to the upstream process to optimize the operation of the wastewater treatment. Because of the looming water scarcity all over the world, supplying safe and reliable drinking water and sustainable development will require the detection and removal of potentially harmful contaminants in water resources (Falconer et al., 2006).

Therefore, extensive research and development in the methodology of micropollutant detection and monitoring are needed.

2.2 Sample preparation

The concentration levels of the suspected mutagens or estrogens in environmental samples are usually too low to be detected in a bioassay. Therefore, it is necessary to concentrate and purify the analytes prior to chemical analysis or bioassay. In chemical analysis, sample preparation, as the foundation step for the experiment, is often the most time-consuming step. A survey showed that sample preparation accounted for nearly 61% of the time required to conduct an analytical task (Bielicka-Daszkiewicz & Voelkel, 2009). Because of the demand to perform an accurate and precise environmental analysis, liquid-liquid extraction (LLE) and solid-phase extraction (SPE) techniques were developed. LLE technique uses two immiscible solvents to partition the analytes from one media to the other. Although LLE has been used as a sample preparation procedure for analysis of trace organics for decades, with the superiority of other simple preparation techniques developed over the past twenty years, it has become less popular over time. In addition, there are many drawbacks of liquid-liquid extraction. For example, the solvents used in LLE must be immiscible with the matrix, which makes the procedure very non-selective. In addition to emulsion formation, difficulty in automation, and time consumption, LLE also requires large volumes of organic solvents, some of which are toxic and can also be expensive. SPE, on the other hand, can overcome all of these drawbacks.

2.2.1 SPE

Solid phase extraction is the technique to clean-up, concentrate and solvent exchange an environmental sample for chemical or biochemical analysis. Solid phase procedure is based on the equilibration of an analyte between the mobile phase (gas or liquid) and the sorbent (Ann & González, 2011). Analytes are partitioned onto a solid sorbent phase mostly from a liquid phase. Since trace

solutes are adsorbed and then desorbed by an on/off mechanism, it can be considered as a form of digital liquid chromatography, a term created by Wells and Michael (Gonzalez, 2001). For purification purpose, there are two possible methods; one simply is the reverse of the other. Either the interferences or the analytes may be sorbed onto the surface of the sorbent and leave the others in the mobile phase, or vice versa. In either case, a distribution coefficient, K_D, can be used to represent the distribution of the analytes between the sample (solvent) and the sorbent, such that:

$$K_D = [\text{analyte}]_{\text{sorbent}}/[\text{analyte}]_{\text{sample}} \text{ (Simpson, 2000)} \qquad \textbf{Eq. 2.1}$$

The percent of analytes being extracted from one phase into another, represented by %E, can also be expressed in term of distribution coefficient, such that:

$$\%E = 100 \times K_D/(K_D+1) \qquad \textbf{Eq.2.2}$$

For a successful solid phase extraction, the distribution coefficient should be as large as possible. Ideally, in a SPE process, K_D for an analyte should be large and the K_D for interferences should be small (or vice versa) (Portugal, 2008). In such a case, one compound (or a specie) will be completely retained in one phase and leave the rest of species in the other phase. Thus, selectivity is obtained. Another parameter, R, is used to indicate the absolute recovery for a SPE process. Similar to the percentage of analytes extracted, R is in form of percentage and the equation can be expressed as:

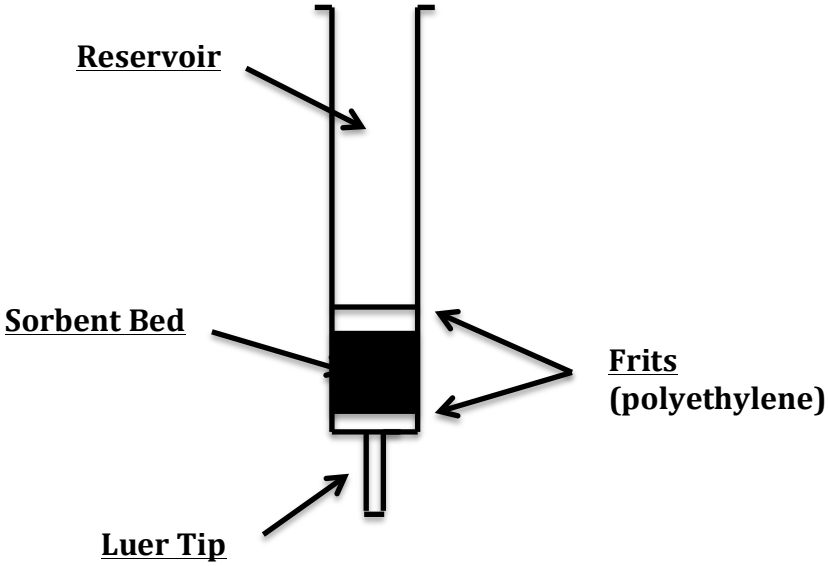
$$\%R = (M_E/M_L) \times 100\% \qquad \textbf{Eq.2.3}$$

where M_E is the amount of analytes eluted from the SPE devices and M_L is the amount of analytes adsorbed onto the SPE devices. The retention properties for the analyte of interest are a function of temperature, the format of SPE, the nature of the mobile phase, and the stationary phase (sorbent). As a typical SPE partition is

conducted under isothermal conditions (room temperature), temperature then becomes a minor factor.

2.2.2 Format of SPE

Over time, SPE has been developed into different formats. The most common format of SPE is in form of a cartridge (column). Sorbent particles (nominally 50 μm in diameter) are packed with two polyethylene fritted disks above a male Luer tip in a disposable short column (generally an open polypropylene syringe barrel) that acts as a reservoir for the environmental samples and solvents, as seen in Figure 2.1(a). After activating the sorbent with solvents, the liquid sample can then be loaded into the column. The analytes are distributed between the liquid and the solid phases where they are retained for the duration of the sampling process by adsorption on the bonded phase molecules of the surface. The analytes must have a greater affinity for the solid phase than for the sample matrix in order to be partitioned between these two phases (Berrueta et al., 1995). Analytes which have been extracted would be afterward isolated from the solid phase by desorption and the analytes would then be recovered by elution with a correspondingly small amount of appropriate solvent (typically two bed volumes) (Poole, 2003; Raisglid, 1996). Since the volume of solvent used in elution of the analytes is far less than the original volume of the sample, the sample is concentrated several times which increases the sensitivity and preciseness of the bioassays as well as chemical analysis.



(a)

(b)

Figure 2.1(a) The SPE column is a common device. A polypropylene syringe barrel contains the sorbent packed between two porous frits.
Figure 2.1(b) The SPE disc is a device in which sorbents are loaded in a membrane. (Sigma-Aldrich, 3M Empore SPE Extraction Disk).

SPE discs were first designed to treat large sample volumes with higher processing rate than columns and to avoid the blockages caused by suspended particles and matrix components. Sorbent particles with 8 to 12 μm in diameter were packed between particle-loaded membranes and immobilized in a web of micro-fibrils, as seen in Figure 2.1 (b) (Berrueta et al., 1995). SPE columns and discs share the same sorbent technology and the only difference between these two devices is the format. Cartridges can be easily fabricated in a laboratory environment, however, discs, so far, can only be produced in a manufacturing setting which results in a limited range of sorbent chemistry selection (Poole, 2003). In addition, cartridges are easier to be scaled up for larger sample loads and to clean up the samples than it is for discs. Because of the low selectivity of sorbents and the difficulty of manufacture, there are not many choices of commercial SPE discs in the market that makes discs significantly more costly than cartridges. Although SPE discs require smaller amount of elutes and can operate at higher flow rates (Thurman & Snavely, 2000), taking the economy and requirement of simple, routine applications into account, cartridge devices are always recommended.

Simplification, miniaturization of sample preparation, and minimization of organic solvent, and sample volumes are the dominant trends in analytical chemistry. Solvent-less sample-enrichment techniques, in which the solutes would be directly extracted from the samples, have been developed over time (Lancas et al., 2009). One example is stir-bar sorptive extraction (SBSE) that was developed in 1999 (Prieto et al., 2010). Stir bars are coated with a layer of polydimethylsiloxane polymer (PDMS) (typically 0.5-1 mm thick) as the extraction medium (David & Sandra, 2007). During the extraction procedure, the trace solutes would be isolated from the environmental matrix and then be extracted and enriched into the coating. Instead of using the solvent to elute the analytes, SBSE introduces the solutes for identification or quantification by thermal desorption (TD) or liquid desorption (LD). TD is used when the SBSE technique is combined with gas chromatograph (GC), and LD process can be applied to high performance liquid chromatography (HPLC), or capillary electrophoresis (CE) (Kawaguchi et al., 2006). Several

environmental and clinical applications indicated that SBSE technique has an acceptable recovery and precise extractions of trace solutes from surface water (David & Sandra, 2007; Guart et al., 2014; Portugal et al., 2008), biological fluid (Kassem, 2010) and wine (Hayasaka et al., 2003; Weldegergis & Crouch, 2008; Zalacain et al., 2007). In addition to being solventless, other advantages of SBSE devices include high feasibility and application to volatile organic compounds (VOCs) and semi-volatile compounds (Kawaguchi et al., 2005; Prieto et al., 2010).

Except stir-bar sorptive extraction, solid-phase microextraction (SPME) as a new solventless sample-enrichment technique that allows the direct extraction of analytes from aqueous matrix has experienced an increasing acceptance on routine analytical procedures (Lancas et al., 2009). SPME, as introduced in the early 1990's by Arthur and Pawliszyn (1990), can be defined as an extraction technique having a very small extracting phase volume compared to the volume of the sample. The principle of SPME is extraction of the analytes from a sample solution onto an optical fiber coated with an absorptive layer of sorbent and the fiber is attached to a holder which controls the contact of the fiber to solution or headspace (see in Figure 2.2). The sorbent coated fiber is exposed to the sample with the analyte of interest for a predetermined period of time and then the sorbed analyte is either desorbed thermally in the injection port of a GC for further chemical analysis, or by using an appropriate solvent to remove the target compounds from the fiber (McClure, 2007). SPME technique can combine sampling, isolation and enrichment in one step (Fatta-Kassinos, et al, 2011).

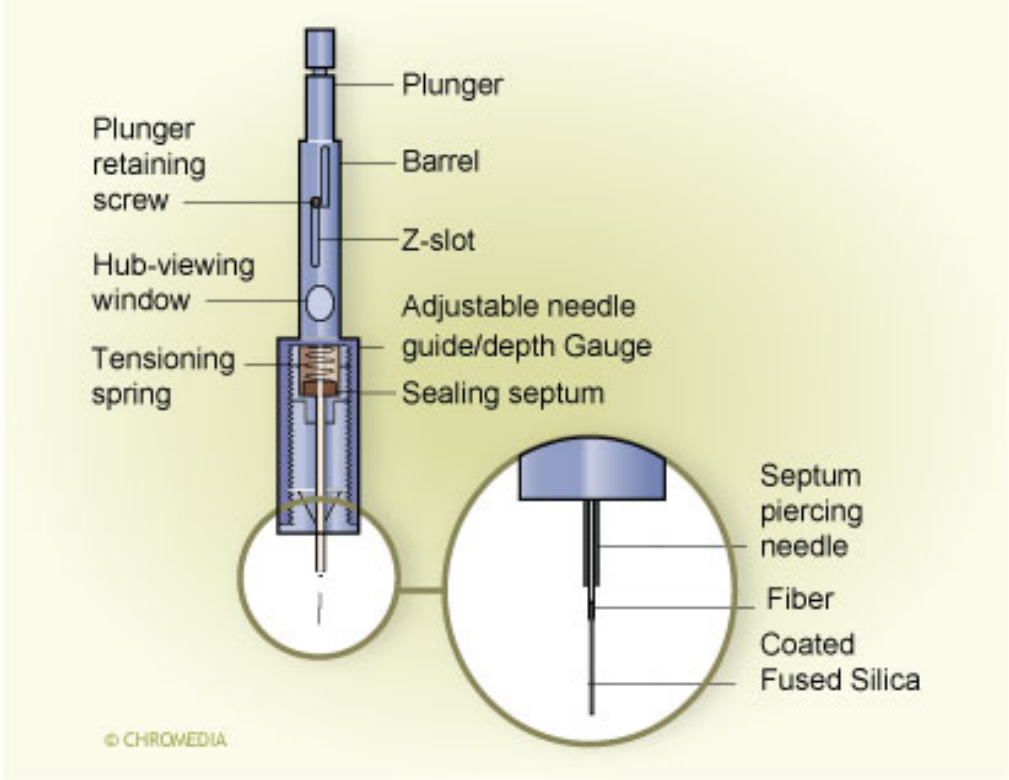


Figure 2.2 Apparatus of the first commercial SPME device (Chromedia, Principles of SPME)

There are three basic modes for fibre SPME: direct extraction, in a headspace configuration, and in a membrane-protected approach (see in Figure 2.3).

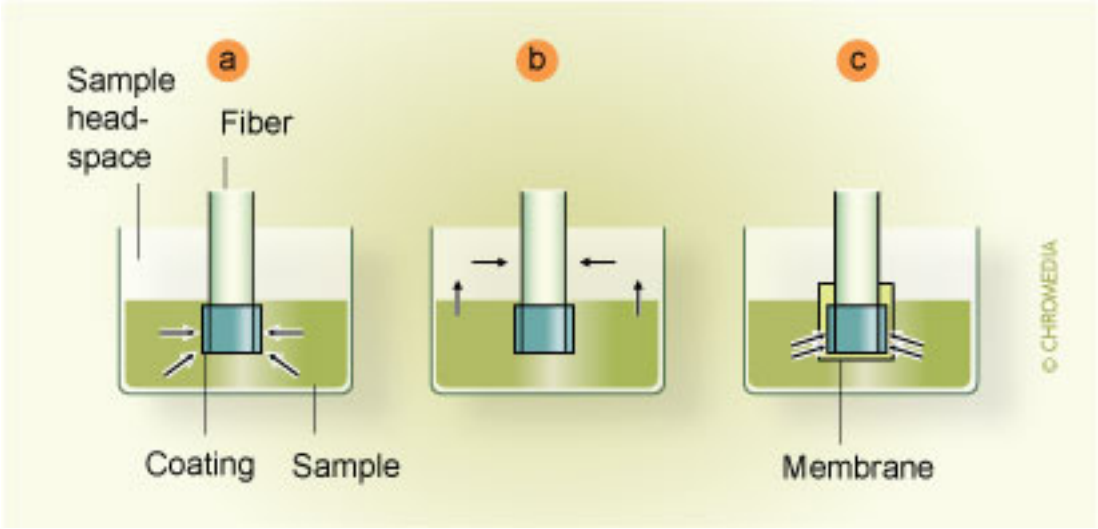


Figure 2.3 Mode of fiber SPME operation: (a) direct extraction, (b) headspace SPME, (c) membrane-protected SPME (Chromedia, Principles of SPME).

For direct extraction mode, the coated fibre is inserted directly into the sample with analytes and the analytes are adsorbed directly from the sample matrix to the extracting phase. In the headspace mode, the analytes have to be transported through the barrier of air before being adsorbed onto the coating which can be used to extract volatile compounds. In order to protect the fiber against damage, the membrane-protected SPME can be used (Vas & Vekey, 2004).

Contrary to traditional SPE methods and to the classic procedures, SPME relies on quantitative but non-exhaustive transference of analytes as the small volume of the extraction phase. The major advantages of the SPME technique are the easy miniaturization and automation. It is also a quick and straightforward approach for on-site analysis (Augusto et al., 2009). However, the extraction happens very slowly and has a considerably low recovery compared to LLE and SPE (Ulrich, 2000). In addition, as SPME requires the application of coating technology during manufacturing, the SPME apparatus is considerably expensive.

In this study, SPE cartridges were selected as the device to extract and enrich the solute from the aqueous samples. SPE cartridges, developed and introduced to the laboratories in the early 1980s, are a more mature technique. Significant amount of sorbent materials have been investigated and are already available in the market. Because of the low cost and high selectivity of sorbent chemistry, the SPE cartridge is more popular than SPE disc or solventless sample enrichment techniques. In addition, SPE cartridge devices have a faster protocol, greater recoveries and more reproducible results (Prieto et al., 2010; Davies, 2010).

2.2.3 Sorbent Selection

In SPE, the solid of sorbent is usually chemically bonded silica particles or small particles of an organic polymer resin with pores to enhance the surface area for interaction between the liquid sample and the extractant (Fritz et al., 1995). Other

sorbents also have been developed such as activated carbon, alumina, silica gel, and magnesium silicate (Berrueta et al., 1995).

Silica, as a basic support material in SPE cartridges, has an average diameter of 50 μm, a surface area of 400-550 m^2/g, an average pore diameter of 60 Å and pore volumes of 0.5-2 mL/g (Gonzalez, 2001). As silica is produced by the polymerization of tetra alkyl orthosilicate under acidic condition, long polymer chains with terminal hydroxyl groups, referred to as silanols, are formed. During the polymerization process, different silanol groups and siloxane linkages are formed and attached to the silica. The pK_a of silanol varies between 4 and 6 in water that results in a weakly acidic group and possible cation exchanger. So, when the pH is higher than 8.0, the surface of silica will be negatively charged. Because of the very polar nature of the bare silica, it is not a good stationary phase for samples with aqueous solvent (solvents for most of the environmental samples are water). Therefore, it needs to be modified to a more hydrophobic sorbent for application to aqueous systems.

SPE can be classified into three major groups based on different modified silicic stationary phases, in which different chemical mechanisms are applied to partition the analytes from a particular matrix. These three groups are: normal phase, reversed phase, and ion exchange. Sorbent selection is based on considerations of the properties of the solution and the target analytes that is summarized in Figure 2.4.

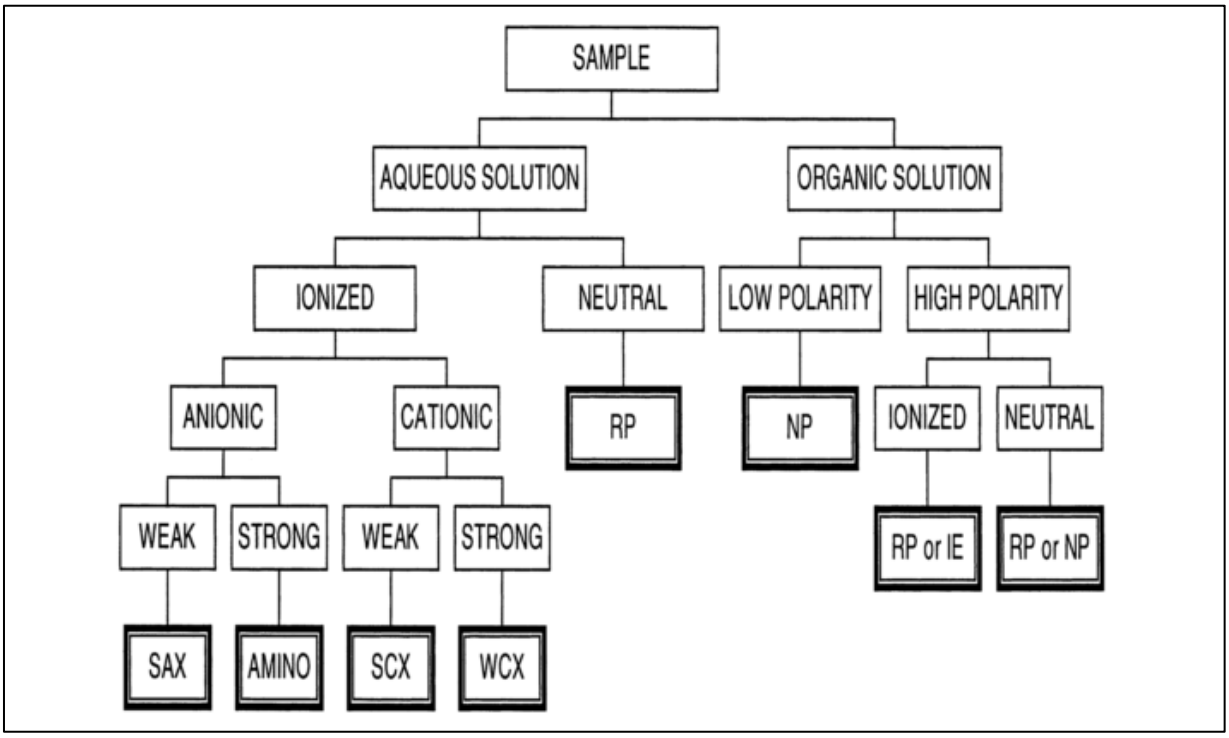


Figure 2.4. Method selection guide for the isolation of organic compounds from solution in which SAX represents strong anion exchanger, SCX represents strong cation exchanger, WCX is weak cation exchanger, RP, NP and IE refer to reversed-phase, normal-phase and ion-exchange sampling conditions, respectively (Poole, 2003).

If the analyte has a strong hydrophobic property, a sorbent can be modified to have a hydrophobic surface to separate the analyte. For a reversed phase separation, the columns are intended to extract nonpolar to moderately polar compounds from a polar or moderately polar matrix (e.g. water) with a nonpolar stationary phase (Roubeuf et al., 2000). The attractive forces between the carbon-hydrogen bonds in the analyte and the functional groups on the sorbent surface separate the analyte from the polar solutions and the analyte is then temporary retained onto the SPE sorbent. This force is also known as the van der Waals force or dispersion force (Biziuk, 2006). Finally, a nonpolar solvent is used to disrupt the forces and desorb the compound from the sorbent. Typical reversed phase materials include carbon-based media, polymer-based media, polymer-coated, and bonded silica media

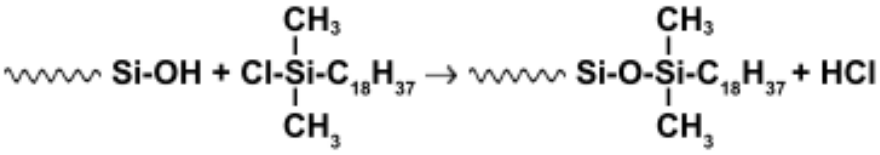
(Biziuk, 2006). C18 columns, as the most widely used and traditional reversed phase extraction device in SPE and HPLC, are utilized to partition dissolved organic compounds such as antibiotics, essential oils, drugs, esters, and water or fat-soluble vitamins from different matrices. Other reversed phase sorbents were also developed for specific needs. For example, ENVI-Chrom P packing with a greater surface area was specially designed to extract polar aromatic compounds from aqueous samples. Some other examples of reversed phase sorbent include C8, C2, cyclohexane (CH), and phenyl (PH) (Raisglid, 1996).

Normal phase SPE, on the other hand, is typically exploited to extract a polar solute from a mid polar to nonpolar matrix such as acetone, hexane and chlorinated solvent with a polar stationary phase (Bulletin 910, 1998). However, since this study focuses on the application of SPE columns on environmental samples, which are normally in aqueous matrices, cartridges from this category were not selected in this work.

In addition to hydrophobic interaction, ionic interaction between an analyte and the sorbent in aqueous sample matrix can also be utilized. Ion exchange SPE can be used to extract compounds with charges in a solution. Anionic analytes can be attracted to the silica surface bonding with an aliphatic quaternary amine group. Cationic compounds are isolated on an aliphatic sulfonic acid group that is bonded to the silica surface. The electrostatic attraction forces between the charged functional group in the compound and the charged group bonded to the silica surface is the primary retention mechanism of ion exchange SPE (Biziuk, 2006). With the development of SPE technology, mixed-mode sorbent systems that are the combinations of reversed-phase and ion-exchange sorbent are available. Some studies have already addressed that mixed-mode sorbents are often more advantageous and provide better separations than reversed phase or ion-exchange SPE alone (Landis, 2007; Mroczek et al., 2002; Clauwaert et al., 2000).

Based on the above information, three commercial cartridges belonging to two different categories were selected in order to evaluate the performance of these cartridges and study the relationships between the sorbents and the physico-chemical properties of target analyte(s). These cartridges are: LC-18 column (500 mg/3 mL) obtained from Supelclean (PA, USA), Oasis MAX (150 mg/6mL) and MCX (150 mg/6mL) obtained from Waters (PA, USA).

The LC-18 cartridge, belonging to reversed phase category, uses octadecyl bonded end-capped silica as its sorbent. The hydrophilic silanol groups at the surface of the raw silica packing (pore size and particle size may be controlled by supplier's manufacturing processes, but it is typically 60 Å pore size, 40 μm particle size) have been chemically modified with hydrophobic alkyl or aryl functional groups by reaction with the corresponding silicates (Bulletin 910, 1998). The reaction can be expressed as following:



Eq. 2.4

In the reaction, the hydrophobic alkyl or aryl functional group substitutes the chlorine on the silicates and finally the new alkyl- or aryl-bonded silicas and hydrochloric acid are formed. The functional group of LC-18 cartridges is displayed below in Figure 2.5.

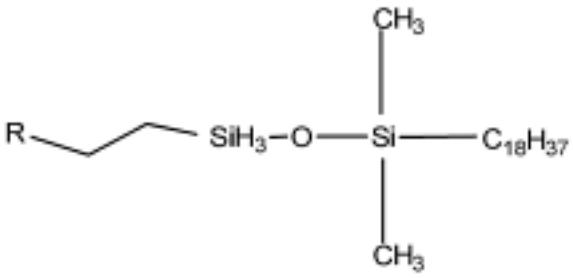
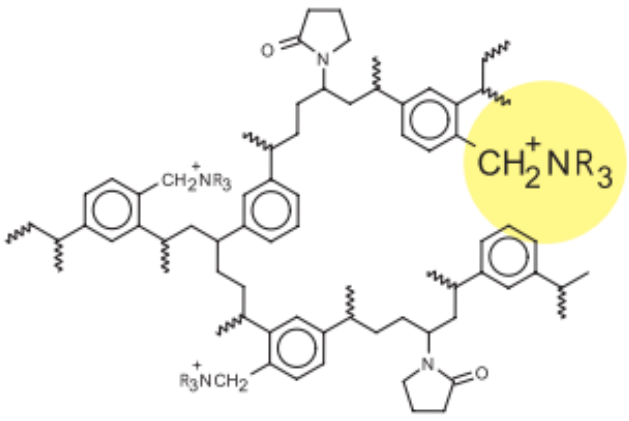


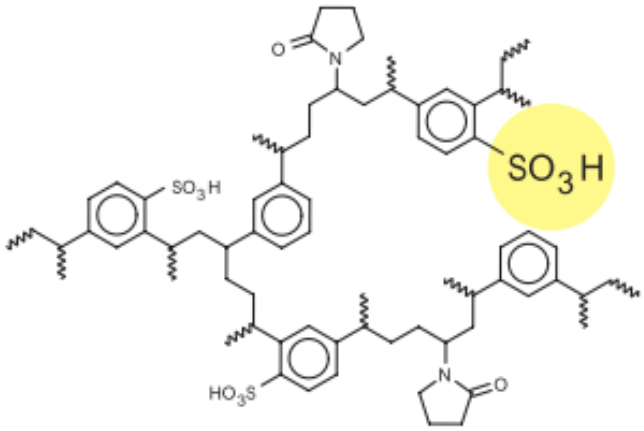
Figure 2.5 The functional group of LC-18 (Supelclean)

Some studies used LC-18 cartridges as SPE devices and found their recovery to be 60.08% to 98.58% for polycyclic aromatic hydrocarbons (PAHs) in water matrix (Kursinszki et al., 2005), 88.7% to 91.5% for caffeine (Ku et al., 1999) and 64.2% to 93.6% for 17β-estradiol (E2) spiked in different matrices (Shi et al., 2011; Hu et al., 2013).

MAX and MCX cartridges are both in the mixed-mode ion exchange category and are synthesized from the reversed phase SPE column-Oasis HLB (Water, USA). MAX (mixed-mode anion exchange) cartridges contain a mixed-mode polymeric (patented) sorbent with both reversed-phase and anion-exchange functionalities. The sorbent with a strong anion-exchange quaternary amine group has an ion-exchange capacity of 0.25 meq/g and is on the surface of HLB sorbent, a poly (divinylbenzene-co-N-vinylpyrrolidone) copolymer (Oasis, 2002). With the modification of the anion-exchange group, the MAX cartridge provides high selectivity for acidic compounds. The Oasis MAX sorbent has a structure as shown in Figure 2.6(a). Whereas, MCX (mixed-mode cation exchange) sorbent with strong cation-exchange sulfonic acid groups (1.0 meq/g of sulfonic-acid-ion-exchange capacity) bonded onto the surface of the Oasis HLB sorbent has dual modes of retention - reversed phase and cation exchange (Oasis, 2002). Because of the sulfonic acid groups, the MCX cartridge provides high selectivity for basic compounds. The structure of Oasis MCX sorbent is shown in Figure 2.6(b).



(a)



(b)

Figure 2.6 The structure of Oasis (a) MAX and (b) MCX sorbent (Waters, Oasis sample extraction products).

The hydrophobic part of the copolymer (divinylbenzene) gives the both MAX and MCX sorbents their reversed-phase characters, while the hydrophilic part (N-vinylpyrrolidone) increases water wettability that allows the sorbent to retain the capacities even when the sorbents run dry (Dobrev & Kaminek, 2002). On contrary to the traditional silica SPE sorbent, Oasis MAX and MCX sorbent are stable from pH 0 to 14, and have two to three times higher capacity due to their larger surface area and the water wettability. The analyte is charged at low pH for MCX sorbent (and at high pH for MAX sorbent) and experiences maximum retention primarily from the ion-exchange mechanism, accompanying with minor reversed phase mechanism. At high pH for MCX (and at low pH for MAX) sorbent, the ion-exchange retention mechanism switches off since the analyte is unionized. Then, reversed-phase retention is the dominant retention mechanism. MAX cartridge is reported to have a recovery of 76% to 100% for antibiotics (Benito-Peña et al., 2006) and 83.4% for estradiol (E2) (Arai et al., 2010). For MCX cartridges, the recovery ranges from 36%

to 106% for different pharmaceuticals, 92% for E2 (Zhang et al., 2011; Castiglioni et al., 2005).

2.2.4 New trends of sorbent in solid-phase extraction

Except the trends in the format modification in SPE technique introduced in Section 2.2.2, the development of new sorbents would improve the sensitivity and the selectivity of the analytical methods. All those new developed sorbents can be classified into following classes:

Surfactant-modified sorbents

When the concentration of surfactant solutions is higher than its critical micellar concentration (CMC), molecules arrange themselves in micelles. However, when the concentration is slightly below the CMC, molecules of ionic surfactants would be adsorbed on the surface of active solids contacting with the solution, forming hemimicelles and admicelles (see in Figure 2.7) which have a monolayer or bi-layer structures on the surface of the solids (Augusto et al., 2013).

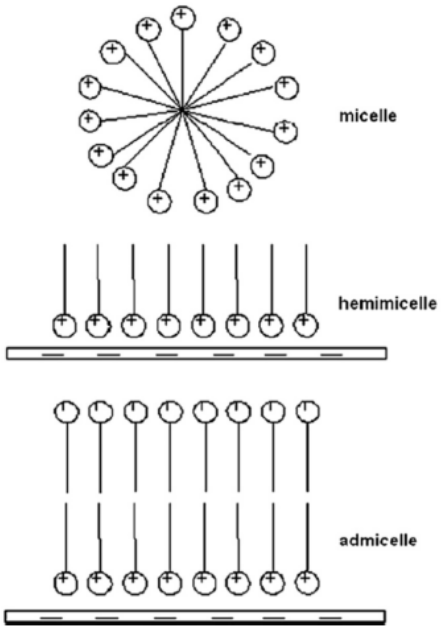


Figure 2.7 Micelles, hemimicelles and admicelles structures (Augusto et al., 2013).

For hemimicelle-based sorbents, as the hydrophobic tail of the surfactant is exposed to the solution, it is easier to retain non-polar analytes on them. On the contrary, admicelles-based sorbents are more suitable for polar compound extraction, as the portion of the coacervates exposed to the sample comprises the ionic tails of the molecules.

Nanostructured materials

The development of namomaterials affects several other fields of technology, including analytical chemistry. The applications of nanomaterials as SPE sorbents were suggested in recent literature. Two most well known sorbents are: electrospun polymer nanofibers (NFs) and carbon nanotubes (CNTs).

Electrospinning is a technique in which a viscoelastic solution is drawn into nanofibers by repulsive electrostatic forces (Chigome et al., 2011). It can be seen in Figure 2.8, the electrospining setup consists of three components: a high voltage power supply, a way to deliver a visco-elastic solution and a means to collect the fibers (Chigome & Torto, 2012). Electrospinning, as one of the nanofiber fabrication methods, is able to easily control the orientation of the nanofibers which has a significant effect on the performance of the SPE devices.

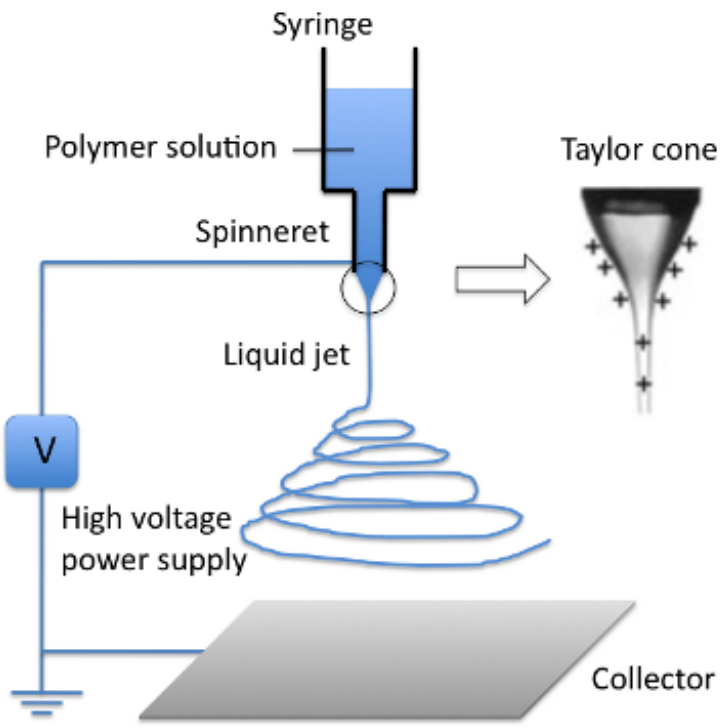


Figure 2.8 The common setup and working principle of electrospinning (Li et al., 2010).

Carbon nanotubes (CNT), an allotropic form of graphitic carbon, were first reported by Iijima in 1991 (Ravelo-Perez et al., 2009). CNT has tubular structures formed by either a single rolled graphite lamella in a cylinder or by several of these single tubes concentrically arranged around a common axis (Figure 2.9) (Augusto et al., 2010, Duran et al., 2009). The adsorptive behavior of CNT is expected to be similar to that of carbon-based alternates, in which weak intermolecular Van der Waals forces hold the large graphitic lamellae together. Therefore, non-polar, polar and even ionic analytes can be strongly adsorbed on to CNTs under the hydrophobic and electronic interactions (Augusto et al., 2010). As CNTs have a large surface-to-volume ratio, it has a much larger adsorptive capacity than other carbon-based adsorbents.

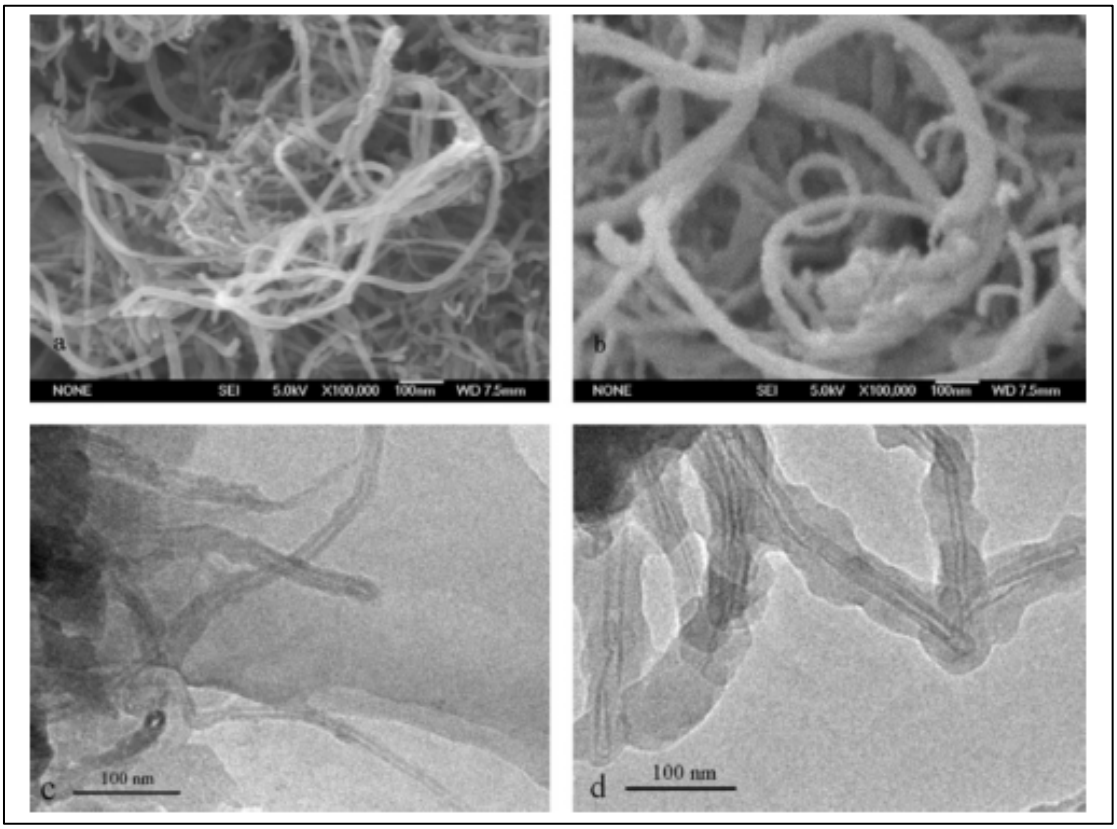


Figure 2.9 Scanning electronic (a and b) and transmission electronic micrographs (c and d) of crude multi-walled carbon nanotubes (MWCNTs) (a and c) and MWCNT-molecularly-imprinted polymer (MIP) (b and d) (Augusto et al., 2013).

At the present time, no commercial cartridges, disks or SPME fibers are available in both surfactant-modified and nanomaterial sorbents because the potential of these sorbents in analytical chemistry has not been fully demonstrated and the capital cost to produce these sorbents in batch is enormous. It is likely that with the development of efficient purification and characterization procedures the commercial SPE cartridges packing with new sorbents will be soon available.

2.2.5 Overview of SPE Procedure

A typical SPE procedure involves the following steps: 1. Column conditioning; 2. Sample loading; 3. Interference removal, and 4. Analyte elution. This procedure is shown in Figure 2.10. The overall analyte recovery is subjected to the variety of the factors in each one of the steps.

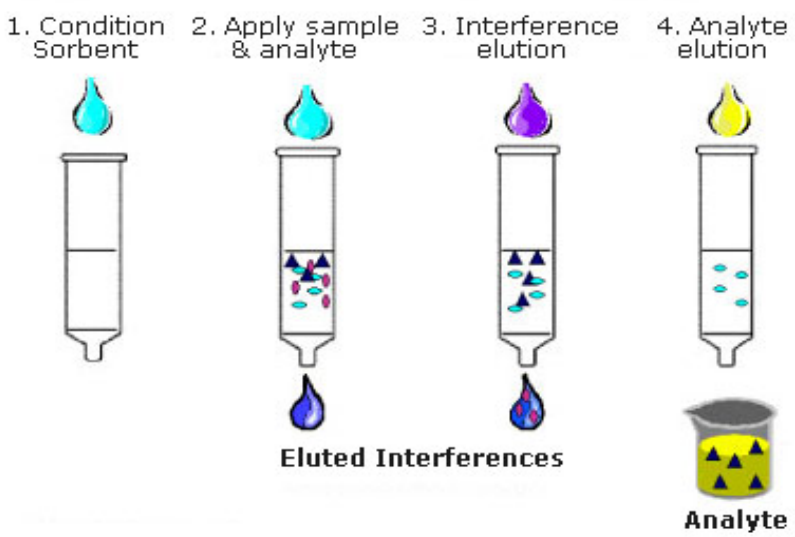


Figure 2.10 Typical procedure of SPE (Crawford scientific, SPE cartridges).

First, the modified silica surface needs to be conditioned in order for it to be active (wetted) and available for the analytes (Berrueta et al., 1995). The long hydrophobic chains will collapse upon themselves. Then, an organic solvent, such as methanol can be used to condition the surface. The purpose of conditioning step is for chain extension. During the extension process, an organic solvent is added to the matrix as a wetting agent to keep the chains fully extended for the interactions between the sorbent and analytes (Figure 2.11). After that, excess organic solvent is removed from the sorbent by Milli Q water to achieve equilibrium. If the solvent used in the conditioning is present during the sample loading, analytes may pass through the solid phase without being extracted from the highly organic mobile phase.

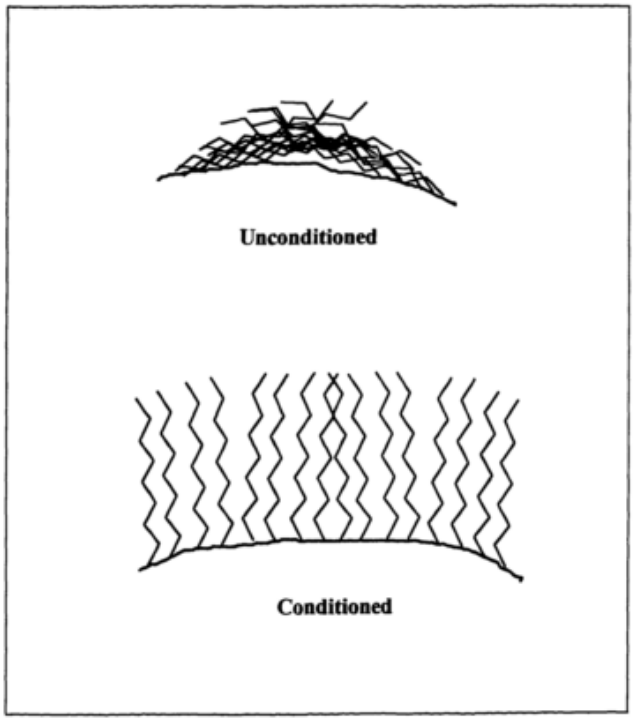


Figure 2.11 Impact of conditioning (Raisglid, 1996).

In the second step, the sample containing analytes of interest is loaded onto the column with vacuum. The loading rate may be varied significantly depending on the nature of the analytes and the retention mechanism of the column. Although the sample with large volume has a high sampling speed, it is still necessary to ensure that the analytes will have enough contact time with the sorbent surface.

An interference removal step usually follows sample loading. In this step, the cartridge would be rinsed with a suitable solvent to remove the interference that may affect accurate determination of the analytes. After that, the cartridge will be left with vacuum open to remove water in the column. Water would also be considered as interference if water miscible solvents were used (Raisglid, 1996).

The final and most important step is elution of the analytes from the sorbent. In order to use minimum volume of elution solvent, an appropriate solvent must be chosen to enhance the interactions between matrix and sorbent or between matrix and analytes, and minimize the interactions between sorbent and analytes. In

addition to solvent selection, sufficient contact time between the sorbent and solvent is important to ensure a high quantitative removal of the analytes from the sorbent.

The efficiency of the extraction is impacted by temperature, sample and solvent flow rates, solvent composition, ionic strength, pH, concentration of analytes, and choice of bonded phase in different steps of SPE procedures. So, during a SPE process, all those factors must be carefully and precisely taken into account.

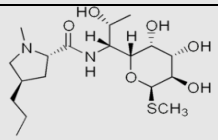
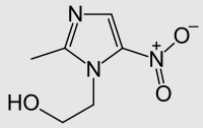
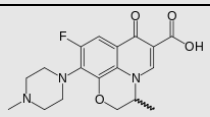
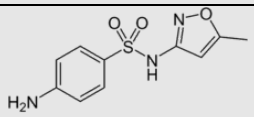
2.3 Model Compounds

Antibiotics, used to manage human as well as veterinary diseases, are reported to be detected in wastewater (Yang et al., 2011; Watkinson et al., 2007; Zhou et al., 2013), groundwater (Barnes et al., 2008; Batt et al., 2006a), drinking water (Focazio et al., 2008), surface water (Yang et al., 2011; Watkinson et al.,2007), sediments (Zhou et al., 2011) and agricultural land (Hu et al., 2010; Karci & Balcioglu, 2009). They are emitted in large quantities during fertilization with manure on agricultural fields and in aquaculture facilities, wastewater influents from hospital and medicine testing laboratories to small sewage treatment plants, discharges into lakes, disposal of unused drugs and so on (Isidori et al., 2005).

Because of the widespread presence of various antibiotics, four suspected mutagenic antibiotics with different physical and chemical properties (shown in Table 2.1) were selected as the model compounds to evaluate the SPE columns and determine adsorption parameters for the analytes on the cartridges and develop relationships with their physical properties. They are: sulfamethoxazole (SMX), metronidazole (MNZ), ofloxacin (OFL) and lincomycin (LCM). All these antibiotics were detected at different concentration levels in various aqueous matrices. SMX was detected at trace levels in some groundwater samples in the United States (Barnes et al., 2008). MNZ and OFL were detected at concentrations of 3.6 to 101 μgL^{-1} and 0.2 to 7.6 μgL^{-1}, respectively at Kalmar County Hospital effluents in

Sweden (Lindberg et al., 2004). LCM was reported at concentrations between 10 and 100 ngL^{-1} at all the sampling sites in the rivers Po and Lambro in Northern Italy (Castiglioni et al., 2004; Isidori et al., 2005). These four antibiotics were selected as they have very diverse solubility, pKa, and log K_{OW} values. These properties might have potential relationships with the performance of the SPE columns. In addition, limited data have been reported on the ecotoxicity, genotoxicity and mutagenicity of these four antibiotics by using bioassays (Isidori et al., 2005; Sekis et al., 2008; Minnich et al., 1976; Reifferscheid & Heil, 1996).

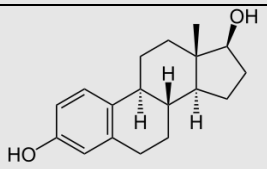
Table 2.1 General properties of LCM, MNZ, OFL and SMX.

Antibiotics	LCM	MNZ	OFL	SMX
Structure				
Chemical Formula	$C_{18}H_{34}N_2O_6S$	$C_6H_9N_3O_3$	$C_{18}H_{20}FN_3O_4$	$C_{10}H_{11}N_3O_3S$
Molecular Mass	406.538 $gmol^{-1}$	171.15 $gmol^{-1}$	361.368 $gmol^{-1}$	253.279 $gmol^{-1}$
Water Solubility	29.3 gL^{-1}	10 gL^{-1}	28.3 gL^{-1}	0.5 gL^{-1}
Acid dissociation constant (pKa)	7.6	2.62	7.9	5.81
Octanol-water Partition Coefficient (log K_{OW})	0.2	-0.1	-0.39	0.89

One of the most studied aqueous estrogenic micropollutants is 17β-Estradiol (E2) due to its widespread use as the active ingredient in birth control pills. E2 as a natural hormone is a compound strongly linked with affecting the fertility and the

development of fish, reptiles and aquatic invertebrates in aqueous environments (González, 2011) was also selected as a model compound. The basic properties of E2 are shown in Table 2.2. Major routes of E2 to enter the aqueous environment are the ineffective removal of pharmaceuticals, endocrine disrupting compounds or their metabolites in a traditional water treatment plant (Falconer et al., 2006; Racz & Goel, 2010; Scruggs et al., 2004) and improper disposal of pharmaceuticals. Falconer et al. (2006) studied the occurrence of E2 in secondary treated effluent and found the concentration to be less than 5 (the minimum limit for reporting) to 20 ngL^{-1}. And the maximum concentration detected in surface water in United States is 200 ngL^{-1} (Chen et al., 2007).

Table 2.2 General properties of E2.

Compound	17β-Estradiol
Structure	
Chemical Formula	$C_{18}H_{24}O_2$
Molecular Mass	272.38 g mol^{-1}
Water Solubility	0.0036 g L^{-1}
Acid dissociation constant (pKa)	10.4
Octanol-water Partition Coefficient (log K_{OW})	4.01

Furthermore, as in Yeast estrogen screen (YES) assay, E2 is used as a standard compound, so it is feasible to test E2 in bioassays and chemical analysis after extraction in SPE columns. Due to its proven estrogenicity, various detection methods, and occurrence in a variety of aqueous pathways, E2 is a very good representative compound for use in this research.

2.4 Mutagenicity Analysis of Water

A mutagenic substance is the one that can cause permanent, nonreversible and propagable changes to the genetic material in the cells of an organism which is a change in inheritable properties of an organism. These mutations can cause alterations in the expression of genes or changes in the structure of gene products (Höfer et al., 2004). Using current analytical approaches there is no possibility of routine examination of the full spectrum of micropollutants present in wastewater (Guzzellaa et al. 2002). This situation has aroused great interest in biological methods of assaying the water consumer's health risk – bioassays (Ohe et al. 2004). The major use of in-vitro mutagenic bioassays is as an initial screening for genotoxic or mutagenic carcinogens, as there is a high degree of correlation between the carcinogenicity of a compound and its mutagenicity (Ames et al., 1975; Ashby & Tennant, 1988). The primary advantage of *in vitro* bioassays is that the investigators can concentrate on a limited number of components instead of a whole living organism. This makes the results much easier to analyze than *in vivo* bioassay. They also decrease the requirement of experienced personnel in the laboratory to handle the living organisms such as small animals for in vivo bioassays. Although many bioassays are developed to determine specific mutagenic mechanisms, only a few have been applied to water quality analysis (Ohe et al., 2004). The most used bioassays (>60%) to test the mutagenicity of the aqueous samples, by far, is the Salmonella assay which will be discussed in the following section. Only few studies found mutagenic responses of SMX, LCM, OFL and MNZ by testing the antibiotics using several in-vitro assays: the Ames test, chronic toxicity testing, chromosome aberration (ABS) assays, the SOS-chromotest and the umu test (Isidori et al., 2005; Reifferscheid & Heil, 1996; Herbold et al, 2001). The use of mutagenic bioassays in water quality analysis can assist inspection for compounds that might result in genetic damage without identifying the mutagenic compound and recognizing the physical and chemical properties of the water. These tests can be utilized as a battery of tests to verify the mutagenicity level of the mutagens in aqueous samples.

2.4.1 The Ames Test

The Ames Assay (*Salmonella typhimurium*/microsome assay) is a widely used and standardized bioassay to determine whether a chemical substance has a high probability of being a carcinogen. Ames test involves determining whether the chemical to be tested causes a histidine-requiring mutant of the gram-negative bacteria *Salmonella typhimurium* that has a base substitution or frameshift mutation in a *his* gene to revert to the *His* phenotype. Each of these bacteria strains tests for a DNA damage; a positive mutagen will cause a reversion of the gene and the Salmonella typhimurium will be able to grow without histidine (Gilmour, 2012). Different mutagenic mechanisms have been studied and developed to be tasted by different Salmonella bacteria strains. Strains TA 1535 and TA 100 are sensitive to base-pair substitutions within DNA; whereas TA 1537, TA 1538, and TA 98 detect frameshift mutations due to a shift at the DNA base code reading frame level (Ames et al. 1985). Some strains that are more sensitive have been developed such as strains TA 97 and TA 102. These bacteria can detect two different types of mutation. For example, TA 98 is a frameshift mutation tester. It will respond when there is an addition or deletion of a number of bases (that is not a multiple of three) in the amino acids (Figure 2.12) that shifts the reading frame of the codons in the mRNA. This insertion or deletion of nucleotides might also result in a protein that is a different length than the original protein, with a new section of seemingly random amino acid attached to the end of the protein that have nothing to do with the sequence of amino acids that was there before. TA 100 responds to a base-pair substitution mutation which involves a replacement of one pair of nucleotides by another (Figure 2.13). This replacement could cause a nonsense mutation which a sense codon is changed to a nonsense (stop) codon that results in the stopping in protein synthesis or a silent mutation which causes no change in the encoded amino acid and gene expression. In case of TA 100, this replacement would result in a missense mutation. A sense codon is substituted with a different sense codon that specifies a different amino acid which could result in an abnormal gene expression (Chigome & Torto. 2011).

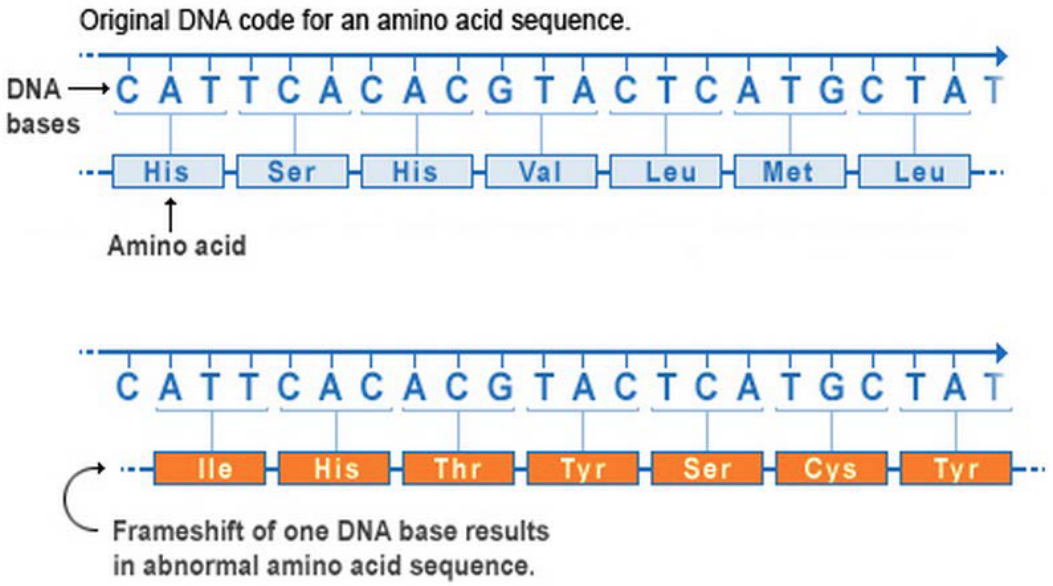


Figure 2.12 Frame-shift mutation mechanism (U.S. National Library of Medicine, Genetics Home Reference, 2010).

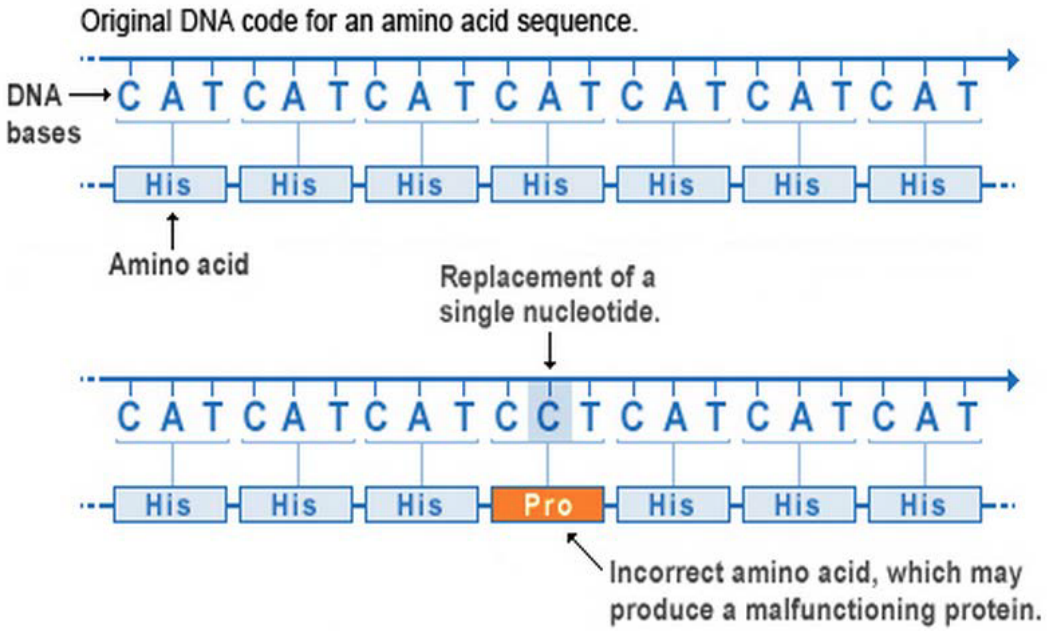


Figure 2.13 Base pair substitution resulting in a missense mutation (U.S. National Library of Medicine, Genetics Home Reference, 2010).

The Ames test was first designed to be conducted in an agar plate. With the improvement of this technology, an alternative method has been developed which is known as the "fluctuation method" (Bridges, 1980). Instead of counting the number of colonies observed in the plates (Ames et al. 1975), the number of yellow wells showed in a 96-microplates is enumerated. If the chemical to be tested causes a histidine-requiring mutant of *Salmonella* bacteria, the dye in the wells will be converted from purple to yellow (Bridges, 1980). The mutagenicity of a substance (represented in certainty in percentage) is proportional to the number of yellow wells enumerated.

The determination of water genotoxicity aims to control the exposure of these mutagenic potentials to the population. In addition to the testing of the genotoxicity of water samples, the Ames assay also has the potential of (1) comparing the final water quality of different treatment processes, (2) helping to identify the suspected carcinogens, and (3) ensuring that the water sample quality is the same for different studies (Claxton et al., 2008). Although Ames assay is an easy and widely used process to check the mutagenicity, it has limitations:

1) Different compounds have different level at which acute toxicity occurs, similarly Ames bioassay only responds to a given concentration. Pre-concentrating procedure might be necessary.
2) The working of Ames assay is based on the mutation of Salmonella typhimurium. So, Ames test might not be adoptable if the test chemicals interact with the bacteria. For example, Ames test cannot be used to detect the mutagenicity of antibiotics with high concentration which would kill the bacteria.
3) Because of the sensitive nature of the Ames assay, two or more bacteria strains with different mutagenic mechanisms are required in the test to obtain the acute genotoxic responses.
4) The mutagenic substance being identified in the Ames test is not necessarily to be carcinogenic. Potential carcinogenicity of the substance requires to be further tested.

2.5 Yeast Estrogen Screen

In addition to the genotoxic chemical compounds, endocrine disrupting compounds (EDCs) are also released daily into water bodies. EDCs have been reported to be detected in wastewater, sediments, drinking water, groundwater and surface water (Eertmans et al., 2003). EDCs can hormonally affect organisms at concentrations as low as nanograms per liter (Campbell et al., 2006). However, Eggen et al (2003) and Sumpter (2005) reported the presence of EDCs in different water bodies worldwide at significantly higher concentrations causing public concern. Some reviews and research found evidence of adverse reproductive outcomes such as infertility, cancers, malformations, and effects on other endocrine systems from long-term exposure to EDCs (Campbell et al., 2006; Diamanti-Kandarakis et al., 2009; Woodruff, 2011). YES assay as a method for EDCs detection is the very first step in wastewater treatment for aquatic environment protection (Spengler et al., 2001).

The YES assay, first developed by Routledge and Sumptar in 1996, is a cellular bioassay to detect the estrogenically active substances in the aqueous samples. This test can be done without having the knowledge of the composition of the pollutants and their concentrations (Gilmour, 2012). The YES bioassay employs a genetically modified strain of yeast *Saccharomyces cerevisiae* in which the chromosome has the human estrogen receptor (hER) DNA sequence and it links to a lac-Z reporter gene (Mcdonnell & Norris, 2014). When an estrogenically active substance is detected, it binds to the hER which causes the expression of lac-Z gene. Lac-Z encodes for an enzyme (β-galactosidase). The presence of β-galactosidase will turn the color of a dye (4-methylumbelliferyl-β-digalactopyranoside) in the test solution from yellow to red (fluorescent 4-methylumbelliferon). The change of color is directly related to the existence of estrogenically active substances. The assay has been used to monitor the removal of estrogenicity after water treatment. In this study, YES assay is used to detect the recoveries of SPE columns by quantifying E2 eluted from the cartridges.

2.6 Summary of Literature Review and Literature Gaps

The broad literature review indicated that although exhaustive scientific studies have utilized SPE columns as a tool to concentrate or purify aqueous samples, limited studies have been conducted on the sorption properties of different commercial SPE columns. Especially, the relationship of the physico-chemical properties of the analytes and the commercial cartridges, are never reported. On the other hand, the effect of water matrix on sample preparation for bioassays using the same SPE cartridges is never reported. These are the objectives of this study, which are elaborated in Chapters 3 & 4. In this chapter, an extensive literature review with respect to various aspects of SPE has been presented. A short background on current literature pertinent to the specific objectives of this work is presented in Chapter 3 and Chapter 4.

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Chapter 3

3 Performance of the Cartridges and their Relationships with the Properties of the Analytes

3.1 Introduction

Pharmaceutical compounds (PhCs), endocrine disrupting coumpounds (EDCs), their precursors, and degradation products are discharged to the environment during their manufacture, use and improper disposal. Although pharmaceuticals, as a new class of contaminants to the aqueous environment, have been released into the environment for decades, with the development of medicine to treat various diseases, the drugs and their mixtures might have increasing impacts on human health. Recently, many studies have been conducted by environmental scientists and government agencies on PhCs and EDCs detection and quantification at trace concentrations. To ensure a successful detection and quantification process, the aqueous samples are required to be extracted and purified. Typically, the extraction of PhCs from waste and environmental water is accomplished using solid phase extraction (SPE) and analysis of water quality is performed using either bioassays with unknown contaminants or a high-performance liquid chromatography (HPLC) with specified target compounds. Processing by SPE allows simultaneous extraction of multiple samples and generally gives good recovery of target compounds (Watkinson et al., 2007), while analysis by bioassays or HPLC allows for high selectivity and sensitivity. As such, these techniques are well suited for the analysis of PhCs and EDCs in the environment.

Much work has been conducted to study the chemical and surface properties of silica that has been modified with alkyl groups that is the sorbent of a reversed phase extraction (Roubeuf et al., 2000; Biziuk, 2006; Raisglid, 1996). However, there is a lack of literature that addresses the properties of a strong anion-exchange quaternary amine group or a strong cation-exchange sulfonic acid group on the end of a hydrocarbon linker as the modified phase. These materials are excellent cation

and anion exchangers with reversed phase properties and are very effective in the separation and isolation of acidic and basic compounds. The typical pH range for mixed mode mechanisms of these strong cation exchangers is 2 - 10 and for anion is 2 – 8. Outside of this range, the Si-O-Si bond linkages may be hydrolyzed. The surface silanols are deprotonated and charged above pH 8, and only the ion exchange capacity of these materials will be the dominant mechanism for analyte retention.

The focus of this study is based on the fact that an analytical method can be developed using SPE followed by bioassays or chemical analysis to detect a wide spectrum of PhCs or EDCs in water at low concentration (ppb and ppt level). The objective of the work presented in this chapter is to compare the performance of three different types of commercial SPE cartridges based on the following parameters: (a) sorption capacity; (b) sorbate per unit of sorbent; and (c) recovery efficiency. Once the better cartridge has been identified and validated, it will be applied for the detection and analysis of mutagenicity and estrogenicity of surface water and wastewater in bioassays and chemical analysis. In this chapter, experimental results for the applications of three commercial cartridges in extracting four antibiotics are presented. The characteristics of the cartridges were presented earlier in Chapter 2 and the physical properties of the cartridges are shown in table 3.1. Both column sorption experiments and batch equilibrium experiments were performed to determine the sorption parameters of LC-18, MAX and MCX cartridges. Subsequently, the relationships between the physico-chemical properties of the analytes and the sorption capacity were also investigated.

3.2 Laboratory experiments – conception and objectives

Two experimental techniques were applied in the laboratory to study the adsorption of the target analytes: 1) batch and 2) column experiments. As discussed earlier the target analytes include the four antibiotics commonly found in different aquatic systems. Henceforth, these target analytes will be called as micropollutants

as all of them are present in water at small concentrations. Typically, batch equilibrium experiments are designed to study equilibrium sorption of the target analytes where all the SPE solids are well mixed in an aqueous suspension. However, since SPE material is typically used in a column format (cartridge) in actual sample preparation, column tests were also performed in the selected cartridges. In the cartridge, the analytes interact with the packed sorbent where not all of the sorbent is exposed to or available for the interaction with the analytes, thus often resulting in early saturation.

3.2.1 Reagents

Lincomycin and ofloxacin were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Metronidazole and sulfamethoxazole were purchased from Sigma-Aldrich (Oakville, Canada). All standards were pharmaceutical grade. Analyte structures were shown in Table 2.1. Stock solutions of antibiotics at a concentration of approximately 400 mg/L were prepared in distilled water and stored in amber vials at 4 °C. The antibiotics solutions were brought to room temperature before use and remade every two to three months. 99.5% acetone was purchased from VWR (Radnor, PA, USA). Methanol and ethanol (HPLC Grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Laboratory-grade water (LGW, 18MΩ) was produced from a Millipore purification system (model Integral 5, EMD Millipore Corporation, Billerica, MA, USA). All reagents were used as received.

3.2.2 Batch sorption experiments

Batch equilibrium experiments in an aqueous system were performed in order to determine the sorption parameters (e.g, C* and its corresponding q_{max}). Usually, a solution of micropollutant(s) is added to water containing a given amount of sorbent. In this process, either the concentration of the micropollutant or the amount of sorbent can be varied. By monitoring the decrease in the aqueous concentration of the solute, the adsorbed amount of the micropollutant is determined.

The isothermal equilibrium parameter C*[ML^{-3}] can be plotted vs its adsorptive capacity q[MM^{-1}]. C* is the aqueous-phase concentration at equilibrium and q can be calculated as:

$$q = \frac{V(C_0 - C^*)}{m} \qquad \textbf{Eq. 3.1}$$

In the equation, C_0 [ML^{-3}] is the initial concentration of the micropollutant in solution and m[M] is the mass of the sorbent in water.

3.2.2.1 Experimental set-up and procedure

All batch experiments were carried out in 500 ml Erlenmeyer flasks. First, the flasks were filled with antibiotics solution with very low concentrations (1 µg/ml, 1.18 µg/ml, 1.76 µg/ml, 2.17 µg/ml, for LCM, MNZ, OFL, and SMX, respectively). Certain amount of sorbent taken from the SPE columns were added to the systems (i.e., 100 mg of MCX and MAX, and 300 mg of LC-18). The systems were mixed on magnetic plate stirrers to keep the sorbent in suspension and be available for the interactions with the micropollutant. Preliminary tests indicated an equilibrium time of 1-2 hours depending on the micropollutant and sorbents ratio. At equilibrium, 2 ml of the sample was withdrawn from the system. The solids were immediately separated from the aqueous solution through filtration using 0.2µm cellulose acetate syringe filters. The absorbance of the samples was measured by a UV-Vis spectrophotometry. A small amount of stock solution with much higher micropollutant concentration was added to the system to achieve a new equilibrium. These procedures were repeated until the q value reached constant regardless of the increasing concentration of the micropollutant solution in the aqueous system (See figure 3.1).

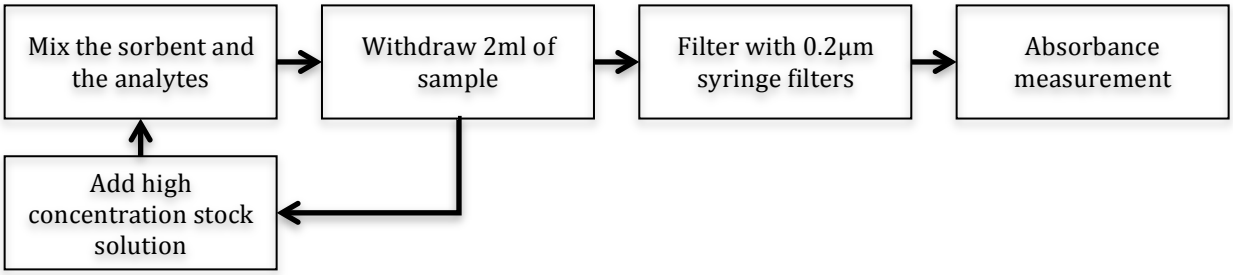

Figure 3.1 Flowchart for batch equilibrium adsorption experiments

3.2.3 Continuous operation: column experiments

The classical laboratory experiment for the simulation of adsorption of an environmental pollutant in the subsurface environment is the column experiment. Generally an aqueous solution with micropollutant(s) was allowed to flow through the column packed with SPE solids from a sample reservoir which is connected by a pump. The commercial columns were first equilibrated with a slightly-polar solvent (i.e. methanol) and deionized water, which wetted the surface and penetrate the bonded phase. A solution with micropollutant was fed into the column. The concentration, C, of the micropollutant appearing in the effluent reservoir was measured over time and the results were plotted in the form of solute breakthrough curve, or relative concentration, C/C_0, versus volume, where C_0 was the influent concentration of the micropollutant.

3.2.3.1 Column apparatus and experimental set-up

The column experiments were performed using three types of commercial SPE cartridge columns with different materials. MAX and MCX columns (Waters) have 150 mg ion-exchanger SPE solids with 80 Å and 79 Å in pore sizes, respectively with 6 ml capacities. LC-18 column (Segma-Aldrich) is a reversed-phase column packed with 500 mg C-18 solids with 55 µm in size and it can take up to 3 ml sample solution. The surface areas of LC-18, MAX and MCX sorbents are 529 m^2/g, 796 m^2/g, and 806 m^2/g, respectively. The breakthrough time of SMX, OFL, LCM and MNZ was measured using an off-line UV absorbance detection set-up which consists of the

solution reservoir, SPE column containing different sorbents, a peristaltic pump, an effluent reservoir, which were all connected with silica tubes (Figure 3.2). Optimized UV absorption wavelengths were obtained by means of scanning the reference solutions. The maximum signal was obtained at UV wavelengths (nm) of 190, 320, 287 and 197 for LCM, MNZ, OFL and SMX, respectively.

Figure 3.2 Experimental set-up for continuous column operation.

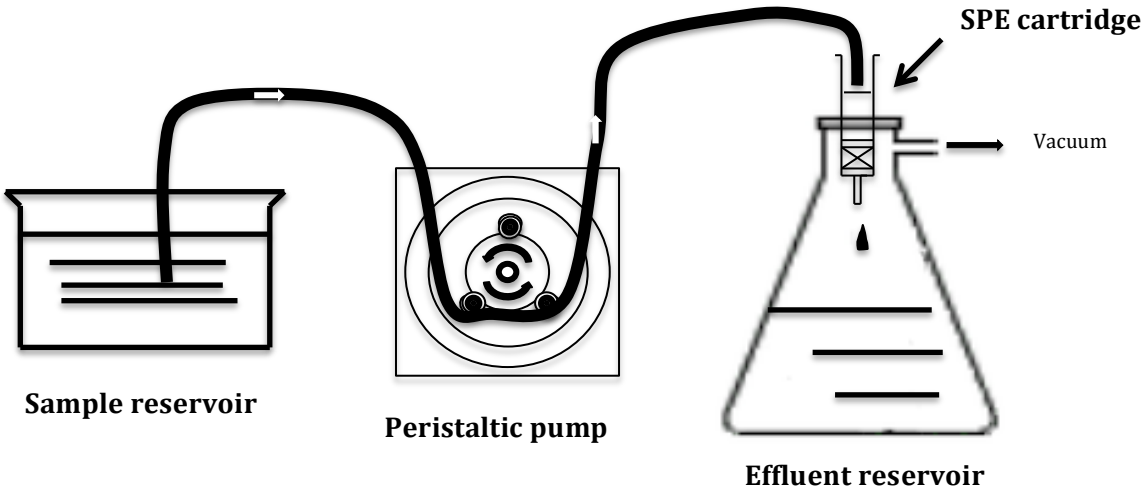

Table 3.1 Physical properties of Oasis MAX, MCX and LC-18 cartridges

	Oasis MAX	Oasis MCX	LC-18
Specific Surface Area (m^2g^{-1})	796	806	529
Average Pore Diameter (Å)	80	79	61
Total Pore Volume (cm^3g^{-1})	1.26	1.26	
Average Particle Diameter (µm)	31.0	29.0	55
Fines Content	<0.1	0.2	
Anion Exchange Capacity (meq g^{-1})	0.2		
Sulfonic Acid Content (meq g^{-1})		1.05	

3.2.3.2 Experimental procedure

5 ml 99% methanol followed by 5 ml deionized water were used to wet the surface and penetrate the bonded phase in MAX and MCX cartridges. For LC-18 cartridge, 2

ml 99% methanol and 2 ml deionized water were used to equilibrate the column. The speed of peristaltic pump was adjusted to supply a constant flow rate of the solutions from the reservoir to the columns between 1.4 and 1.5 ml/min. Dilution of the effluent was performed prior to the UV absorbance measurement to follow the linearity of Beer-Lambert's Law.

3.3 Results and Discussions

3.3.1 Adsorption isotherms of antibiotics

3.3.1.1 Effect of concentration

The removal of antibiotics by MAX, MCX and LC-18 sorbents at different initial concentrations keeping the doses of sorbent was investigated. The percent removal of antibiotics decreased with increasing concentration due to lower availability of the sorbent. Figure 3.3 describes the effect of antibiotics initial concentrations on the removal percentage by different sorbents. However, the amount of antibiotics adsorbed per unit sorbent mass increases with the increase in initial antibiotics concentration due to the decrease of uptake resistance of solute from solution of antibiotics (refer Figure 3.4). For example, the increase in initial concentration from 2.2 ppm to 65.3 ppm resulted in a decrease from 89.9% to 32.8% in adsorption of SMX in MAX cartridge while the adsorption of SMX per unit weight of adsorbent increased from 1.9 to 126.3 mg g^{-1}. The phenomenon is consistent to the trend reported in various studies (Stephen et al., 2005; Azam et al., 2009).

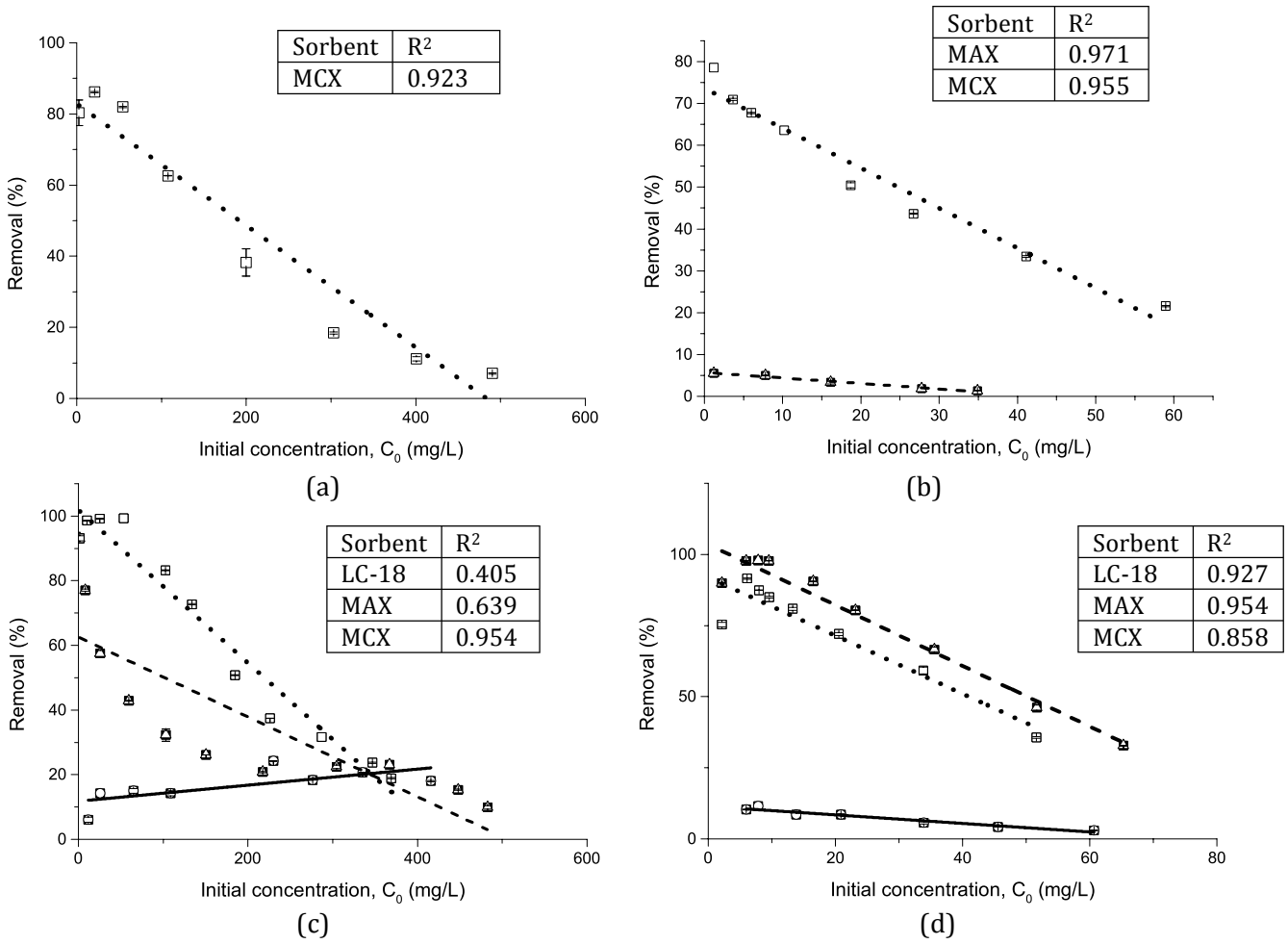

Figure 3.3 Removal profiles of (a) LCM, (b) MNZ, (c) OFL and (d) SMX in LC-18 (represented by solid line), MAX (represented by dash line) and MCX columns (represented by dot line). In some graphs, the errors are too small to show.

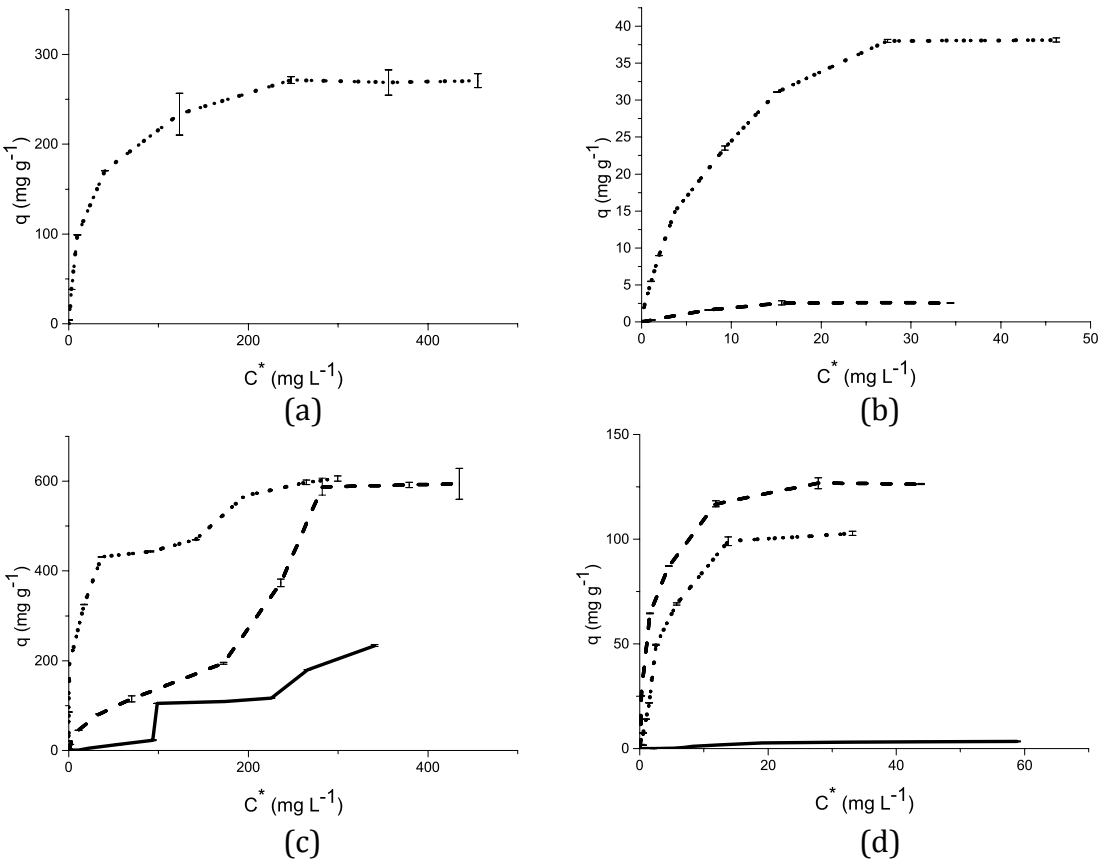

Figure 3.4 Adsorption isotherms of (a) LCM, (b) MNZ, (c) OFL and (d) SMX in LC-18 (represented by solid line), MAX (represented by dash line) and MCX columns (represented by dot line). In some graphs, the errors are too small to show.

It can be seen that most of the micropollutants followed Lamgmuir isotherm for all three sorbents. The equilibrium adsorption capacity is presented in Table 3.2.

Table 3.2 The equilibrium uptake capacities and extent of adsorption of LCM obtained at different initial concentrations.

C_0 (mg L^{-1})	MCX	
	q (mg g^{-1})	% adsorption
20	38.18	86.15
100	170.74	62.63
200	233.64	38.26
400	268.92	11.13
500	270.81	7.03

Table 3.3 The equilibrium uptake capacities and extent of adsorption of MNZ obtained at different initial concentrations.

C_0 (mg L^{-1})	MAX		MCX	
	q (mg g^{-1})	% adsorption	q (mg g^{-1})	% adsorption
1	0.26	5.50	1.86	78.62
10	1.83	4.32	14.98	63.59
20	2.47	3.31	31.08	50.43
30	2.56	1.52	32.08	45.72
40	2.54	0.63	38.00	33.41
60	2.56	0.38	38.12	21.60

Table 3.4 The equilibrium uptake capacities and extent of adsorption of OFL obtained at different initial concentrations.

C_0	LC-18		MAX		MCX	
(mg L^{-1})	q (mg g^{-1})	% adsorption	q (mg g^{-1})	% adsorption	q (mg g^{-1})	% adsorption
2	0.64	54.28	3.27	93.11	3.28	93.11
100	22.99	14.22	115.13	32.25	325.44	83.27
200	109.05	24.21	181.97	22.20	457.85	43.45
300	179.99	20.66	373.43	22.39	587.83	28.67
400	233.72	18.03	590.52	13.36	606.27	18.92
500	233.71	16.43	594.41	9.90	606.02	9.53

Table 3.5 The equilibrium uptake capacities and extent of adsorption of SMX obtained at different initial concentrations.

C_0	LC-18		MAX		MCX	
(mg L^{-1})	q (mg g^{-1})	% adsorption	q (mg g^{-1})	% adsorption	q (mg g^{-1})	% adsorption
10	1.08	13.00	25.20	97.75	21.81	85.00
20	2.69	8.55	85.09	85.37	69.10	72.04
30	3.05	5.65	103.83	70.57	99.05	59.17
50	3.33	4.02	126.78	46.15	102.72	35.65
60	3.38	3.00	126.32	32.76	102.68	30.73

3.3.1.2 Adsorption isotherms

Adsorption isotherm helps to study the relationship between the amount of a substrate adsorbed onto the adsorbent at constant temperature and its concentration in the equilibrium solution. It provides essential physico-chemical data for assessing the applicability of the adsorption process as a complete unit

operation (Aydın & Baysal, 2006). Two famous models used to investigate the adsorption process are Langmuir and Freundlish isotherm models (Chan et al., 2008; Lata et al., 2008). Some parameters in those models can be construed further to investigate the sorption mechanisms, surface properties and an affinity of the adsorbent (Nayak & Singh, 2007). The application of Langmuir isotherm is based on the assumption that the adsorbent sites are monolayer. The adsorption process only occurs at specific homogenous sites on the adsorbent surface with energy level evenly distributed (Mohd Din et al., 2009). Once the activated site is occupied by the adsorbate, no further adsorption could take place at the same site. Freundlish isotherm, on the other hand, was developed on the assumption that the adsorption takes place on heterogeneous sites with uneven distribution of energy level (Mohd Din et al., 2009). The Freundlish studies reversible adsorption and is not restricted to the formation of monolayer (Mall et al., 2006; Ng et al., 2002). The linearized form of Langmuir and Freundlich isotherm models can be represented by the following equations:

Langmuir isotherm: $$\frac{C^*}{q} = \frac{1}{K_L} + \left(\frac{a_L}{K_L}\right) C^* \qquad \textbf{Eq. 3.2}$$

Freundlich isotherm: $$logq = logK_F + \left(\frac{1}{n}\right) logC^* \qquad \textbf{Eq. 3.3}$$

where q is the amount of adsorbate adsorbed at equilibrium (mgg^{-1}), C^* is the equilibrium concentration of the adsorbate solution (mgL^{-1}), K_L (Lg^{-1}) and a_L (Lmg^{-1}) are Langmuir isotherm constants. Ideally for Langmuir isotherm, plots of C^*/q versus C^* gives a line with a_L/K_L as its slope and $1/K_L$ as intercept. K_L/a_L also has a relation to the maximum adsorption capacity at monolayer, Q_e (mgg^{-1}). As for Freundlich, after plotting log q versus log C^*, two heterogeneity factors can be determined: the slope 1/n (dimensionless) and the intercept K_F (mg g^{-1})(L $mg^{-1})^{1/n}$ which are also known as the Freundlich constants.

Figure 3.5 and 3.6 exhibit the linear plots of Langmuir and Freundlich for the model micropollutants adsorption onto MAX, MCX and LC-18 sorbents. The values of R^2, a measure of goodness-of-fit of linear regression, given in Table 3.6-3.9 indicates that

all the micropollutants adsorption in this study can be better fitted into Langmuir isotherm than Freundlich isotherm; a possibility of monolayer antibiotics formation on the adsorbent surface. The adsorption power was affected by the fact that different intensity and uneven distribution of active functional group may result in differences in the energy level of the active sites available on the sorbent surface. In mixed mode ion exchanger sorbent, active sites with lower energy level will form monolayer coverage due to electrostatic forces (Mohd Din et al., 2009).

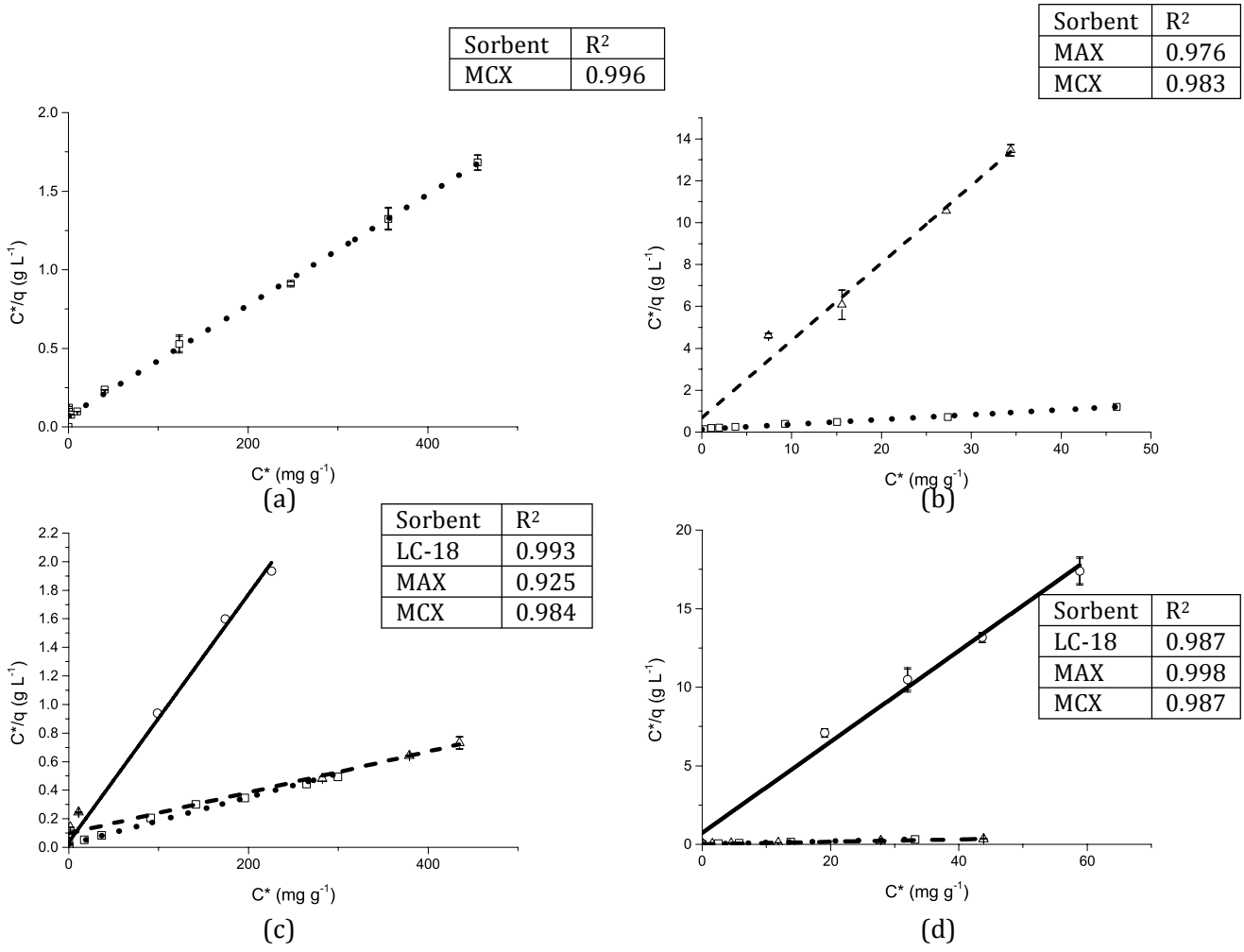

Figure 3.5 Langmuir isotherm plots of antibiotics:(a) LCM, (b) MNZ, (c) OFL and (d) SMX in LC-18 (represented by solid line), MAX (represented by dash line) and MCX columns (represented by dot line). In some graphs, the errors are too small to show.

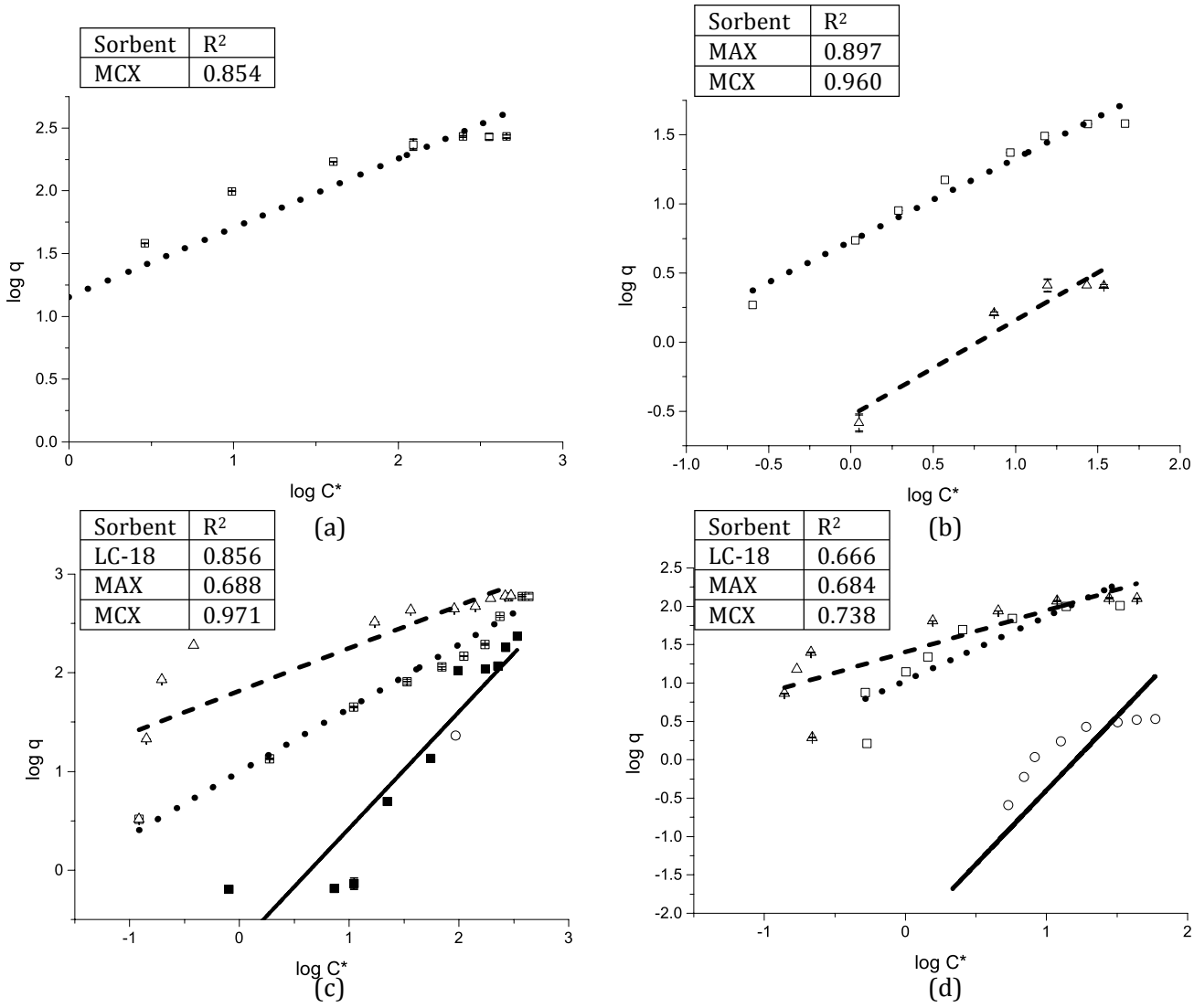

Figure 3.6 Freundlich isotherm plots of antibiotics:(a) LCM, (b) MNZ, (c) OFL and (d) SMX in LC-18 (represented by solid line), MAX (represented by dash line) and MCX columns (represented by dot line). In some graphs, the errors might be too small to show.

Table 3.6 Langmuir and Freundlich coefficients for LCM on MAX, MCX and LC-18

Adsorbent	Langmuir isotherm parameter				Freundlich isotherm parameter		
	Q_e (mg g^{-1})	K_L (L g^{-1})	a_L (L mg^{-1})	R^2	K_F (mg g^{-1})(L mg^{-1})$^{1/n}$	n	R^2
MCX	285.71	11.69	0.041	0.999	14.34	1.82	0.875

Table 3.7 Langmuir and Freundlich coefficients for MNZ on MAX, MCX and LC-18

Adsorbent	Langmuir isotherm parameter				Freundlich isotherm parameter		
	Q_e (mg g^{-1})	K_L (L g^{-1})	a_L (L mg^{-1})	R^2	K_F (mg g^{-1})(L mg^{-1})$^{1/n}$	n	R^2
MAX	3.50	0.35	0.099	0.946	0.29	1.45	0.923
MCX	44.84	6.30	0.14	0.995	5.38	1.67	0.965

Table 3.8 Langmuir and Freundlich coefficients for OFL on MAX, MCX and LC-18

Adsorbent	Langmuir isotherm parameter				Freundlich isotherm parameter		
	Q_e (mg g^{-1})	K_L (L g^{-1})	a_L (L mg^{-1})	R^2	K_F (mg g^{-1})(L mg^{-1})$^{1/n}$	n	R^2
LC-18	119.05	11.68	0.098	0.999	0.17	0.85	0.870
MAX	569.23	28.57	0.046	0.998	9.91	1.55	0.974
MCX	625	42.37	0.068	0.985	65.66	2.31	0.719

Table 3.9 Langmuir and Freundlich coefficients for SMX on MAX, MCX and LC-18

Adsorbent	Langmuir isotherm parameter				Freundlich isotherm parameter		
	Q_e (mg g^{-1})	K_L (L g^{-1})	a_L (L mg^{-1})	R^2	K_F (mg g^{-1})(L mg^{-1})$^{1/n}$	n	R^2
LC-18	4.91	0.22	0.044	0.940	0.40	1.77	0.872
MAX	140.85	35.21	0.25	0.923	25.34	1.84	0.723
MCX	113.64	36.63	0.32	0.998	19.88	1.78	0.857

In Table 3.6-3.9, the Q_e values, the maximum adsorption capacities at monolayer, of MCX columns for LCM, MNZ and OFL adsorption were higher than the other sorbents which were 285.7, 44.84 and 625 mg g^{-1}, respectively. For SMX, MAX had a slightly higher Q_e than MCX column. It can be concluded that MCX was the better sorbent of the three selected sorbents based on the maximum adsorption capacity.

Table 3.10 exhibits the q_{max} values from the adsorption isotherms (Figure 3.4) which represents the maximum weight of sorbate per unit of sorbent retained in the columns. Comparing the values of q_{max} obtained from direct plots and Q_e calculated from isotherm fittings, it showed the same trend that the absorption capacities reduced as the pKa value of model compounds decreased (pKa values: OFL > LCM>SMX>MNZ). OFL has the highest q_{max} values in all sorbents. As OFL has the highest pKa value (pKa = 7.9) among the antibiotics, both ion exchange and reversed-phase characteristics influenced the adsorption procedures. OFL also has the lowest log K_{OW} value (log K_{OW} = -0.39) which indicates that it can be considered relatively hydrophilic and polar. This suggested that all the selected sorbents could be used to extract hydrophilic micropollutants from aqueous solutions. However, for LCM, another hydrophilic and polar micropollutant (pKa = 7.6 and log K_{OW} = 0.2) having a higher adsorption capacity than SMX (pKa = 5.81 and log K_{OW} = 0.89) in MCX cartridge, could not be retained in MAX and LC-18 sorbents indicates that the

sorption capacity was not a function of hydrophilicity and octanol-water partition coefficients, but only a function of pKa values.

Table 3.10 Q_{max} values of LCM, MNZ, OFL, and SMX on different sorbents

Sorbents	Antibiotics			
	LCM	MNZ	OFL	SMX
	q_{max} (mg g^{-1})			
LC-18	No adsorption	No adsorption	116.62	3.36
MAX	No adsorption	2.56	594.41	126.33
MCX	270.81	38.06	606.27	102.06

3.3.2 Breakthrough of the cartridges

The performance of the SPE columns was compared based on some critical SPE parameters: breakthrough volume, retention factor, elution volume, and recovery efficiency, which depend on the properties of the SPE bed.

The effect of sample volume on SPE recovery is important in environmental sampling. Because of the low level of the contaminants in the environment, SPE columns are expected to treat a large volume of sample. Once the retention mechanism, the sorbent and an elution solvent are decided, it was necessary to perform a breakthrough experiment to compare the breakthrough volumes of the model compounds in different cartridges. The breakthrough is the maximum volume of the sample that may be passed through the sorbent before the analyte of interest is no longer retained (Thurman, 1998). As the equilibrium concentration is different for all compounds and cartridges, adsorbed weight (W) was used to replace breakthrough volume as one of the comparison parameters. Adsorbed weight represents the binding amount of the target analytes on the sorbent which was estimated by either subtracting the amount of eluted analytes from the total

amount of analytes passed through the cartridge or integrating the area under the breakthrough curve (Figure 3.6).

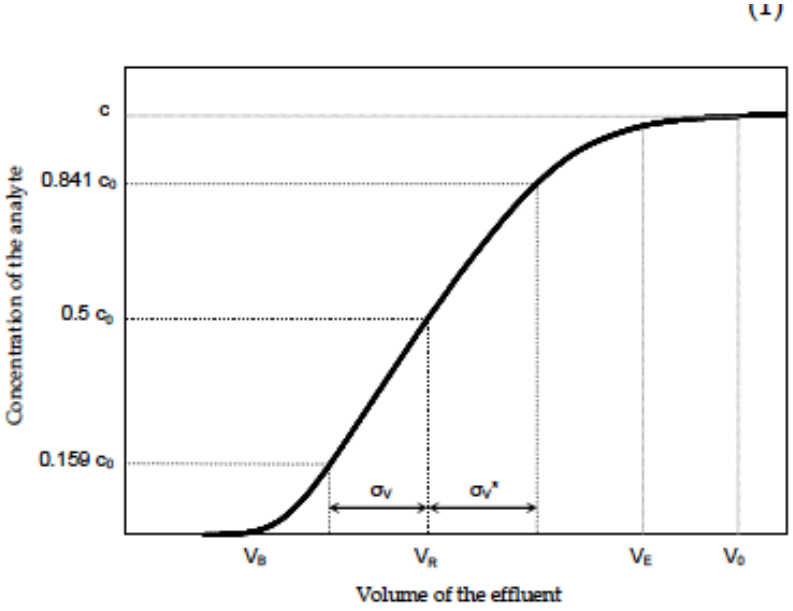

Figure 3.7 Typical representation of the breakthrough curve (Bielicka-Daszkiewicz & Voelkel, 2009).

Figure 3.7 shows a typical representation of the breakthrough curve (i.e. concentration of the analyte at the outlet of the SPE column vs. sample volume percolated through the system), where C_0 is the initial analyte concentration in the sample. V_B is the breakthrough volume, V_R is the chromatographic elution volume, and V_C is the sample volume when the concentration of the analyte at the outlet equals to C_0.

When a sample spiked with traces of a solute having an initial UV absorbance A_0, is percolated through a SPE cartridge, a breakthrough curve can be observed, beginning at a volume, V_B is usually defined at 1% of initial sample concentration up to a volume, V_E is defined at 99% of sample concentration where the effluent has the same concentration as that of the spiked water sample (Hennion, 1999). The

breakthrough curves of each antibiotic in LC-18, MAX and MCX cartridges are shown in Figure 3.8.

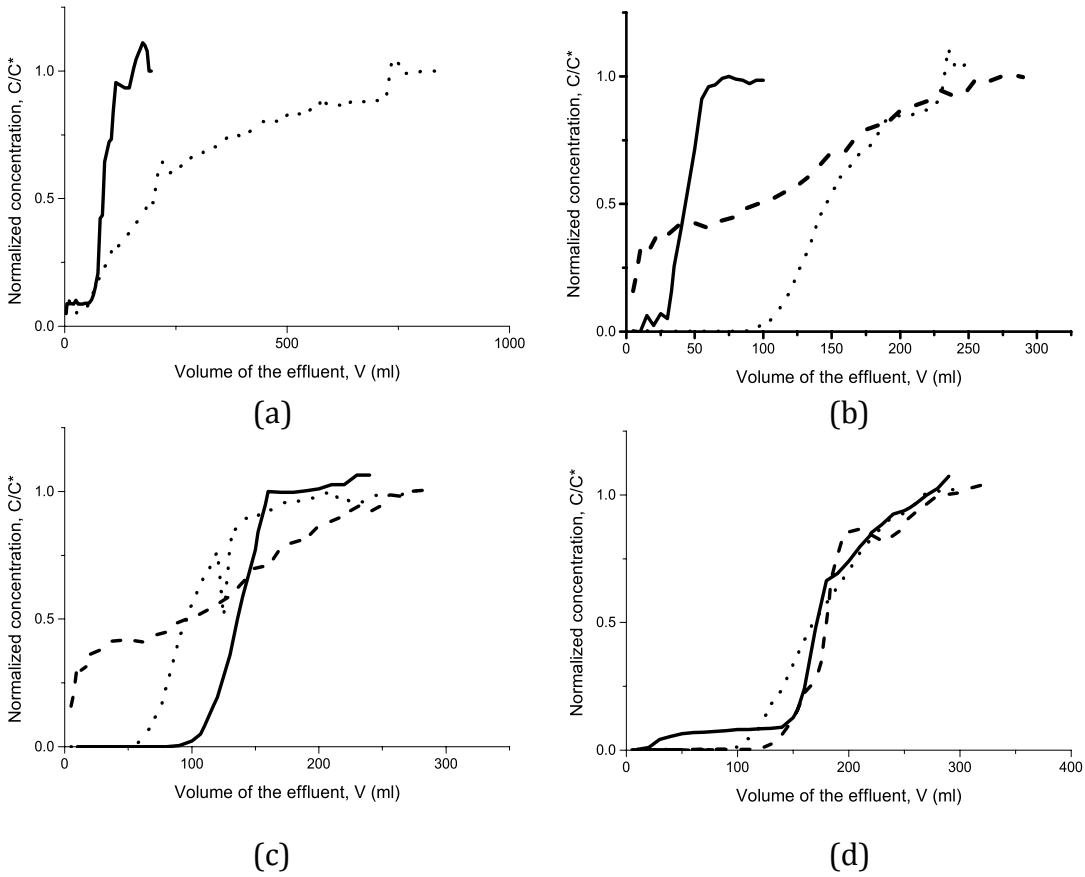

Figure 3.8 Breakthrough curves for (a) LCM, (b) MNZ, (c) OFL and (d) SMX in LC-18 (represented by solid line), MAX (represented by dash line) and MCX columns (represented by dot line).

Extraction parameters of sulfamethoxazole (SMX), metronidazole (MNZ), ofloxacin (OFL), and lincomycin (LCM) from water samples using LC-18, MAX and MCX sorbents are presented in Tables 3.1-3.4. Significant similarities were found when these data were analyzed. For all analytes, better efficiency of SPE columns represented by the number of theoretical plates corresponded to the highest adsorptive capacity. The equation used to calculate the theoretical plates number (N) can be presented as follow:

$$N = \frac{V_R(V_R - \sigma_V)}{\sigma_V^2} \qquad \textbf{Eq. 3.4}$$

Oasis MCX was found to be the most efficient sorbent for both metronidazole and lincomycin: the numbers of theoretical plates are 25.27 and 21.95, respectively and maximum adsorbed amounts are equal to 9.04 mg and 30.25 mg, respectively. The most efficient sorbent for sulfamethoxazole is Oasis MAX where the number of theoretical plates is 41 and the adsorptive weight was 18.4 mg. Although Oasis MCX has a lower number of theoretical plates for SMX, it has a similar adsorbed weight (W = 18.01 mg) as Oasis MAX column. LC-18 column was the most efficient sorbent for Ofloxacin (N= 44.08 and W = 55.1 mg).

Table 3.11 Parameters determined for SMX on different sorbents.

Sorbent	C_E (ppm)	V_R (ml)	V_B (ml)	V_E (ml)	σ_v (ml)	σ_v^* (ml)	W (mg)	N
LC-18	101.7	171	137	267	17	48	17.619	4.04
MAX	101.7	180	128	210	26	15	18.359	41.01
MCX	101.7	168	88	278	40	55	18.006	13.44

Table 3.12 Parameters determined for MNZ on different sorbents.

Sorbent	C_E (ppm)	V_R (ml)	V_B (ml)	V_E (ml)	σ_v (ml)	σ_v^* (ml)	W (mg)	N
LC-18	50.58	43	3	83	20	10	2.187	2.47
MAX	10	53	33	103	10	25	0.586	22.79
MCX	50	146	93.4	240	26.3	47	9.044	25.27

Table 3.13 Parameters determined for OFL on different sorbents.

Sorbent	C_E (ppm)	V_R (ml)	V_B (ml)	Equilibrium V_E (ml)	σ_v (ml)	σ_v^* (ml)	W (mg)	N
LC-18	408.8	136	98	168	19	16	55.089	44.08
MAX	101.07	96	1.7	294	90.7	99	9.599	0.0618
MCX	404	96	56	168	20	36	42.255	18.24

Table 3.14 Parameters determined for LCM on different sorbents.

Sorbent	C_E (ppm)	V_R (ml)	V_B (ml)	V_E (ml)	σ_v (ml)	σ_v^* (ml)	W (mg)	N
LC-18	37.27	190	26	713	82	261.5	6.895	3.05
MAX	NA	NA	NA	NA	NA	NA	NA	NA
MCX	352	86	53	132	16.5	23	30.245	21.95

As the pH of SMX, MNZ, OFL and LCM solution used in the experiments varied from weak acidic to neutral (pH range from 5.45 to 7.33), the analytes experienced both reversed-phase and ion exchange mechanisms in MAX and MCX cartridges. LCM has a pK_{a1} of 7.6 suggesting the presence of the cationic species of LCM at pH 4.7 (Figure 3.9) (Tölgyesi et al., 2012). As the pH of LCM was not high enough to be charged for MAX sorbent and its poor retention of the analyte on reverse-phase packings (Bergwerff et al., 1998; Carson & Heller, 1998; Haagsma et al., 1993), LCM can not be retained on MAX column (see Table 3.15).

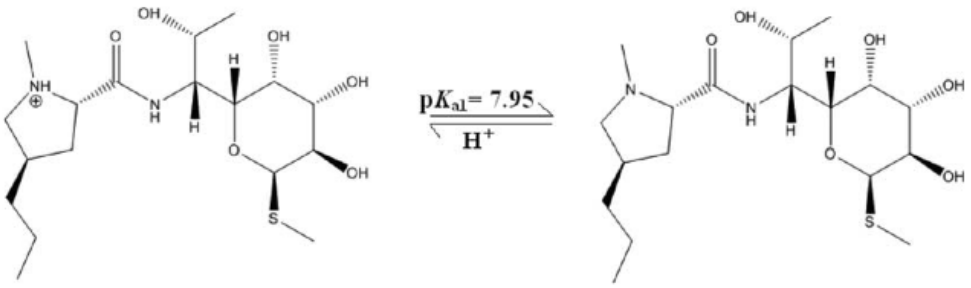

Figure 3.9 Cationic species in lincomycin present at pH 4.7.

For SMX and MNZ, MAX and MCX sorbents have better efficiencies than LC-18 sorbent indicated by a larger number of theoretical plates. Some studies have the same outcome that mixed-mode sorbents are more advantageous than reversed phase or ion-exchange SPE alone (Landis, 2007; Mroczek et al., 2002; Clauwaert et at., 2000). However, LC-18 was a better column to retain OFL, and MCX was better for LCM. In addition, the adsorbed weight of OFL was the highest among all the antibiotics in all the cartridges making it to be the easiest compound to be extracted.

Mixed-mode Cation eXchange (MCX) sorbent has a better extraction efficiency than the others.

Table 3.15 Adsorptive capacities (mg g^{-1}).

	LC-18		MAX		MCX	
	Column	Batch	Column	Batch	Column	Batch
LCM	13.79	NA	NA	NA	201.63	285.71
MNZ	4.37	NA	3.91	3.5	60.29	44.84
OFL	110.18	119.05	63.99	569.23	281.7	625
SMX	35.24	4.91	122.39	140.85	120.04	113.64

Table 3.15 compares the adsorptive capacities obtained from batch equilibrium and column sorption experiments. It can be observed that LC-18 cannot extract LCM and MNZ and has a much lower adsorptive capacity for SMX in column than in the batch. Table 3.15 also presents that LCM and MNZ cannot be adsorbed on LC-18 sorbent in the batch, but can be retained in the cartridge format. In the batch experiments, LC-18 could not be kept in suspension as it is very light and would float at the surface, therefore, reducing the interaction with the adsorbates in batch operation. MAX and MCX sorbents had comparable or much larger adsorptive capacities (especially for OFL) in batch experiments than in the columns because in the batches, the liquid phase can fully contact and attach to the active sites on the sorbents with mixing. On contrary, some channeling might occur during the column operation and thus not exposing all of the sorbent materials to the solution yielding lower adsorption capacity values.

3.3.3 Recovery studies

When the sorbate and sorbent reached equilibrium in the columns, the analytes were eluted by different amount of 100% acetone (typically 5 ml for MAX and MCX, and 3 ml for LC-18 columns). The eluent was evaporated to dryness under nitrogen and resuspended in distilled water. The concentrations of the samples were

determined by measuring the absorbance in a UV-Vis spectrophotometer. In order to study the recovery of columns, the antibiotics were passed through the columns until their maximum adsorption capacities were reached. Tables 3.16 -3.19 show the recoveries of each column and the amount of solvent it required. C_E represents the amount of antibiotics being eluted from the column per ml of solvent. As OFL has the highest adsorption capacity in all types of sorbent, it required higher amount of solvent to be desorbed and eluted from the columns. The recovery of test compounds in LC-18 varied from 72% ~104%. The recovery reported by other studies fell into this range (Kovalczuk et al., 2008; Batt et al., 2008; Kursinszki et al., 2006). The recovery of antibiotics in MAX and MCX sorbents were near 100% that indicates that mixed-mode sorbents have higher recovery than reversed phase alone, which was found earlier in the literature (Culleré et al., 2010; Fontanals et al., 2010; Benito-Peña et al., 2006).

Table 3.16 Recovery of LCM in MCX and LC-18 columns

LC-18			MCX		
V_{elute} (ml)	C_E (mg/ml)	Recovery (%)	V_{elute} (ml)	C_E (mg/ml)	Recovery (%)
5	2.619	88.286	5	0.036	0.640
			25	0.540	38.952
			80	0.610	101.000

Table 3.17 Recovery of MNZ in MAX, MCX and LC-18 columns

LC-18			MAX			MCX		
V_{elute} (ml)	C_E (mg/ml)	Recovery (%)	V_{elute} (ml)	C_E (mg/ml)	Recovery (%)	V_{elute} (ml)	C_E (mg/ml)	Recovery (%)
5	0.362	104.220	5	0.330	97.709	5	0.271	14.798
						25	0.345	98.297

Table 3.18 Recovery of OFL in MAX, MCX and LC-18 columns

LC-18			MAX			MCX		
V_{elute} (ml)	C_E (mg/ml)	Recovery (%)	V_{elute} (ml)	C_E (mg/ml)	Recovery (%)	V_{elute} (ml)	C_E (mg/ml)	Recovery (%)
5	3.043	39.757	5	0.410	38.938	5	0.234	11.215
25	1.010	72.741	25	0.340	103.531	25	0.225	22.004
						180	0.238	100.200

Table 3.19 Recovery of SMX in MAX, MCX and LC-18 columns

LC-18			MAX			MCX		
V_{elute} (ml)	C_E (mg/ml)	Recovery (%)	V_{elute} (ml)	C_E (mg/ml)	Recovery (%)	V_{elute} (ml)	C_E (mg/ml)	Recovery (%)
5	3.826	77.912	5	5.963	102.741	5	4.543	108.159

Comparing the recovery values, it seems LC-18 had the worst performance while MAX and MCX had comparable performances. In addition, maximum concentration of the eluent (C_E) could be achieved for MAX for SMX elution.

3.4 Error analysis

Both batch adsorption experiment and continuous operation were repeated three times to investigate the accuracy of the data. Table 3.20 shows the relative standard deviation of adsorption capacities and recoveries of different micropollutants from different sorbents in each experiment.

Table 3.20 Relative percentage standard deviation of the experiment data.

	Batch sorption (Q_e)			Continuous operation (q_{max})			Recoveries		
	LC-18	MAX	MCX	LC-18	MAX	MCX	LC-18	MAX	MCX
LCM	NA	NA	2.83	15.87	NA	4.16	1.92	NA	0.69
MNZ	NA	1.95	0.73	28.41	14.35	9.06	1.22	0.68	1.36
OFL	0.92	5.77	0.99	2.52	16.36	8.41	3.92	1.05	1.82
SMX	4.87	0.21	0.96	1.75	2.71	2.28	2.15	0.81	1.07

It was indicated in Table 3.20 that the performance of the selected sorbents was very consistent and stable. Especially for MCX sorbent, it had a lower relative standard deviation compared with MAX and LC-18 sorbents which suggests that MCX has more steady performance than the other sorbents. Overall, the results obtained in this stage of study were very reliable and promising based on the low relative standard deviation.

3.5 Conclusions and Summary for Solid Phase Extraction Application

SPE, as a well-established technique, has many advantages over other sample preparation methods and it has been used for the analysis of numerous different classes of compounds in a variety of matrices. For environmental samples, reversed-phase and ion-exchange sorbents are largely used for the determination of organic micropollutants in aqueous environmental samples.

The study in this chapter demonstrated the comparison of two mixed-mode sorbents: Oasis® MAX and MCX and one reversed-phase sorbent: Supelco® LC-18. For MAX and MCX sorbents, the adsorption processes were monolayer as indicated by the correlation coefficients of Langmuir. In addition, the increase of maximum adsorption capacity per unit sorbent of the compounds in a column followed the trend of the increase of pKa values of the compounds.

Oasis® MCX sorbent had higher recoveries and adsorption capacities for micropollutants in distilled water than other commercially available sorbents such as Oasis® MAX and LC-18. And for a more general conclusion, mixed-mode sorbent was better than reversed-phase sorbent both in recovery and adsorption capacity for the four tested compounds which are all fairly hydrophilic and polar.

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Chapter 4

4 Assessment of the Mutagenicity and Estrogenicity of River Water and Wastewater Secondary Effluent Following SPE treatment

4.1 Introduction

The expanding application of bioassays to monitor water quality is due to the concern over the occurrence of a large number of suspected mutagenic or estrogenic chemical substances found in different water matrices such as surface water, ground water, wastewater effluents, and even in drinking water. There are two types of bioassasys: *In vivo* and *in vitro* bioassays.

In vivo tests, as known as "Direct Toxicity Assessment (DTA)", can be conducted either in the laboratory or in the field (in situ bioassay) by conducting tests on whole and living organisms (Murphy et al., 2009). They measure changes on parameters such as growth rate, feeding activity, reproduction, and mortality. They also measure the effects based on more specific biochemical endpoints (Margot et al., 2013). Sometimes, *in vivo* bioassays might be employed over *in vitro* bioassays in order to observe the overall effects of the mutagenicity or carcinogenicity of the micropollutants in wastewater on a living subject. In vivo test reflects the complexity of contaminant responses in the living organisms. However, it is more logistically difficult to conduct and it also has too many uncertainties that may result in hard-interpreting results.

In vitro test systems, also known as "bioanalytical tools", based on particular cellular mechanisms, measures cellular effects specific to groups of mutagens with similar modes of action (Margot et al., 2013). They usually use part of the organisms such as cell culture or transgenic bacteria or yeast to detect changes in receptor activation or enzyme function such as genotoxicity, mutagenicity or endocrine secretion (Margot et al., 2013). Although the mechanistic assays, which use the cell lines, usually have minimal metabolic capacity that makes them hard to show the effects

of bioactivation of toxicants in the animal, in vitro bioassays are less time and resource consuming (Asker, 2011). The primary advantage of in vitro bioassays is that the investigators can concentrate on a limited number of components instead of a whole living organism. This makes the results much easier to analyze than in vivo bioassays. They also decrease the requirement of experienced personnel in the laboratory to handle the living organisms such as small animals for in vivo bioassays.

Two very important toxicity measures are to monitor estrogenicity and mutagenicity of a substance. The most widely used in vitro bioassay for testing estrogenicity is the Yeast Estrogen Screen (YES) test which uses a strain of yeast *Saccharomyces cerevisiae* that respond to estrogenically active substances. The strain is genetically modified to harbor a human estrogen receptor (hER) expression cassette and a reporter gene. The presence of estrogenic substance changes the receptor and enables the estrogen receptor complex binding to the estrogen-responsive element. Finally, β-galactosidase is produced and it metabolizes 4-methylumbelliferyl-β-digalactopyranoside. The estrogenic activity can be expressed by estradiol equivalent concentration (EEQs) which can be determined by measuring the absorbance of the dye.

The Ames fluctuation test, as the most commonly used bioassay for mutagenicity testing, uses a variety of modified *Salmonella typhimurium* strains that respond to different mutagenic mechanisms. The test uses genetically defective *Salmonella* strains unable to synthesize histidine, an enzyme *Salmonella* requires to grow. When the tested substance triggers a reversion mutation the bacteria can then produce histidine for survival. Based on the statistical deviation of the sample relative to the background and positive control, the determination of the probabilistic mutagenicity of the contaminants can be made (Ashby & Tennant, 1998). As there is a strong correlation between mutagenicity and carcinogenicity, a substance which has a positive response in Ames test warrants further investigation using other *in vivo* or *in vitro* tests such as human carcinogenic tests.

The mutagenicity of river water, as a source of surface water has been extensively evaluated, as it is a main influent source to drinking water treatment facilities. In various articles, it was addressed that wastewater, especially hospital and industry wastewater, are the major discharge sources of mutagenic and estrogenic substances due to laboratory activity and commercial production all over the world (Tabrez et al., 2010; Vargas et al., 1993; Jolibois et al., 2003; Bistan et al., 2011; Citulski et al., 2001). Thus, it is important to monitor the quality of the effluents from the wastewater treatment facilities. Solid phase extraction is an efficient technology to concentrate and extract the potential mutagenic and estrogenic substances in surface water and wastewater samples in order to make the sample concentration lower than the detection limit in the bioassays. Meanwhile, bioassays and chemical analysis could evaluate the recovery of the SPE columns if the amount of target substance is known. In this chapter, 17β-Estradiol (E2) was spiked into river water and wastewater in order to imitate aqueous samples with estrogenic substance in low concentration. Initially, an attempt was made to concentrate the positive mutagen Sodium azide for *Sal* TA 100 and 2-nitrofluorene for *Sal* TA 98 from different water samples, however, the experiments were not successful due to low solubility and high toxicity of both the compounds and low resolution in UV-Vis spectroscopy for chemical analysis. Although, E2 is estrogenic, it is not toxic to handle at different concentrations, and was used as the model compound to determine the matrix effect on the SPE performance.

4.2 Materials and methods

4.2.1 Chemicals

17β-Estradiol (E2) (MW: $C_{18}H_{24}O_2$, CAS: CAS 50-28-2) was purchased from Sigma-Aldrich (Oakville, Ontario, Canada) with 98% purity. The stock at a concentration of approximately 200 mg/L were prepared in ethanol (99.5 % purity) purchased from Fisher Scientific (Ottawa, Canada) and stored in amber vials at 4 °C. The standard was brought to room temperature before use and freshly prepared every two to

three months. Acetonitrile (minimum 99.8%) was obtained from Caledon Laboratories (Georgetown, Ontario, Canada). HPLC grade methanol and ethanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). 99.5% acetone was purchased from VWR (Radnor, PA, USA). No further purification was required for all the reagents. A Nanopure Ultrapure Water System (model Integral 5, EMD Millipore Corporation, Billerica, MA, USA) provided nanopure (LGW, 18MΩ) water used in the experiments.

4.2.2 Sample collection and preparation

Surface water grab samples (large volumes) were collected from a stream (which is hydrologically connected to the Thames River in London, Ontario, Canada) in a glass container that had been thoroughly washed and rinsed before use. Secondary effluent wastewater samples with large volumes were taken from Adelaide Pollution Control Center in London, Ontario. Each sample was collected in a 4-L glass bottle which had been washed and rinsed thoroughly with ultra-pure water.

Upon return to the laboratory, the samples were immediately filtered through 0.2µm Supor 200 filters (PALL, Mississauga, Ontario, Canada) to remove all solid particles and microorganisms to minimize possible biological degradation and the possibility of blockage in the SPE column. Water samples were stored at 4 °C in the dark for no longer than three days before use. From this large batch, a portion of surface water and secondary effluent was spiked and the respective blank water matrix (e.g. non-spiked) was taken from the same batch. E2 standard solution was spiked into both river water and wastewater samples to simulate an aqueous concentration of 3 µg/L E2.

Solid phase extraction was performed by following the SPE protocol from Waters. Briefly, the SPE was effectuated using Waters Corp. Oasis MCX 6 mL cartridge (Mississauga, Ontario, Canada) with 150 mg of sorbent material. The cartridges were pre-conditioned with 5 mL of methanol followed by 5 mL of ultra-pure (Milli Q)

water. SPE cartridges were then submerged in a sample reservoir filled with spiked and non-spiked water samples. A peristaltic pump was used to extract the sample out of the cartridges from the reservoir at a flow rate of 1.35 to 1.5 mL per minute (Figure 4.1). After the extraction was complete, 5 mL of Milli Q water was passed through the SPE column to remove the impurities and interference. The retained E2 was eluted into a 20 mL glass vial using 3 mL of acetonitrile at a rate of about one drop every 5 to 6 seconds by vacuum. The eluent was evaporated to dryness using a nitrogen evaporation system operating at 50 ± 5 °C. The samples were then reconstituted by adding 3 mL of Milli Q water. Finally, E2 samples were sterilized by filtering through sterile MicroLiter PTFE syringe filters purchased from VWR (Mississauga, Ontario, Canada) before being applied for analysis.

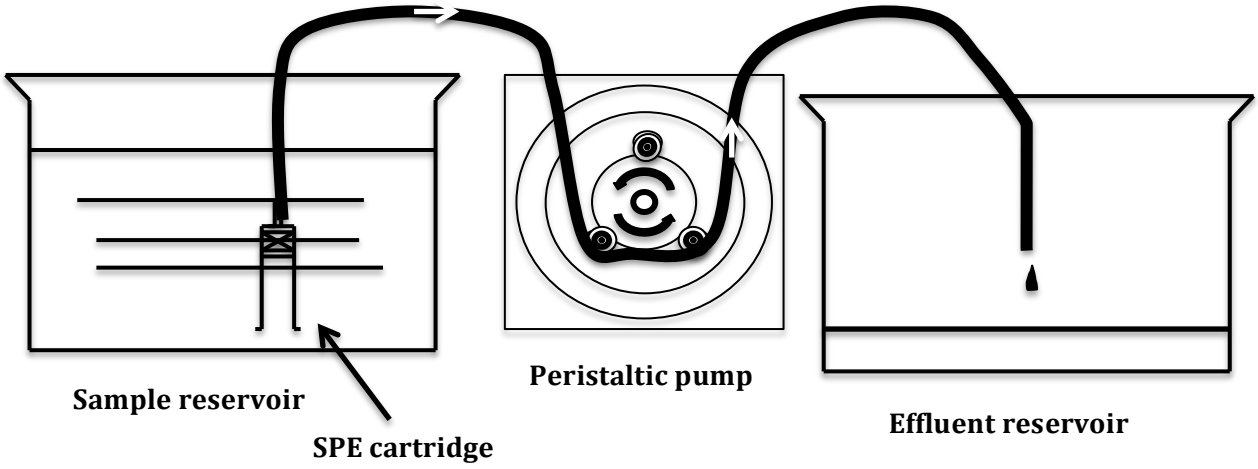

Figure 4.1 Experimental set-up of large sample extraction.

4.2.3 Instrumental analysis

The estrogenic substance E2 was chemically analyzed by a high performance liquid chromatographic (HPLC) system (Agilent 1260 Infinity series consists with Quaternary pump: G1311B, Auto sampler: G1329B and diode array detector: G1315C, Agilent, Clara, USA). E2 was separated from different matrices on an Agilent Poroshell 120 EC-C18 reversed phase (4.6 × 50 mm, 2.7 µm) analytical

column (Agilent Technologies, Clara, USA). The mobile phase was a mixture of acetonitrile and Milli-Q water (55:45, v/v) and its flow rate was set at 0.8 mL/min. The injection volume was 20 µL from 2 mL amber HPLC vials, capped and sealed with PTFE lids. The separated E2 was detected by a UV spectrophotometer at a wavelength of 210 nm. Figure 4.2 shows the flow diagram of the experiment procedures.

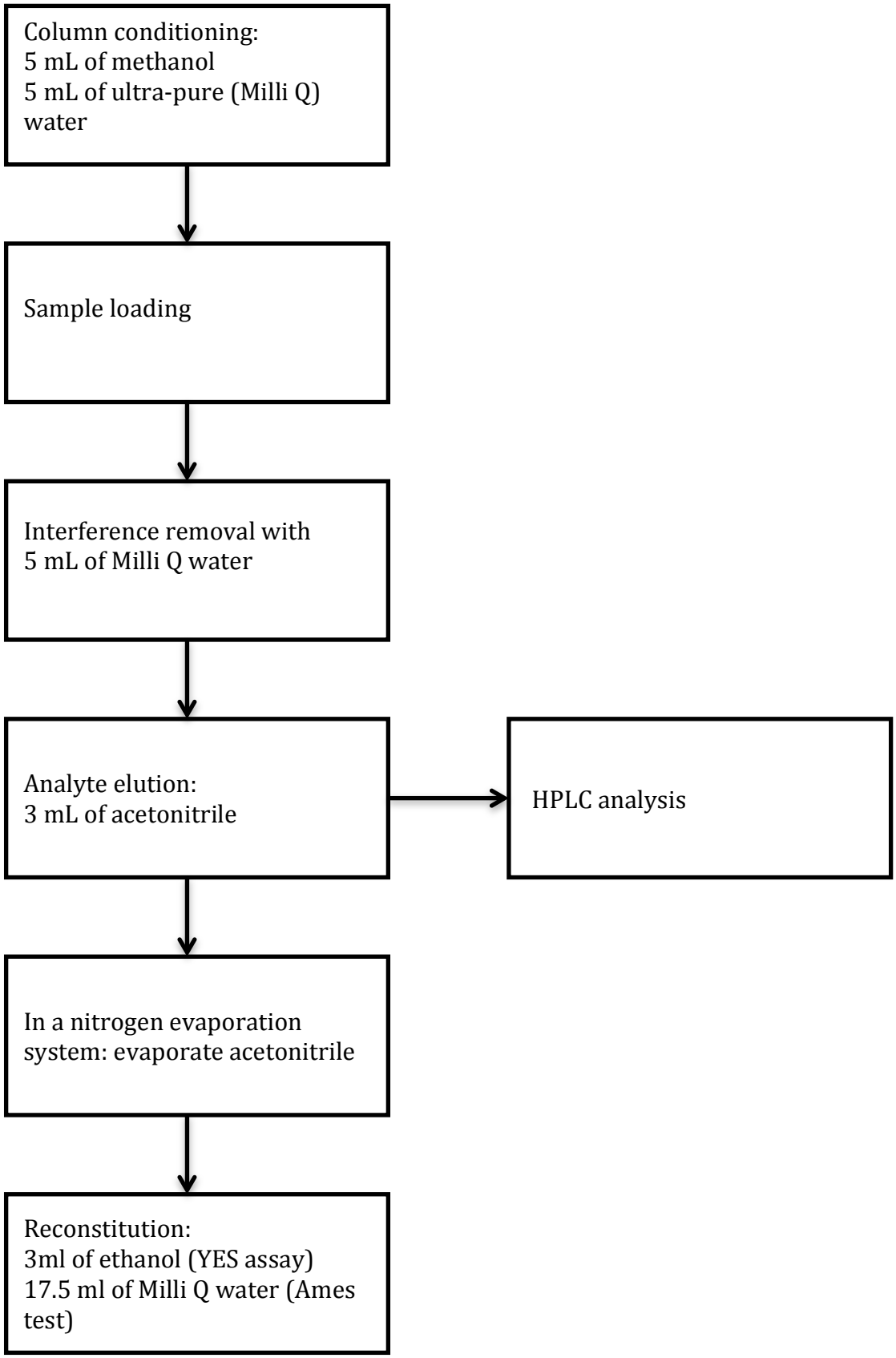

Figure 4.2 Flow chart for SPE procedure for bioassays and HPLC analysis

4.2.4 Ames fluctuation assay

The mutagenicity of the water was determined by using the Ames Assay (Ames et al., 1975). The test employs two *Salmonella typhimurium* strains with different mutation mechanisms: TA 97 and TA 98 which carry a mutation in the operon coding for histidine biosynthesis. All the bacteria and reagents for Ames test were supplied by Environmental bio-detection product inc. (EBPI) (Mississauga, ON, Canada). Reverse-mutation assays were performed using the "Fluctuation method". Instead of counting the number of colonies observed in the agar plates the method originally designed by Ames, the number of yellow wells showed in a 96-microplates is enumerated (if the chemical to be tested causes a histidine-requiring mutant of Salmonella bacteria, the color of the dye in the wells will be converted from purple to yellow.)

A 17.5 mL sample was filtered through 0.22 µm PTFE membrane filter, mixed with 2.5 mL of reaction mixture (consists of 72.1% Dacis Salts solution, 15.8% Glucose, 7.9% Bromocresol Purple, 4% Biotin and 0.2% Histidine) and 10 µL of the *Salmonella* strain cultured overnight (16 to 18 hours at 37 °C) with an optical density of 0.5 to 1 at 600 nm. The positive control was prepared by adding 0.1 mL of standard mutagen (9-aminoacridine and 2-nitrofluorene) to 2.5 mL of the Reaction Mixture, 17.4 mL sterile distilled water, and 10 µL of bacteria. The background was prepared by mixing 17.5 mL of sterile distilled water, 2.5 mL of the Reaction Mixture, and 10 µL of bacteria. The blank (the sterility check) was prepared by adding 17.5 mL of sterile distilled water to 2.5 mL of the Reaction Mixture only. After the solution had been well mixed in centrifuge tubes and transferred into reagent reservoirs, 200 µL of the mixtures were dispensed into each well in a 96-microtitre plates (Corning Costar, USA) by a multichannel pipette. The plates were then covered with lids and put into an air-tight plastic bag to prevent evaporation. The plates had to stay in a 37°C incubator for five days before the yellow wells could be enumerated.

The level of the mutagenicity of the water matrix after extraction from MCX cartridges was determined visually by enumerating the number of wells changed from purple to yellow as a positive reaction. The "Background" plate showed the level of spontaneous mutation of the assay organism. The test results correspond to the total number of positive wells (yellow color) scored in a 96-microtitre plate for the sample plate in comparison to the background plate. Mutagenicity of a test substance (and certainty in percentage) is proportional to the number of yellow wells enumerated. The statistical significance of the results is determined by comparing the standard test tables provided by EBPI.

4.2.5 Yeast Estrogen Screen assay

4.2.5.1 YES assay Procedures

Recombinant yeast cells (Saccharomyces cerevisiase) were provided by Trojan UV (Ontario, Canada). The YES assay was performed as previously described in Routledge and Sumpter (1996). Briefly, 250 µL concentrated yeast stock from a cryogenic vial was seeded into the growth medium in a conical flask. Growth medium consists of glucose, L-aspartic acid, vitamin solution, L-threonine solution, copper sulfate solution and minimal medium. The whole culture was incubated at 28 °C, 180 rpm for approximately 24 hours or until turbid, on an orbital shaker. The following day, assay medium was prepared by adding 2 mL of the 24-h yeast culture and 0.5 mL Chlorophenol red-β-D-galactopyranoside (CPRG, Sigma-Aldrich, Oakville, Ontario, Canada) solution (10 mg mL^{-1}) to 50 mL growth medium (approximately 4 × 10^7 yeast cells in the medium). For a standard test, E2 stock, at a concentration of 54.48 µg/L, for the standard curve was prepared using absolute ethanol. E2 stock solution was diluted in absolute methanol by a twofold serial dilution method and the concentration of 12 dilutions of E2 in the plate was in the range of 54.48 µg/L to 26.6 ng/L. 10 µL of the E2 standard dilutions were transferred, in triplicate, into the wells in a 96-microtitre plate (Corning Costar, USA) and allowed to dry (approximately 20 min). One or two rows of the blank were prepared by adding 10 µL of the absolute ethanol to 190 µL of the assay media to terminate the growth of

the yeast cells. 100 µL out of 3 mL of reconstituted extraction samples were twofold serially diluted in two rows of the microplates using ethanol, and were left to completely evaporate. Upon the dryness of the standard and sample wells, 200 µL of the seeded assay medium was added to each well. The plates then were sealed with autoclave tape and shaken vigorously for 2 min in a plate shaker (VWR). Subsequently, the plates were incubated at 32 °C in a naturally ventilated heating cabinet for three days with 2 min vigorously shaking every day. In day four, the plates were shaken for 3 min, and left for approximately 1 hour to allow the yeast to settle.

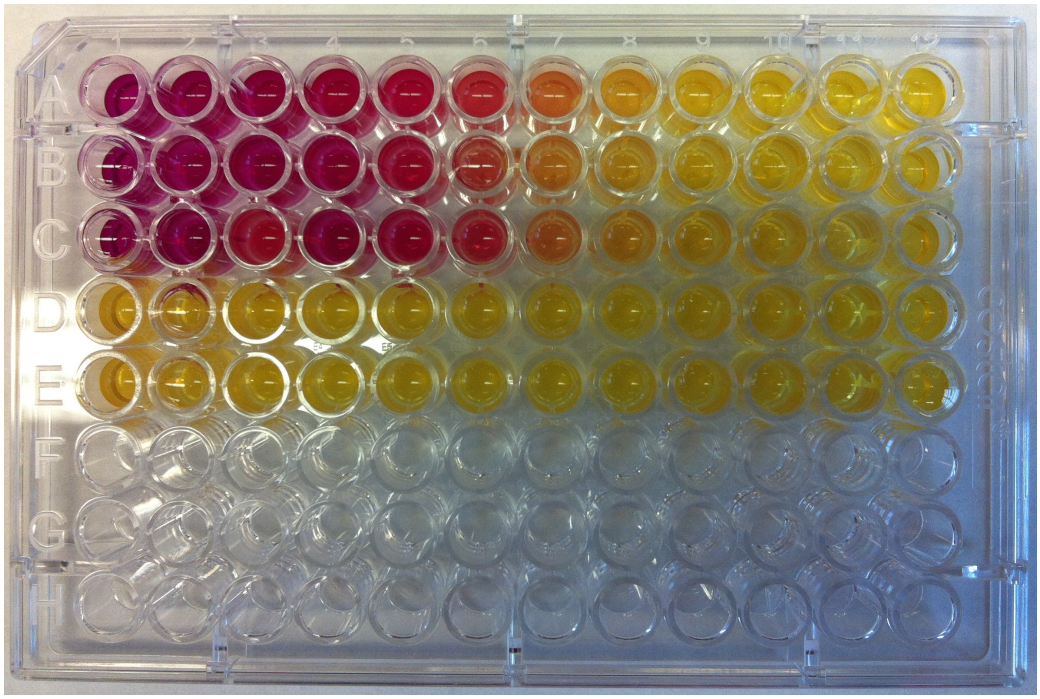

Figure 4.3 Photo of YES assay plate. Yellow well indicates that no estrogenicity was detected. Other well with color changing from orange to purple represents the normal growth of yeast. First three rows were E2 standard and last two rows were blank.

4.2.5.2 YES assay Calculation and Sample Response

The estrogenic activities can be expressed by estradiol equivalent concentration (EEQs). The absorbance of samples at 540 nm and 620 nm and the blank (medium)

at 620 nm were measured in a plate reader (Tecan Infinite 200 PRO, Switzerland). In order to correct for turbidity, the data need to be processed with the following equation:

Corrected value = chem. abs. (540 nm) - [chem. abs. (620 nm)-blank abs. (620 nm)]

Eq. 4.1

A response of a proper concentration can be interpolated into a dose-response curve (using 17β-estradiol (E2) as reference compound) (Figure 4.4). The curve was fitted to the Eq. 4.2, using Origin Labs software (Northampton, USA).

$$response = a + \frac{b-a}{1+10^{(\log EC50 - logC)\times m}} \qquad \textbf{Eq. 4.2}$$

where **a** is the baseline response (bottom), **b** is the maximum response (top), **C** is the concentration, **m** is the Hill slop, and **EC50** is the half-maximal effect concentration. Hill slope quantifies the steepness of the curve and is also known as the slope factor (Fent et al., 2006).

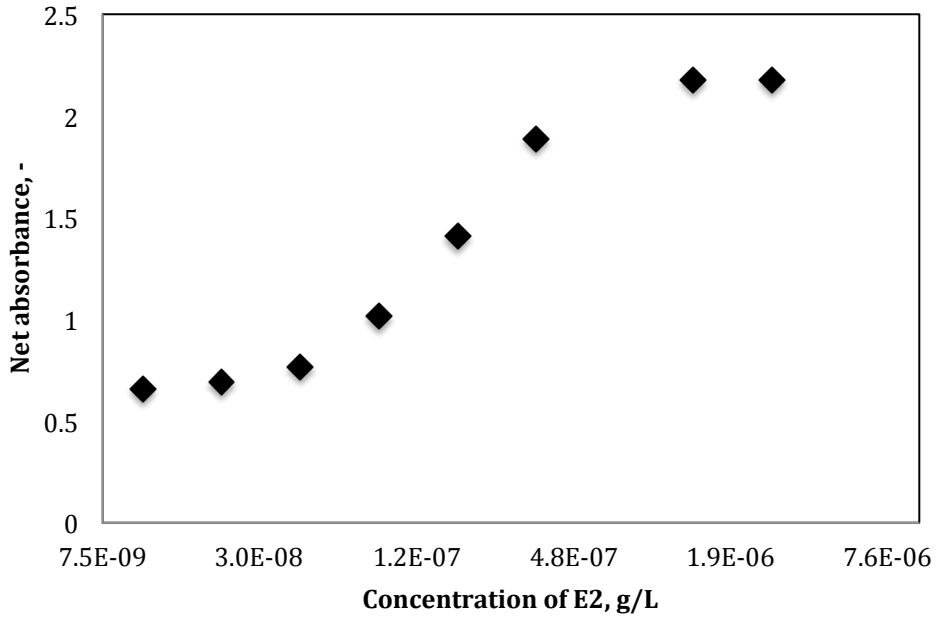

Figure 4.4 Estradiol (E2) dose-response curve using ethanol.

The data were processed as per the methodology explained by Huber (Gilmour, 2012). The curve constants a, b, m and EC50 were determined by the non-linear

curve fitting for the standard. The corrected absorbance calculated by Eq. 4.1 verse concentration factor of the sample was plotted and fitted into Eq. 4.2 with fixed a, b and m obtained from standard curve fitting. If a concentration gives a response that can be fitted into the linear part of the dose-response curve, it is considered as a suitable concentration (Bistan et al., 2012). Finally, EEQs are the quotients of $EC50_{17\beta\text{-estradiol}}$ and $EC50_{samples}$.

4.3 Results and Discussion

4.3.1 Determination of Estradiol in liquid chromatography and YES assay

The quantitative parameters of the proposed HPLC method were calculated under the optimized conditions described in Section 4.2.3. The calibration curve was obtained by plotting the peak areas of E2 against the concentration of the E2 in the Acetonitrile sample. The linear range was obtained between 1 – 100 mg L^{-1}, with a correlation coefficient of 0.99996 by using a weighted linear regression method. With this HPLC method, the limit of detection of E2 was 1 mg L^{-1}. When the concentration of E2 is below that limit, too much noise would appear. The calibration equation is shown in Table 4.1 where Y is the area of the peak and X is the amount of E2 being detected.

Table 4.1 Main method parameters of the HPLC method

LOD (limit of detection) (mg L^{-1})	1
Regression equation	Y = 67537.714 X (Eq. 4.3)
DLR (mg L^{-1})	1 – 100
R^2	0.99996
Retention time (min)	1.253

Surface water with 2L in volume from Thames River and 6L of wastewater from Adelaide Pollution Control Center were extracted and concentrated in Oasis MCX columns. The final volumes of the tested samples were 3mL.

The presence of hormones 17β-estradiol within the limit of detection was not observed in river water and wastewater samples by HPLC analysis. Either the concentrations of E2 in concentrated water samples were lower than the LOD (1 mgL^{-1}) in this HPLC method or there was no E2 present in the Thames River.

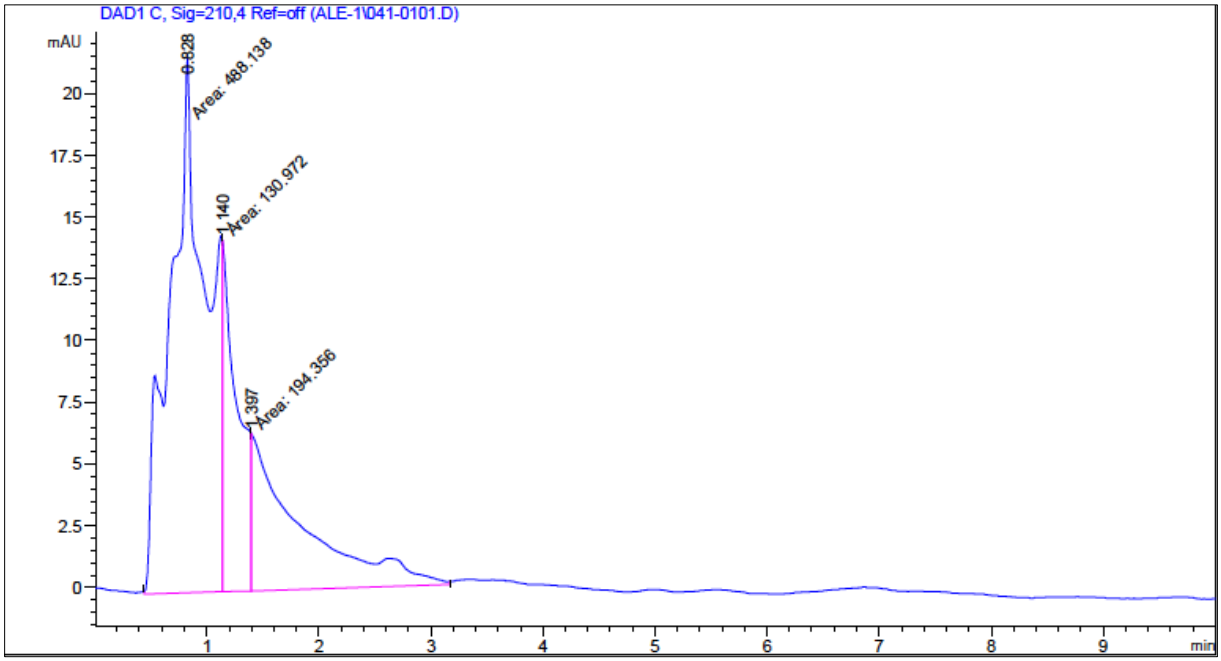

Figure 4.5 Chromatograms of the surface water collected in Thames River. The split peak corresponds to the E2 peak detected in Figure 4.7.

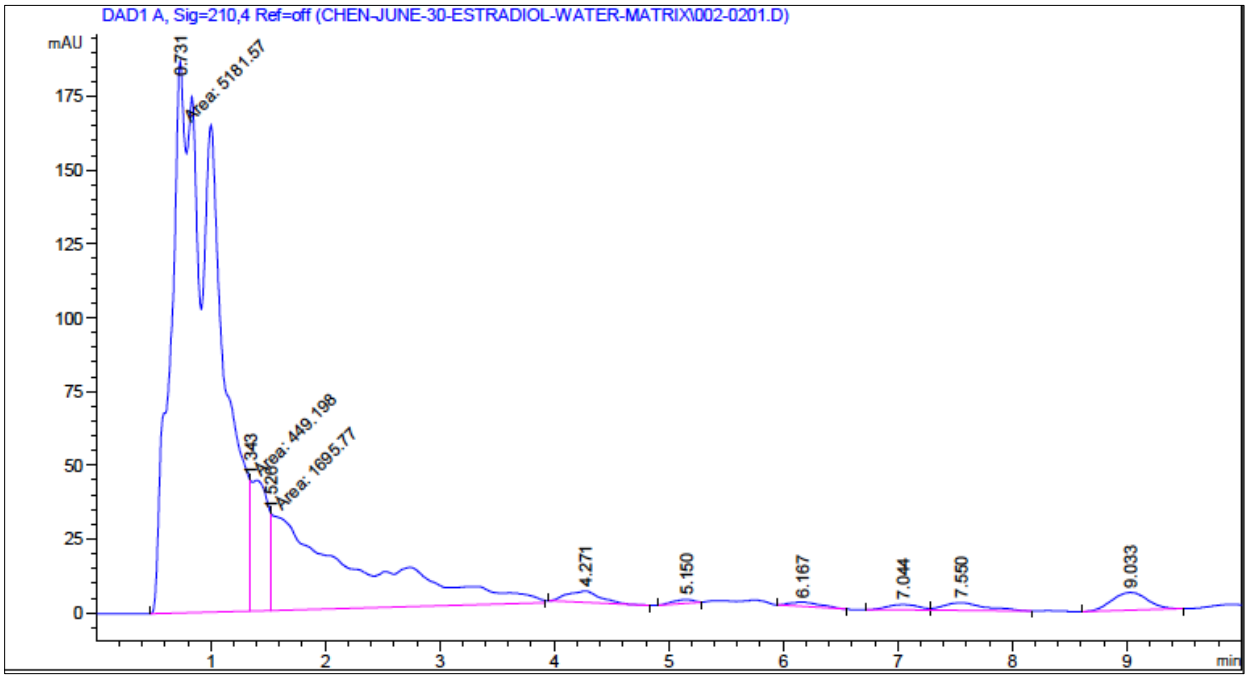

Figure 4.6 Chromatograms of the wastewater collected in Adelaide Pollution Control Center. The split peak corresponds to the E2 peak detected in Figure 4.8.

To further determine the presence of estrogenic substance in these water samples, Yeast Estrogen Screen (YES) assay was performed. However, the response given by the results in the YES test cannot be fitted into the linear part of the dose-response curve. These results indicated that the concentrations of E2 presented in concentrated surface water and wastewater samples were lower than the lowest limits of quantification (LLOQ) for which were 0.34 ngL^{-1} E2 equivalent for surface water and 0.68 ngL^{-1} E2 equivalent for WWTP effluents (Krein et al., 2012).

It can be concluded that for E2 detection, YES assay was more sensitive than HPLC analysis. Otherwise, HPLC was capable to separate the compounds in the water samples and more accurate in quantification. Both HPLC analysis and YES assay suggested that no E2 could be detected after 600 times concentration for surface water and 2000 times concentration for wastewater.

4.3.2 Recovery test of Oasis MCX in surface water and wastewater matrices

After selecting the Waters Oasis MCX based on the results of Chapter 3, the performance of the established method was tested in the more relevant environmental matrices such as surface water and wastewater. As revealed in section 4.3.1, the concentration of background E2 in the extracted surface water and wastewater samples were lower than the detection limits. 17β-estradiol standard was spiked into 2L of surface water and 6L of wastewater samples. The spiked aqueous samples were extracted by Oasis MCX cartridges and finally eluted to a 3mL sample with E2 concentration of 2 mgL^{-1}. The elutes were tested in HPLC and YES assay to determine the recovery of MCX cartridges.

4.3.2.1 Recovery in liquid chromatography

From the chromatograms of surface water and wastewater samples shown in Figure 4.5-4.8, it is likely that aqueous samples experienced some matrices effects as a large amount of interferences passed through the MCX sorbent simultaneously with E2 standard; much of that likely being co-extracted and then co-eluted with methanol and acetone. The spike recoveries of 2 river water samples ranged from 106 -109%. As shown in Figure 4.5 and 4.6, some unidentified interference had been detected in the same retention time and wavelength. In order to quantify the E2 standard in surface water and wastewater samples, the area of those unknown substances in Figure 4.5 and 4.6 were subtracted from the area of the peaks of E2 in Figure 4.7 and 4.8. Table 4.2 revealed the peak areas, corresponding concentrations and the recoveries in surface water samples.

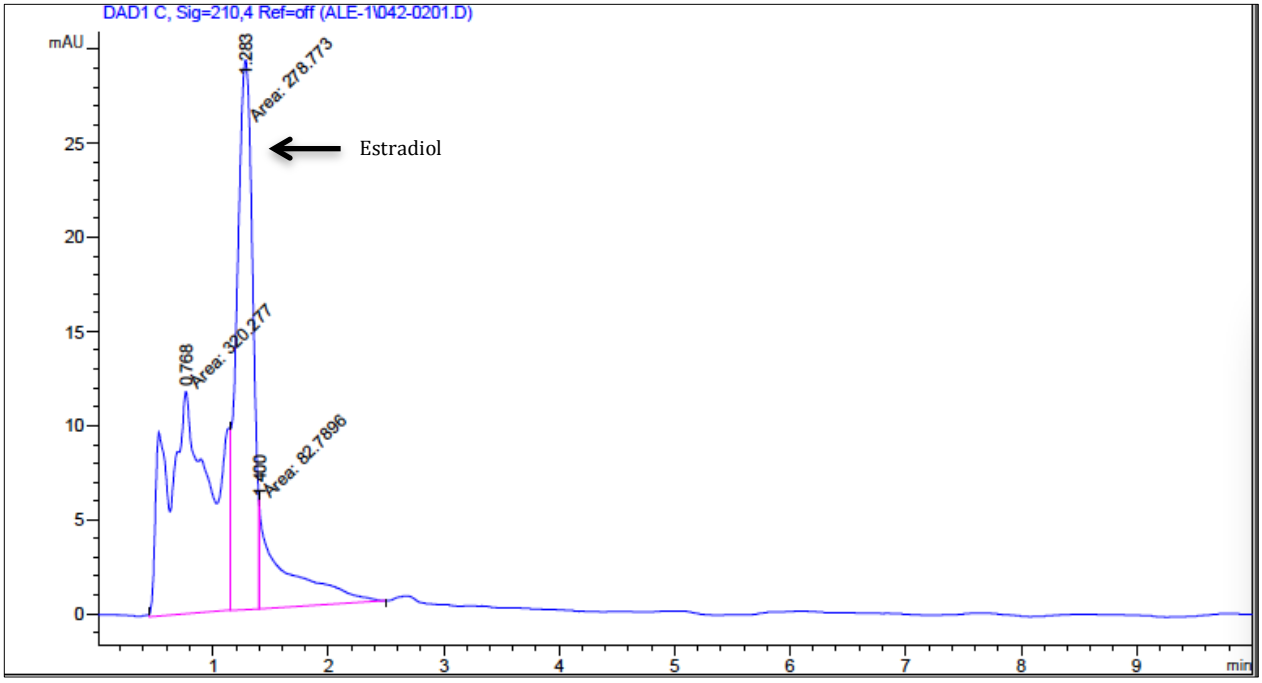

Figure 4.7 Chromatograms of the surface water spiked with E2 standard.

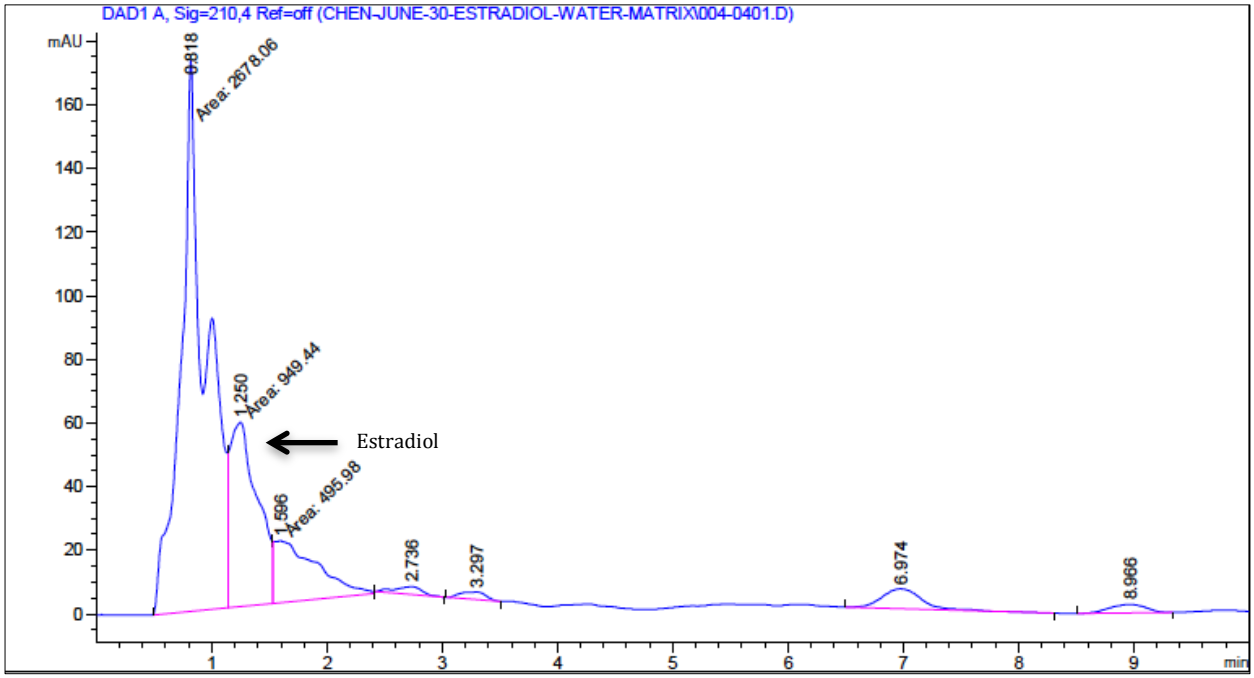

Figure 4.8 Chromatograms of the wastewater spiked with E2 standard.

Table 4.2 shows the recovery of E2 standard in Oasis MCX cartridges in surface water samples measured by HPLC. A_{sp} represents the peak areas of spiked E2 standards, A_{nsp} was the area of non-spiked sample integrated in the same retention time as the spiked samples, $C_{theoretical}$ is the theoretical concentration of E2 after SPE treatment in the surface water samples and C_{E2} indicates the concentration of E2 being detected and calculated in Eq 4.3.

Table 4.2 Recovery of E2 in Oasis MCX sorbent from HPLC analysis.

A_{sp}	278.773	2650.02
A_{nsp}	130.972	1212.37
$C_{theoretical}$, mgL^{-1}	2	20
C_{E2}, mgL^{-1}	2.188	21.29
Recovery	109.4%	106.4%

As elaborated in Chapter 3, Oasis MCX cartridges had a nearly 100% recovery for polar and hydrophilic sample extraction in distilled water matrix. In this chapter, HPLC analysis indicates that Oasis MCX is highly efficient for pre-concentration of relatively nonpolar and hydrophobic substance E2. Surface water, as a matrix with high dissolved organic concentration (TOC ≈800 mg/L), seems to have insignificant influence on the MCX sorbent. On the other hand, the HPLC signal of E2 spiked in wastewater was quite broad and could not be deconvoluted for accurate analysis. Since it was difficult to define the border of the peak, quantification of E2 in concentrated wastewater samples indicated a recovery of 300% indicating significant interference from the water matrix.

4.3.2.2 Recovery in YES bioassays

For further E2 concentration verification, the concentrated spiked E2 samples in different aqueous matrices were applied in YES assay. As no detectable estrogenicity was found in both concentrated surface water and wastewater samples, the positive responses in the YES assay were from the spiked E2. The calculated EEQs were the

actual concentrations of E2 being assessed in YES assays. Table 4.3 presents the EEQs of spiked and concentrated surface water and wastewater samples and the calculated recoveries of MCX sorbents.

Table 4.3 Estrogenic activity of E2 in surface and wastewater samples determined by YES assay and the recovery of MCX sorbent.

	River water		Wastewater
$C_{theoretical}$, mgL^{-1}	2	20	2
EC50	0.000573	0.0000595	0.00197
EEQ, mgL^{-1}	2.191	21.12	1.693
Recovery	109.55%	105.6%	84.65%

It can be seen in Table 4.3 that the recoveries of MCX sorbent for E2 extraction from surface water obtained in YES assays were quite consistent with the recoveries measured in HPLC analysis, which were in the range of 105% to 110%. The more-than 100% recovery could be explained as experimental error, since the volume as small as 30 µL of E2 standard was added into 2L of surface water and 6L of wastewater to make the concentration of E2 in the water samples to be 3 µg L^{-1} and 1 µg L^{-1}, respectively.

YES assays confirmed that E2 can be extracted by MCX sorbent from different aqueous matrices and the recoveries were acceptable. In addition, the effect of matrix was minimal for YES assay as compared to the HPLC method. HPLC analysis could not resolve the impact of wastewater matrix on the SPE sorbent, since various compounds in the matrix were co-extracted and co-eluted with the target analytes which resulted in large interference in the chromatograms. However, using the non-

spiked wastewater sample as the blank, the concentration of spiked E2 can easily be determined by the YES assay. These two methods also demonstrated that surface water matrix has insignificant influence on the performance of SPE cartridges. On the other hand, under the impact of wastewater matrix, the recovery of MCX sorbent was reduced by at least 15%. The recoveries determined in HPLC and YES assays substantiated that despite the diversity and complexity of surface water and wastewater matrices, MCX sorbent was able to successfully extract and recover the target analyte, E2.

4.3.3 Mutagenicity of Wastewater in London

While it had been demonstrated by YES tests that there were minor estrogenic substance in London's wastewater, the mutagenicity of wastewater matrix was unknown. Ames test, as a quick bioassay can provide valuable information about the safety of resultant water after MCX sorbent extraction. The results of the *Salmonella* mutagenicity assay for wastewater in TA 98 and TA 100 *Salmonella* strains are summarized in Figure 4.9. The level of mutagenicity is determined by the statistical deviation of the number of reverts relative to the background. If the deviation is more than 15%, the sample can be considered mutagenic. A 3L of secondary effluent was passed through the MCX cartridge and finally reconstituted in distilled water to make a 17.5mL sample to be tested with a single bacterial strain in Ames test. It can be seen in Figure 4.9 that both non-extracted and extracted wastewater samples had some positive responses in Ames assay. However, the numbers of deviations were not large enough to conclude that the raw and SPE concentrated wastewater were mutagenic.

There is an increase in the number of positive response wells after extraction. Hence, it is likely that the detection of positive mutagenicity occurred after the extraction of a larger amount of wastewater in MCX sorbent.

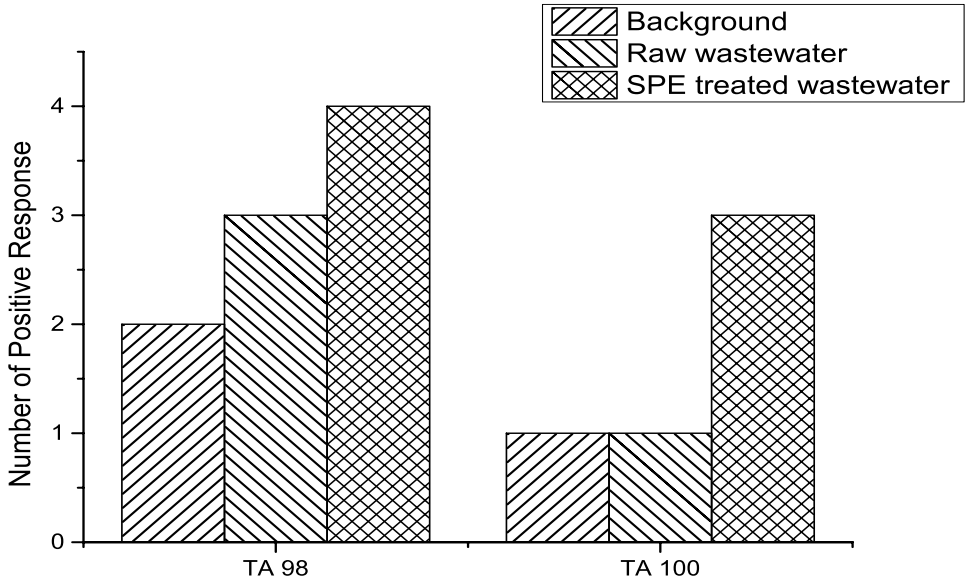

Figure 4.9 Mutagenicity analysis using Ames test for concentrated surface water and wastewater in City of London.

As the level of mutagenicity in Ames test was expressed as the clear significance (either 95%, 99% or 99.9%), distinct from YES assay, it was unable to quantify the mutagenic substances in the tested samples. Therefore, Ames assay is unqualified to verify the recovery of MCX cartridge for target analyte extraction.

In addition, the wastewater samples were collected and extracted in June. Some literatures suggested that the mutagenicity of wastewater varies depending on the time of sampling (Atasoy et al., 2012; Jolibois & Guerbet, 2005; Jolibois et al., 2002). Furthermore, the flowrates of wastewater are diverse in a year or even in a day in the wastewater treatment plant. (i.e. in a day, the peak hours appear right before

noon and at 8 p.m. and in a year, the flowrates of domestic wastewater in summer is higher than in winter). The high flowrates might result in the higher possibility of mutagenicity in wastewater samples (Jolibois et al., 2002). Therefore, further tests need to be conducted for the samples collected in different time of the day, and year to determine the mutagenicity of wastewater in Adelaide Pollutant Control Center.

Conclusions

As demonstrated in Chapter 3, Oasis MCX is an ideal mixed-mode ion exchanger SPE sorbent for water sample extractions. In this chapter, the effects of background water quality on the performance of SPE for known analytes were examined. First, two water matrices: surface water and wastewater were collected and pre-concentrated in MCX cartridges. By testing with HPLC and YES assay, the background concentrations of E2 in surface water and wastewater were found lower than the limit of detection. E2 recovery from spiked surface water and wastewater samples using MCX sorbent was in the range of 85%-109%.

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Chapter 5

5 Conclusions and Recommendations

5.1 Conclusions

From the first stage of research in Chapter 3, the major conclusions are as follow:

(i) Mixed-mode ion exchanger sorbents were better suited for extraction of polar and hydrophilic compounds as compared to reversed-phase only sorbents.

(ii) In column sorption experiments, Oasis MCX was the most efficient sorbent for metronidazole and lincomycin extraction. Oasis MAX was better in sulfamethoxazole extraction. Ofloxacin was better to be extracted in LC-18 sorbent. The efficiency of sorption was correlated to the acid dissociation constants, pKa, of the compounds. The compounds with neutral pKa values were removed better in LC-18 sorbent.

(iii) Lincomycin could not be retained on MAX sorbent because the pH of the solution was not high enough to be charged for MAX sorbent and its poor retention of lincomycin on reverse-phase packings.

(iv) The capacity of the sorbents for target analytes can be determined by routine laboratory batch and column experiments. In most cases, the maximum capacity determined by batch and column tests matched closely; deviation occurred only when the sorbents were difficult to keep in suspension.

(v) The packing format of SPE cartridge ensured good contact between the analytes and the sorbent.

The following conclusions can be drawn based on the results presented in Chapter 4.

(i) The presence of 17β-estradiol was not observed in river water and wastewater samples within the limit of detection by HPLC analysis.

(ii) YES assays confirmed that the concentrations of E2 presented in concentrated surface water and wastewater samples were lower than 0.34 ngL^{-1} E2 equivalent for surface water and 0.68 ngL^{-1} E2 equivalent for WWTP effluents.

(iii) HPLC analysis verified the recovery of Oasis MCX sorbent was approximately 100% in surface water matrix. However, quantification of E2 standard in wastewater matrix was difficult as too much interference was co-extracted and co-eluted.

(iv) YES assays confirmed the recovery of E2 in Oasis MCX from wastewater matrix was 84.65%. The efficiency of the SPE sorbents decreased as the complexity of water matrices increased.

(v) Ames assay was not an effective tool to determine the quantitative performance of the SPE cartridges, while YES assay can determine this effectively.

5.2 Recommendations

The results obtained from each stage of the study were very promising. Some recommendations are presented below for further investigations.

(i) As for commercial SPE cartridges, limited amount of sorbent is packed in the open polypropylene syringe barrel, the total volume of sample that can be loaded into the cartridge is also limited. Therefore, the commercial cartridge could only be used in a laboratory scale. For a larger scale use such as biomonitoring, online SPE could be coupled with HPLC to monitor the substance right away.

(ii) In this study, solvents used in SPE procedures were recommended by the cartridge manufacturer. To optimize the SPE performance, conditioning solvent, sample loading rates, and composition of the elute solvents can be further investigated.

(iii) Further testing of various micropollutants with different properties in SPE cartridges should be carried out to determine the effect of polarity, ionic state, solubility and hydrophobicity (log K_{OW}) of the compounds.

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