Monitoring of Estrogen Hormones in Songkhla Lake using HPLC-UV

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ABSTRACT

A simple and environmental friendly method for the extraction and preconcentration of trace estrogen compounds (estrone and 17β-estradiol) from water samples has been developed. Polypyrrole (PPY), easily synthesized by chemical oxidation of monomer in nonaqueous solution, was applied as a sorbent for micro-solid phase extraction. This micro-solid phase extractor was prepared by using tea bag filter paper as a protecting membrane. It was cut and made into a miniature envelope (1.2 cm×1.0 cm), with 15 mg of polypyrrole packed inside. Parameters influencing the extraction efficiency were optimized. In the extraction process the sample solution was stirred to move the tea bag around the entire sample solution and desorption was achieved by sonication. The extract was then analyzed by high performance liquid chromatography with UV detection. Under the optimized conditions, the proposed method provided the linearity in the range of 25.0 μg L^{-1} to 1000 μg L^{-1} with the limit of detection and limit of quantification for both analytes of 10.0 μg L^{-1} and 25.0 μg L^{-1}, respectively.

This method was used to monitor some areas around Songkhla Lake for wet and dry seasons. There was no anlytes detected. However, the proposed method provided good recoveries and precision. The recoveries ranged from 80.0 ± 1.8 % to 116.16 ± 0.90 % with the RSD of 0.10 to 9.0 %.

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List of Abbreviations

ANOVA	Analysis of variance
AOAC	Association of Analytical Communities
C2	Ethylsilane modified silica
C8	Octadecylsilane modified silica
CPE	Cloud point extraction
DI	Deionized water
DLLME	Dispersive liquid liquid micro extraction
E1	Estrone
E2	17β-estradiol
E3	Estriol
EDCs	Endocrine disrupting compounds
FDA	Food and Drug Administration
GC-MS	Gas chromatography-mass spectrometry
GPS	Global positioning system
HETP	Height equivalent to a theoretical plate
HF-LPME	Hollow fiber-Liquid phase micro extraction
HPLC-DAD	High performance liquid chromatography with diode array detector
HPLC-UV	High performance liquid chromatography with ultraviolet detector
IUPAC	International Union of Pure and Applied Chemistry
LC-MS	Liquid chromatography-mass spectrometry
LLE	Liquid liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
MWCNT	Multiwalled carbon nanotube
µ-SPE	Micro-solid phase extraction
PPY	Polypyrrole
R^2	Coefficient of determination
RSD	Relative standard deviation

List of Abbreviations (continued)

SBSE	Stir bar sorptive extraction
SDME	Single drop micro extraction
SPE	Solid phase extraction
SPME	Solid phase micro extraction

CHAPTER 1

Introduction

1.1 Background and the rationale

Steroid hormones, sex hormones excreted by humans and animal, have been detected in effluents of sewage treatment plants and surface water (Desbrow *et al.*, 1998). The natural steroids of major concern are mainly estrogens which include estrone (E1) and 17β-estradiol (E2) because they display their physiological effect at a lower concentration than other steroids (Barel-Cohen *et al.*, 2006). These natural steroid hormones are released into the aquatic environment almost all the time by urine of all species, sexes and kinds of farm animals in the form of runoff from cattle pasture, fishpond effluent, fields fertilized with chicken manure and effluent from coop and barn and the other dominant source is sewage effluent from human (Barel-Cohen *et al.*, 2006; Briciu *et al.*, 2009). Some negative impacts of these estrogens have been reported. They have been suspected of having adverse effect on the endocrine system in wildlife and human (Gross-Sorokin *et al.*, 2005). Many studies have suggested that estrogens from treated waste water can cause male fishes feminization, decreased sperm counts, prostate cancer, breast cancer, sexual disruption, reproductive malfunction, developmental and behavioral abnormalities at very low concentrations (1 pg mL^{-1}) in aquatic environment (Deksissa, 2008).

The data of estrogens presence in natural water have been reported in some researches and the high levels of estrogens were noted in polluted river. The concentrations of 112 ng L^{-1} for E1 and 200 ng L^{-1} for E2 were detected in surveyed of 139 polluted rivers and stream in the US (Kolpin *et al.*, 2002). Tiber river water in Italy was reported to have the concentration of E1 and E2 at 0.11 and 1.5 ng L^{-1}, respectively (Baronti *et al.*, 2000). In addition, the levels of E1 from 0.8-3.6 ng L^{-1} and E2 from 0.6-3.1 ng L^{-1} were found in seven French rivers (Cargouët *et al.*, 2004). Moreover, the concentration of 11.9 ng L^{-1} for E1 was obtained from two rivers in

North-East of Spain (Lopez de Alda *et al.*, 2002). Still, there was no reported information of such estrogens in the river or lake in Thailand.

Songkhla Lake or Thale Sap Songkhla in southern Thailand is an important marine ecosystem for aquaculture, surrounded by agricultural area and factories. Major sources of agricultural pollution such as shrimp farms, pig farms, contaminate the lake with wastewater, pesticides, and other toxic substances. The other important sources of pollutants are human communities around the lake (Pornpinatepong *et al.*, 2010). Therefore, estrogens could also be presented in this lake. To the best of our knowledge there has not been any data on these estrogens in Songkhla Lake, so it is important to determine these compounds level to get the baseline data for future monitoring.

Analytical techniques that have been used for estrogens analysis are gas chromatography coupled with mass spectrometry (GC-MS, or GC-MS-MS) (Okeyo and Snow, 1998; Xiao *et al.*, 2001; Cargouët *et al.*, 2004; Carpinteiro *et al.*, 2004; Basheer *et al.*, 2005; Lei *et al.*, 2009; Zhou *et al.*, 2009; Kanimozhi *et al.*, 2011; Xu *et al.*, 2013), liquid chromatography coupled with mass spectrometry (LC-MS, or LC-MS-MS) (Beinhauer *et al.*, 2014; Isobe *et al.*, 2003; Laganà *et al.*, 2004; Chen *et al.*, 2007; Kuster *et al.*, 2008; Kumar *et al.*, 2009; Guo *et al.*, 2013; Wang *et al.*, 2013a), and high performance liquid chromatography with diode array detection (HPLC-DAD) (Vallejo-Rodríguez, 2011; Wang *et al.*, 2015). Even though these techniques are robust but it is also costly and does not allow many laboratories to acquire such instrumentation to develop similar methodologies (Vallejo-Rodríguez, 2011). At a lower cost, high performance liquid chromatography with UV detection is a possibility and the use of this available analytical instrument could be justified. However, the expected concentrations of estrogens in water environment were very low and cannot be directly detected with the analytical method. Therefore, a sample pretreatment step is required before analysis.

Liquid- liquid extraction (LLE), solid phase extraction (SPE) and cloud point extraction (CPE) are usually used in sample pretreatment, however SPE and LLE techniques require a large sample volume of toxic organic solvent and also time

consuming (Wang *et al.*, 2006). While CPE is not suitable for the analysis using GC and HPLC because some of the surfactants used for the extraction are incompatible with the instrument (Farajzadeh *et al.*, 2009; Hadjmohammadi and Ghoreishi, 2011). Therefore, in the last decade micro-solid phase extraction (µ-SPE) based on the packing of sorbent in a sealed porous polypropylene membrane envelope has been employed in analytical chemistry. It was confirmed to be an effective pretreatment technique for various applications with several good properties i.e., fast to prepare, accurate, inexpensive, easy-to-handle, use small volume of solvents and small amount of sorbents without special auxiliary device; the device only consists of the sorbent and enveloped membrane for the extraction (Basheer *et al.*, 2008). However, there are still some disadvantages related to the polypropylene membrane such as the low extraction rate caused by the low wettability of the hydrophobic membrane and also longer extraction time due to the small pore size of the membrane (Basheer *et al.*, 2006; Basheer *et al.*, 2007). A cellulosic tea bag filter paper, with its hydrophilic nature, high air permeability and fast water absorption (2 s), could overcome these drawbacks. The target analyte could easily diffuse through the tea bag filter paper and reach the sorbent, thus, enhances the extraction efficiency. In addition, tea bag filter paper also has a high tensile strength and high porosity that can provide a highly protective and durable layer for solid sorbent. It is also non-toxic and very cheap (Pelden *et al.*, 2014).

To effectively extract the target analyte by the µ-SPE technique the choice of sorbent is also an important factor. Various polymeric commercial and nano-material sorbents have been applied. Polypyrrole (PPY), an environmental friendly and easy to synthesize polymer, has been widely used for the extraction of different types of compounds. It can extract aromatic compounds through the $\pi-\pi$ interaction and other hydrophobic compounds through hydrophobic interaction. Due to its anion exchange property, it can also extract anion species (Wu and Pawliszyn, 2001; Bagheri and Mohammadi, 2003). Polypyrrole functionalized OH with tea bag filter paper has previously been used for the extraction of butachlor residue in water samples (Pelden *et al.*, 2014). However, PPY has never been applied with tea bag filter paper as a miniaturized µ-SPE. This would be the first time that a miniaturized

μ-SPE with tea bag filter paper packed polypyrrole was explored for the extraction of estrogens.

This work investigated the extraction efficiency of the μ-SPE with tea bag filter paper using polypyrrole sorbent for the extraction of two estrogens (estrone and 17β-estradiol) in water sample and analyzed by HPLC- UV. This technique was then applied to monitor these estrogens in some areas around Songkhla Lake for four months, the months of November and December 2014 mark as the wet season and months of February and March 2015 as the dry season.

1.2 Steroid hormones

Steroid hormones are a group of lipophilic biologically active compounds which are synthesized from cholesterol. They are excreted by the adrenal cortext, ovaries, testis and placenta in human and animals, they can be divided into two groups depend on their biological activity and pharmacological effect. The first group is sex steroids that are the hormones producing sex differences or supporting the reproduction, there are estrogens, gestagens and androgens. The second group is corticosteroids which including glucocorticosteroids that regulate many parts of metabolism and immune function, and the third is mineralocorticosteroids which regulate blood volume and electrolyte content (Ying *et al.*, 2002; Noppe *et al.*, 2008; Wang *et al.*, 2011).

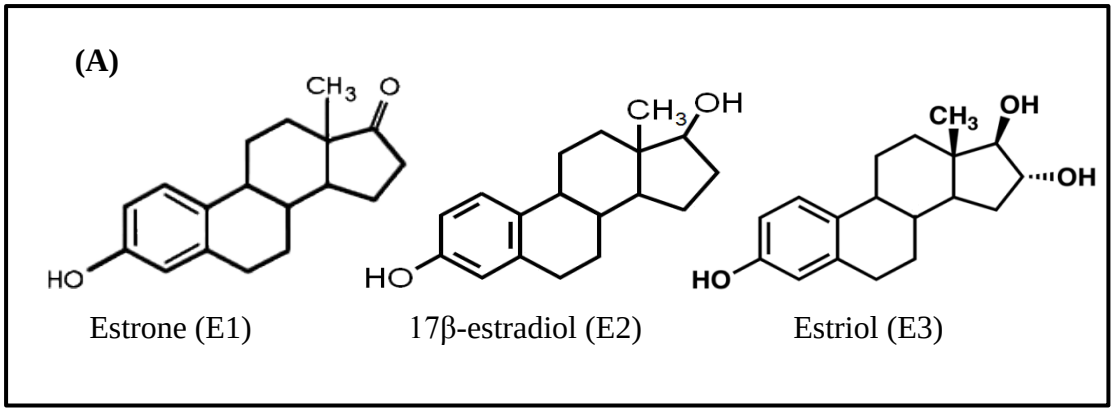
Estrogens which consist of estrone (E1), estradiol (E2) and estriol (E3) are natural female sex steroid hormones (structures are shown in Figure 1.1A), which are important for stimulating the development of women reproductive tissues and the secondary sexual characteristics such as breasts, skin and brain (Ying *et al.*, 2002).

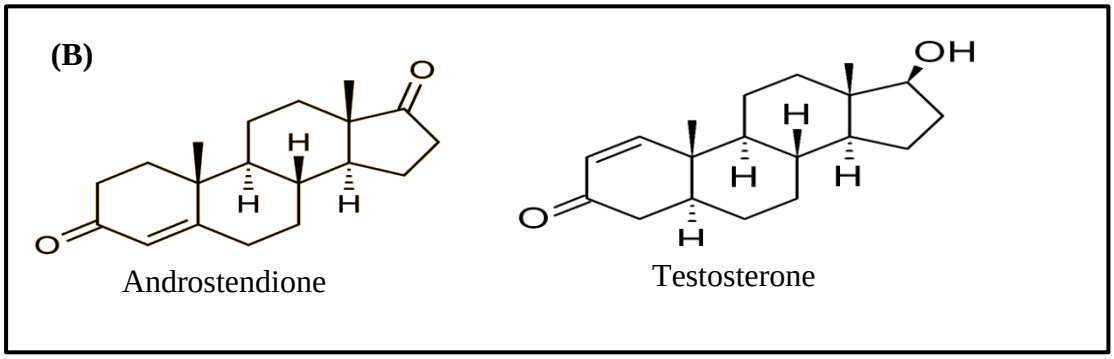
Androgens are the male sex hormones which are produced in testis and in fewer amounts in the ovaries. It plays an important role in the development of male sex organs and male secondary sex characteristics. The most important androgens are testosterone and androstenedione (Figure 1.1B) (Ying *et al.*, 2002; Noppe *et al.*, 2008).

Gestagens also called progestins or progestagens are a group of hormones including the progesterone (Figure 1.1C); that is secreted by the corpus luteum and it performs as the hormonal balancer of estrogens for maintaining pregnancy (Noppe *et al.*, 2008).

Steroid hormones that are biosynthetically present in the body are called endogenous steroid hormones and for the foreign compounds, either naturally or synthetically produced are called exogenous steroid hormones (Aufartova *et al.*, 2011). Exogenous steroid hormones can acts as the endocrine disrupting compound (EDCs) which can interfere with the usual functioning of the endocrine system in humans and animals by acting like natural endogenous steroid hormones. There are four ways that these compounds can act as the endocrine disruption: (i) they may mimic or behave as natural hormones by binding to a receptor leading to a similar response by the cell (agonistic response), (ii) they may antagonize endrogenous hormones by preventing binding of natural hormones (antagonistic response), (iii) by altering the pattern of synthesis and metabolism of natural hormones, and (iv) by modifying the function and production of hormone receptor levels (Markey *et al.*, 2002). Among these compounds, natural estrogens hormone (E1 and E2) are the most potential endocrine disruption compounds which causing the effect in aquatic organism event at trace level concentration (Ying *et al.*, 2002; Zuehlke *et al.*, 2005).

Based on the water circulation system, estrogens may occur in all water bodies. The presence of these natural estrogens in the water of the environment, even in low amount has become a worldwide concern because of its potential risk to human life and wildlife (Briciu *et al.*, 2009).





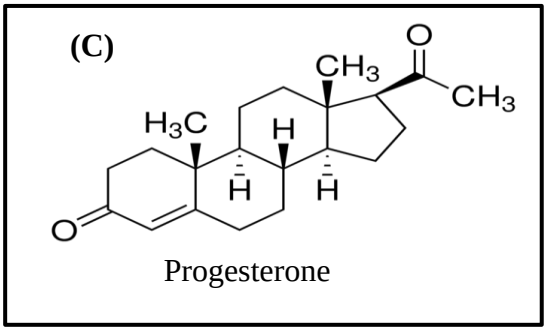


Figure 1.1 Structure of (A) natural estrogens (B) androgens (C) progestagen

1.3 Sources of estrogens

Natural estrogens enter the aquatic environment *via* several pathways (Figure 1.2) including wastewater treatment effluent, untreated discharges, runoff from soil which have large amounts of animal waste and bio solids from livestock applied in agriculture field (Lopez de Alda *et al.*, 2002; Wang *et al.*, 2006; Deksissa, 2008; Liu *et al.*, 2012). The cattle and poultry manure have been reported to be a source of 17β-estradiol into the environment (Wang *et al.*, 2006).

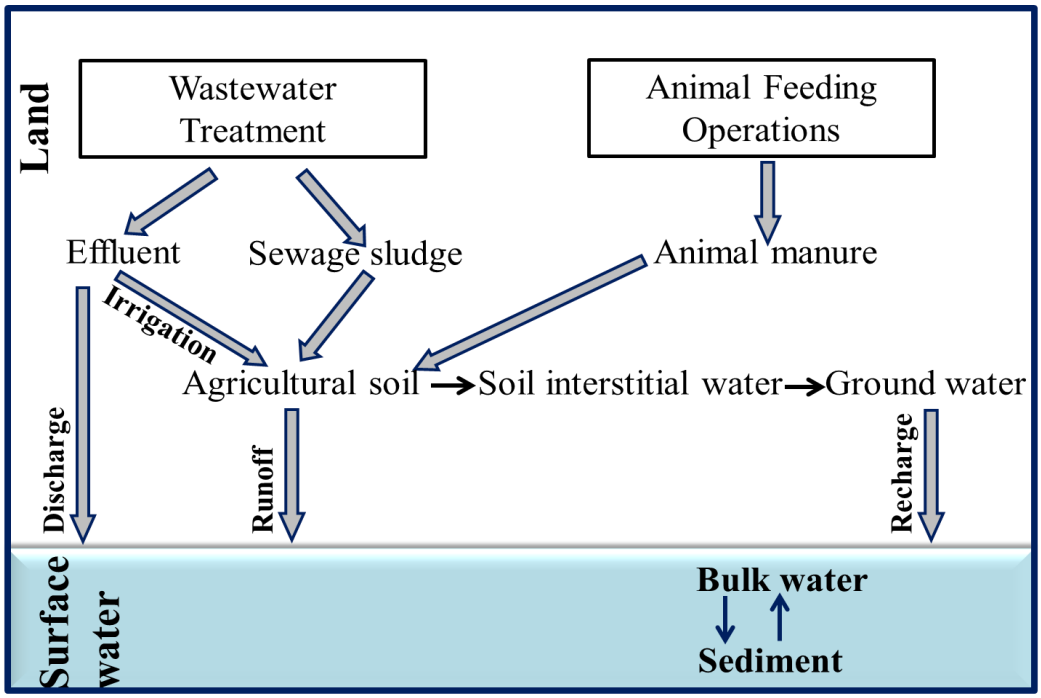


Figure 1.2 Main sources of estrogen contamination in aquatic environment (modified from Deksissa, 2008).

Humans excrete the natural estrogens E1, E2 and E3 into the aquatic environment through urine and feces into effluents of wastewater treatment plants. E1 is the predominant hormone in menopausal women, E2 is the primary metabolite in reproductive woman and the highest potency and E3 is the metabolite of E1 and E2. The average daily excretion of the natural estrogen hormones is given in Table 1.1 (Johnson *et al.*, 2000).

Livestock also excrete the same natural estrogens (E1, E2 and E3) as humans and there have been reports of the increasing of estrogen level in surface and groundwater downstream from farms and agricultural land. The amounts of estrogens released from several types of livestock are shown in Table 1.2 (Lange *et al.*, 2002; Johnson *et al.*, 2006; Wise *et al.*, 2010).

Table 1.1 Estimates of estrogen excretion by humans (per person) in μg day^{-1}

Category	**E1**	**E2**	**E3**	**Total**
Males	1.6	3.9	1.5	7
Menstruating females	3.5	8	4.8	16.3
Menopausal females	2.3	4	1	7.3
Pregnant women	259	600	6000	6859

Table 1.2 Estimated total daily estrogen excretion of different livestock species

Species	**Type**	**Total estrogen excreted in urine (μg day^{-1})**	**Total estrogen excreted in feces (μg day^{-1})**	**Total estrogen excreted per day (μg day^{-1})**
Cattle[a]	Calves	15	30	45
	Cycling cows	99	200	299
	Pregnant[b]	320-104320	256-7300	56-111620
Pig[b]	Cycling sow	82	21	103
	Pregnant	700-17000	61	_
Sheep[a]	Cycling ewes	3	20	23
	Rams	3	22	25

[a] Data are estimated as total of E1, E2, 17 α -E2 and E3 and include hormones from veterinary treatment (Lange *et al., 2002)*

[b] Pig and pregnant cattle data are from Johnson *et al*., 2006

1.4 Physicochemical properties of estrogens

The solubility of estrogens in water is very low. A log k_{ow} parameter measures the hydrophobicity of the hormones and their ability to bind to the organic colloids and macromolecules in water by partitioning between octanol and water. Compounds with log $k_{ow} > 2.5$ are expected to accumulate in solid phases than being soluble in the aqueous phase (Nghiem *et al.*, 2004; Deksissa, 2008; Schafer *et al.*, 2011). The log k_{ow} of estrogens are 3.94 for E1 and 3.43 for E2, this can be indicated that estrogens are hydrophobic compounds.

E1 and E2 have very low volatility as their vapor pressures are very low (Table 1.3) which shows that these compounds are not easily loss by the volatilization process (Deksissa, 2008). pk_a indicates the acid dissociation constant at which the hormones lose a hydrogen atom and become negatively charged. At pH higher than pk_a the estrogens have the negative charge. Estrone can be either a proton-donor or a proton-acceptor compound because of its –OH and –CO functional groups, while estradiol can only act as a proton-donor molecule due to its –OH functional group (Nghiem *et al.*, 2004). All parameters related to characteristic of estrone and estradiol is summarized in Table 1.3.

Table 1.3 Physicochemical properties of estrone and 17β-estradiol

Estrogens	**Water solubility (mg L^{-1} at 20°C)**	**Vapour pressure (mm Hg)**	**log k_{ow}**	**pk_a**	**Dipole moment (Debye)**
Estrone (E1)	13[a]	2.3×10^{-13}	3.94[a]	10.4[a]	2.1[b]
17β-estradiol (E2)	13[a]	2.3×10^{-13}	3.43[a]	10.4[a]	2.2[b]

[a] Data are from Nghiem *et al*., 2004 and Deksissa, 2008

[b] Data are from Schafer *et al*., 2011

1.5 Estrogens toxicity

Exogenous estrogens or environmental estrogens show their effect by binding to the estrogen receptor, antagonizing the natural hormone, as the result it is altering and modifying the endogenous estrogens function (Brian *et al.*, 2005). E1 and E2 are the most potent endocrine disrupters that may impact the normal functioning of the endocrine system at a very low concentration (Barel-Cohen *et al.*, 2006). As the potency of estrogens are typically measured in relation to E2 which have a value of 1 and E1 is estimated to have the relative potency of 0.2-0.4, so E2 is more potent than E1. These values have been determined by estrogen receptor binding in *vitro* assays or vitellogenin (female yolk protein which is synthesized by the liver in response to estrogens) induction in male juvenile fish (Wise *et al.*, 2010). The possible effect of these substances may be related with the increasing incidence of breast cancer, testicular cancer and decline of sperm counts in adult men, male reproductive disorder (Deksissa, 2008). It has been shown that male fishes exposed to sewage effluent which contained the estrogens (E1 and E2) can be suffered from the feminization effect and for the river which obtain significant amounts of sewage effluent cover number of intersex fishes (have both parts, male and female) (Xiao *et al.*, 2001; Deksissa, 2008).

1.6 Analytical method

The analytical techniques of choice for quantifying estrogens have been GC and LC which are the primary methods for the separation of steroids. Different types of detector for the determination of estrogens have been used such as mass spectrometry detector (MS-MS, MS), fluorescence detector (FD) and ultraviolet detector (UV) (Alda and Barceló, 2001; Isobe *et al.*, 2003; Wang *et al.*, 2006; Kumar *et al.*, 2009; Zhou *et al.*, 2009; Vallejo-Rodríguez, 2011; Wang *et al.*, 2011; Tomšíková *et al.*, 2012; Guo *et al.*, 2013). By considering the low LOD (ng L^{-1}), GC-MS-MS, GC-MS, especially LC-MS and LC-MS-MS are the methods of selection in steroid-hormone analysis because of their sensitivity and selectivity. However, since the analysis of estrogens with GC-MS involves derivatization prior to detection and also limited by the volatility of the compounds, LC-MS and LC-MS-MS have become

widely used for the determination of estrogens in the environmental samples (Tomšíková *et al.*, 2012). Nevertheless, the drawback of LC-MS and LC-MS-MS is the matrix effect of the environmental samples can result in signal suppression or signal enhancement of the target analytes (Stafiej *et al.*, 2007; Tomšíková *et al.*, 2012). In addition, these instruments are still very expensive and consequently not widely distributed (Stafiej *et al.*, 2007).

In this study, the application of HPLC with UV detection and appropriate preconcentration step were employed for trace analysis of estrogens.

1.7 Sample preparation

The analytes in the environment usually exist within a complex matrix. Most of the analytical instruments cannot perform directly with such sample matrix. Therefore, sample preparation, a series of steps for cleaning up sample matrix to isolate and enrich the analytes of interest to a suitable concentration level and decrease the interference of matrix, is required (Altun, 2008).

Different sample preparation techniques have been reported for the extraction of estrogens from various matrix, including, liquid liquid extraction (LLE) (Hadjmohammadi and Ghoreishi, 2011; Xu *et al.*, 2013; Li *et al.*, 2014; Wang *et al.*, 2015), conventional solid phase extraction (SPE) (Isobe *et al.*, 2003; Beck *et al.*, 2005; Kumar *et al.*, 2009; Vallejo-Rodríguez, 2011; Zheng *et al.*, 2011; Guo *et al.*, 2013), cloud point extraction (CPE) (Wang *et al.*, 2006), solid phase micro extraction (SPME) (Okeyo and Snow, 1998; Basheer *et al.*, 2005) and stir bar sorptive extraction (SBSE) (Hu *et al.*, 2012). Recently, micro-solid phase extraction (μ-SPE) has been introduced as an environmental friendly method for the extraction of various target analytes from complex sample without additional sample clean up. This method has also been applied for the extraction of estrogens in ovarian cyst fluid samples (Kanimozhi *et al.*, 2011).

1.7.1 Liquid liquid extraction (LLE)

Liquid liquid extraction (LLE) or solvent extraction is the traditional and the most widely used sample pretreatment technique for separation and

preconcentration of analytes from aqueous matrix samples. The classical LLE separation depends on the equilibrium distribution between two immiscible phases (aqueous or donor phase and organic or acceptor phase) which requires the matching polarity of the analytes and extraction solvent by using separatory funnel (Altun, 2008; Zhang *et al.*, 2012). The analytes are extracted into the organic phase, and then it is transferred, evaporated to dryness and re-dissolved prior to analysis (Altun, 2008).

This method can provide large sample capacity and the clean organic extract can be directly analyzed. However, it still has some drawbacks such as time consuming, labor intensive, use large volume of expensive and environmental harmful organic solvents (Altun, 2008; Zhang *et al.*, 2012). To overcome these drawbacks a modern technique to LLE, liquid phase micro extraction (LPME), has been established. In LPME, the extraction normally occurs in a small volume of water-immiscible solvent or acceptor phase from the donor aqueous phase. It can be divided into four main types (Sarafraz-Yazdi and Amiri, 2010; Stoytcheva, 2011):

- Dispersive liquid liquid micro extraction (DLLME)
- Hollow fiber liquid phase micro extraction (HF-LPME)
- Single drop micro extraction (SDME).
- Cloud point extraction (CPE)

Among these miniaturized techniques, DLLME, HF-LPME and CPE have been used for the extraction of estrogens in aqueous sample and milk sample (Wang *et al.*, 2006; Hadjmohammadi and Ghoreishi, 2011; Xu *et al.*, 2013; Socas-Rodriguez *et al.*, 2014; Wang *et al.*, 2015). However, these techniques still have some drawbacks such as, CPE use the surfactants for the extraction, so the choices of the surfactant often cause problem in the analysis of the analytes using GC and HPLC because some surfactants are incompatible with the instrument (Hadjmohammadi and Ghoreishi, 2011). HF-LPME also has some disadvantages, such as, long extraction time, low reproducibility of the extraction due to the creation of air bubbles on the surface of hollow fiber while longer stirring. In real samples the adsorption of hydrophobic compounds on the fiber can block the pores which interrupt the transferring of the analytes to the extraction solvent inside the fiber which results in low extraction efficiency (Sarafraz-Yazdi and

Amiri, 2010). The drawbacks of DLLME technique are the use of toxic organic solvents (Gure *et al.*, 2015) and low partition coefficient of the analytes into the extraction solvent because of the interruption from the disperser solvent (Sarafraz-Yazdi and Amiri, 2010).

1.7.2 Solid phase extraction (SPE)

Solid phase extraction (SPE) is the method use for isolation and concentration of target analytes by solid support (sorbent). The extraction is based on the partitioning of the analytes from the sample matrix (liquid) into a solid phase (Camel, 2003; Altun, 2008). The basic procedure of SPE is to place the sorbent in an open polypropylene or polyethylene syringe, tube or cartridge (Figure 1.3) which at the bottom covers by porous polypropylene filter paper that can allow the liquid to flow freely but not the sorbent. The top of sorbent is also covered by polypropylene filter paper to protect the sorbent from spreading away while flowing the sample. The liquid sample is passing through the SPE column and the analytes will retain on the sorbent, then the retained analytes is recovered upon elution.

SPE technique involves four sequential processing steps as illustrates in Figure 1.4. First, the solid sorbent is conditioned with the suitable organic solvent to remove impurities which have randomly collected while the cartridge is exposed to the laboratory environment or present in the cartridge supplied by manufacturer. This is followed by the same solvent as the sample solvent to allow the sorbent to be solvated. Second, a sample is loaded into the SPE column. In this step the analytes will interact with the sorbent and some of the matrices may also be retained on the sorbent but some matrices that have low interaction will pass through, *i.e.*, some matrices are isolated from the target analytes in this step. Third step is the washing with appropriate solvents to wash out the interference on the solid sorbent but not the target analytes. The last step is the elution; the analytes are eluted out from the sorbent with a suitable solvent.

SPE technique is mostly used for the extraction of estrogens; however, it requires a large amount of organic solvent, sample volume and is time consuming due to its many steps. It is also costly because commercial sorbents are used.

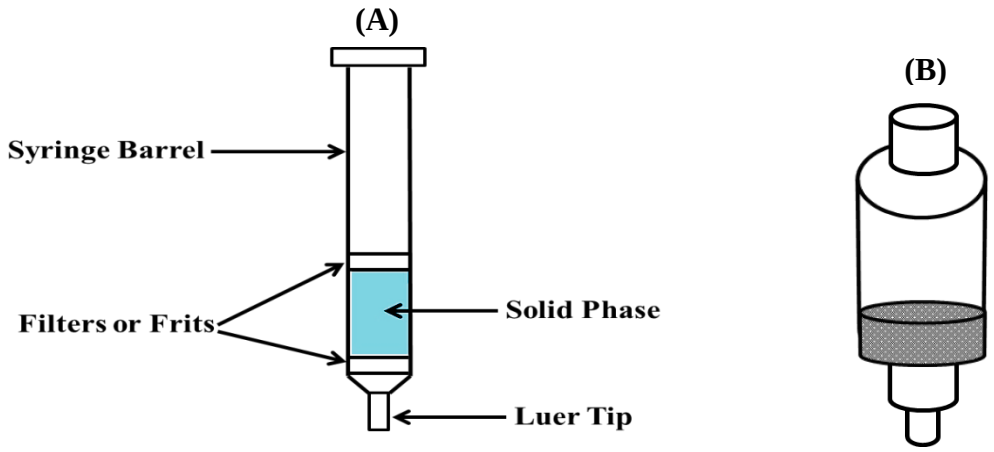


Figure 1.3 Solid phase extraction (A) syringe and (B) cartridge (modified from Altun, 2008; Stoytcheva, 2011).

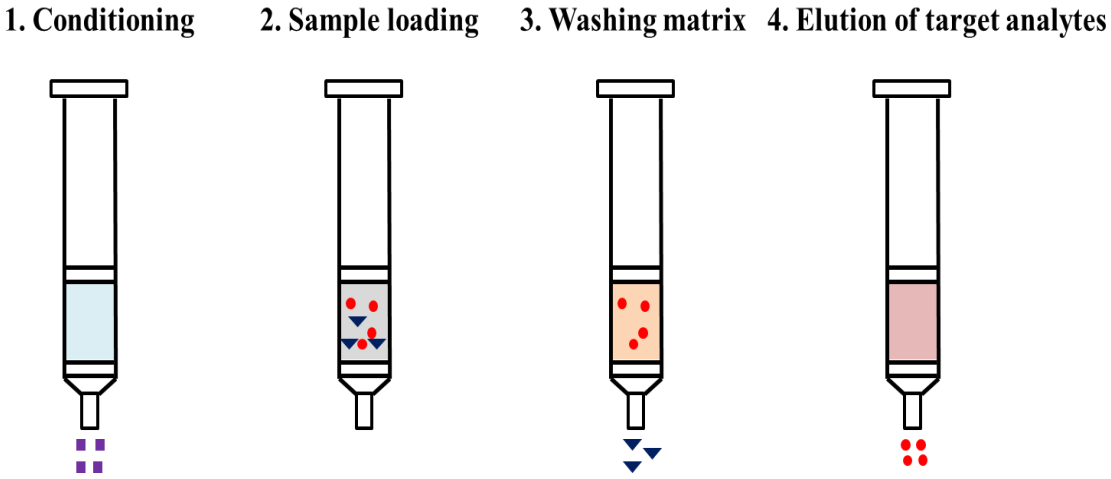


Figure 1.4 Four processing steps in SPE technique (modified from Altun, 2008).

1.7.3 Solid phase micro extraction (SPME)

Solid phase micro extraction (SPME) is the sample preparation technique that has been considered as a miniaturization of the SPE technique (Lord and Pawliszyn, 2000). In SPME, a short piece of fused-silica fiber is coated with an appropriate sorbent (the extraction phase) and the fiber is retracted into the needle which inserts on a syringe (Figure 1.5). When in use, the fiber is exposed to the

headspace of the sample or directly immersed in a liquid sample. The extraction is an equilibrium process that based on the partitioning of the analyte between the sample and the coating sorbent on the silica fiber. Then the concentrated analytes on the fiber is desorbed by thermal desorption with GC or desorbed with LC eluent using a static or dynamic mode (Vas and Vekey, 2004). However, there are still some disadvantages such as life shortage of SPME fiber owing to its fragility, sample carry-over effect and expensiveness of the fiber.

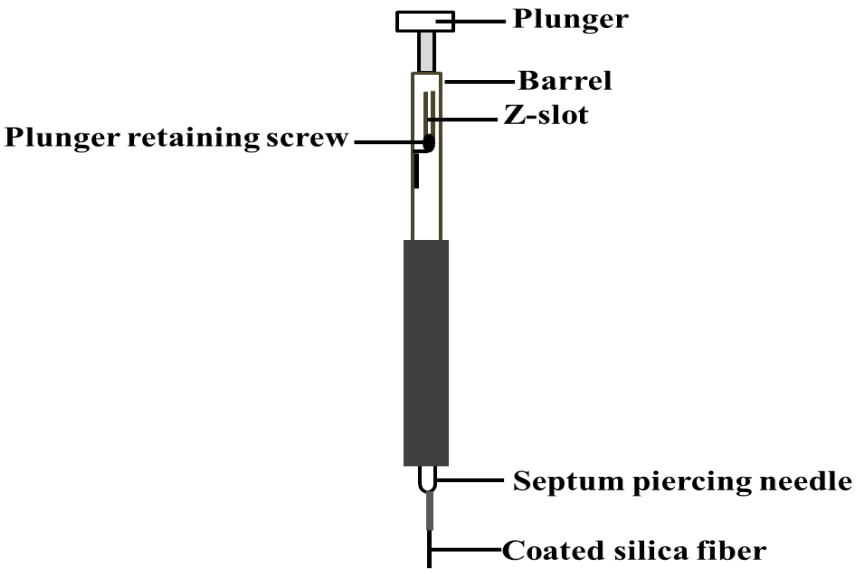


Figure 1.5 The schematic of SPME device (modified from King *et al.*, 2003).

1.7.4 Stir bar sorptive extraction (SBSE)

Stir bar sorptive extraction have been used for the extraction of organic compounds in aqueous, food, biological and environmental samples. Similar to SPME, SBSE is an equilibrium technique between the analytes in the sample and the sorbent polymer coated on a stir bar that stirs for a given time. The amount of the extracting phase coated on the stir bar is higher than that in SPME for 50 to 250 times which could provide significant increase in the recovery and extraction capacity. After extraction, the analytes are desorbed by thermal desorption into injector port of GC or

by liquid desorption for HPLC (Figure 1.6) (Pavlović *et al.*, 2007; Hu *et al.*, 2012; Jos *et al.*, 2013).

SBSE has been developed and successfully applied for trace analysis of various target analytes in environmental or biological samples (Pavlović *et al.*, 2007). The main disadvantage of SBSE is the high extraction time requires during sample pretreatment (Jos *et al.*, 2013).

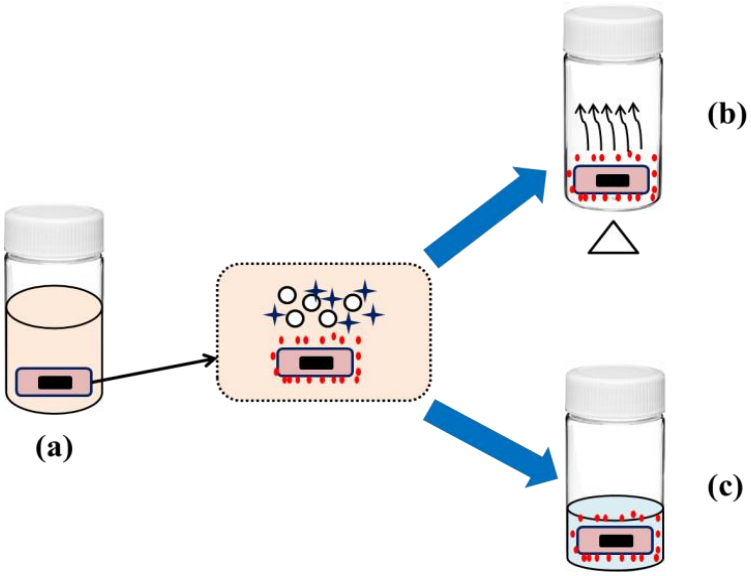


Figure 1.6 Stir bar sorptive extraction method (a) extraction of analytes on sorbent coated stir bar (b) thermal desorption into GC injector (c) liquid desorption for HPLC.

1.7.5 Micro- Solid phase extraction (µ-SPE)

To reduce organic solvent consumption, sorbent usage and sample handling, recently, µ-SPE has been developed and proven to be advantageous over the conventional SPE for the extraction and preconcentation of analytes in complex matrix samples (Khayoon *et al.*, 2014). µ-SPE based on packing material in a sealed porous polypropylene membrane envelope has been applied in the analysis of different kinds of analytes in various samples including biological, food and environmental samples (Basheer *et al.*, 2006; Basheer *et al.*, 2007; Ahmadi *et al.*,

2008; Basheer *et al.*, 2008; Ge and Lee, 2011; Kanimozhi *et al.*, 2011; Huang *et al.*, 2012; Wang *et al.*, 2013a; Wang *et al.*, 2013b; Khayoon *et al.*, 2014). However, due to the small pore size of hydrophobic polypropylene membrane and low wettability when exposed directly to the water sample, this technique still provided some disadvantages of long extraction time and low extraction efficiency which results from slow diffusion of the analytes to the sorbent through the membrane (Pelden *et al.*, 2014).

From a previous research (Pelden *et al.*, 2014), tea bag filter paper which made from cellulose (Figure 1.7) has been applied as the µ-SPE membrane instead of polypropylene for the analysis of butacholor in water sample. It has indicated some excellent properties such as cheaper cost, strong tensile strength that can provide a highly protective layer for solid sorbent, good heat-seal ability, fast water absorption, high porousity and relatively non toxic (more details in Table 1.4). A hydrophilic nature of the tea bag filter paper causes it adsorb water rapidly and with its high porousity, the analytes could easily diffuse through the tea bag filter paper and reach the sorbent. These could over come the longer extraction time and low extraction efficiency of the polypropylene membrane.

For µ-SPE, the choice of sorbent is a critical factor. Many porous sorbent such as multiwalled carbon nanotube (MWCNT), ethylsilane or octadecylsilane modified silica (C_2 or C_{18}), molecular imprinted polymer, sulfonated graphene sheet, nanotube array and conductive polymers have been chosen as the sorbent for µ-SPE. Zolitic imidazolate framework-8 was used as sorbent of µ-SPE to preconcentrate estrogen from water samples (Wang *et al.*, 2013a). In another research, C_2 was selected as the µ-SPE sorbent for the extraction of estrogens in ovarian cyst fluid samples (Kanimozhi *et al.*, 2011).

Polypyrrole is one of the most widely used sorbents which has been demonstrated that it could efficiently extract the aromatic compounds *via* π-π and hydrophobic interactions because of conjugated π structure in polypyrrole (Bagheri and Mohammadi, 2003). PPY has never been applied with tea bag filter paper as a

miniaturized µ-SPE; therefore it is interesting to apply PPY for estrogens extraction in this work.

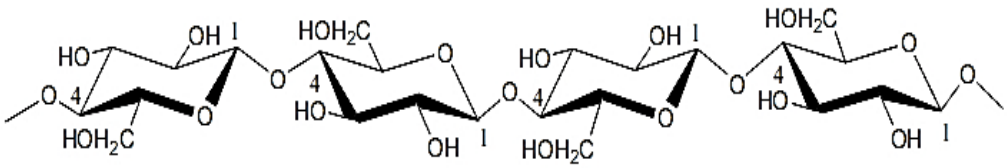


Figure 1.7 Structure of cellulose (Han and Rowell, 1996).

Table 1.4 Properties of tea bag filter paper (Dahiya *et al.*, 2004)

Description	**Properties**
Color	White color
Toxicity	Non toxic
Meterial	Cellulose
Width	120 mm, 125 mm, 484 mm
Thickness	16.5 gsm
Category	Filter paper
Taste	Tasteless
Food grade standard	Strong tensile strength
High air permeability	Fast water absorption
Feature	No additive
Heat-seal ability	Good heat-seal ability (120 to 160°C)
Time of water pass	2.0 second
Air permeability	Perfect air permeability
Ventilation	Good ventilation

1.8 Objective

The main objective of this work is to investigate the extraction efficiency of the μ-SPE made from polypyrrole sorbent packed in tea bag filter paper sachet for the extraction of two kinds of estrogens (estrone and 17β-estradiol) in water sample and analyzing by HPLC- UV. This technique was then applied to monitor these estrogens in some areas around Songkhla Lake.

1.9 Benefits of the project

It is expected that the proposed method will be a simple and fast sample preparation method with high efficiency for the extraction of estrogens in real samples. Moreover, from the monitoring, the baseline data of these two estrogens in Songkhla Lake will be established for further evaluation.

CHAPTER 2

Experimental

2.1 Chemicals and materials

2.1.1 Standard chemical

- Estrogens
  - Estrone (E1): $C_{18}H_{22}O_2$ 99.0% purity, Sigma-Aldrich (St. Louis, MO, USA)
  - 17β-Estradiol (E2): $C_{18}H_{24}O_2$ 98.0% purity, Sigma-Aldrich (St. Louis, MO, USA)

2.1.2 General Solvents and chemicals

- **Solvents**
  - Methanol (CH_3OH): HPLC grade, LAB-SCAN (Bangkok, Thailand)
  - Acetonitrile (CH_3CN): HPLC grade, LAB-SCAN (Bangkok, Thailand)
  - Acetone (CH_3COCH_3): AR grade, LAB-SCAN (Bangkok, Thailand)
  - Ethanol (C_2H_5OH): AR grade, LAB-SCAN (Bangkok, Thailand)
  - Ethyl acetate ($C_4H_8O_2$): AR grade, Merck (Darmstadt, Germany)
  - Ultrapure water (resistivity, 18.2 MΩ cm) was obtained from a Maxima ultrapure water system (ELGA, Buckinghamshire, England).
- **Chemicals**
  - Ferric chloride anhydrous ($FeCl_3$): Fluka (Buchs, Switzerland)
  - Pyrrole monomer: 98% purity (w/v), Sigma-Aldrich (St. Louis, MO, USA) was distilled prior to use.
  - Formalin (HCHO): 37% (v/v), Loba Chemie (Mumbai, India).

2.1.3 Materials

- The cellulosic tea bag filter paper was from C.T. Super Sales and Service Ltd. (Bangkok, Thailand)
- Whatman® filter paper No. 42 (pore size of 2.5 µm): Ligand (Bangkok, Thailand)
- Whatman® filter paper No. 3 (pore size of 6.0 µm): Ligand (Bangkok, Thailand)
- Glass microfiber filter GF/F (pore size of 0.70 µm): Ligand (Bangkok, Thailand)
- Nylon membrane filters (pore size 0.20 µm): Ligand (Bangkok, Thailand).

2.2 Instruments and apparatus

2.2.1 High performance liquid chromatography with ultraviolet detector

❖ HPLC-UV system

- Pump (515 HPLC Pump, Waters, New York, USA)
- Detector (Spectromonitor 3100, MILTON ROY, Missouri, USA)
- Recorder (e-corder 401, Denistone East, Australia)
- 6 ports valve injector (injection volume of 20 µL, Schenkon, Switzerland)
- VertiSepTM UPS C18 HPLC column, 4.6×150 mm, 5 µm (VERTICAL, Bangkok, Thailand)
- VertiSepTM UPS C18 guard column, 4.6×10 mm, 5 µm (VERTICAL, Bangkok, Thailand).

2.2.2 Apparatus

- Amber vial 2.0 mL (Agilent technology, Missouri, USA)
- Analytical balance (Sartorius, New York, USA)
- Amber vial 15.0 mL (LB Sci, Missouri, USA)
- Buchi Heating Bath B-490 (Tokyo, Japan)
- Evaporator (Buchi Rotavapor R-200, Tokyo, Japan)

- Glassware such as beakers, cylinders, volumetric flasks, glass rods, forceps, spatulas
- Magnetic stirrer (IKA^{R}, Selangor, Malaysia)
- Magnetic stirrer (Heidoiph, type: MR 3001, Schwabach, Germany)
- Microliter pipette 1000 µL, 5000 µL (Eppendorf, Humburg, Gemany)
- Oven (Fisher, Scientific, *Leicester*, UK)
- Refrigerator (Hitachi, Tokyo, Japan)
- Syringe 1.0 mL (Nipro, Bangkok, Thailand)
- Ultrasonic bath (Units Model Elmasonic S 100H, Singen, Germany)
- Vacuum pump (Gast manufacturing, Michigan, USA)
- Vortex Genic-2 (Scientific Industries, New York, USA)
- Impulse sealer (Goldex, Seoul, South Korea)

2.3 Standard solution preparation

A stock solution of each estrogen was prepared in methanol at a concentration of 1000 mg L^{-1} for 25.0 mL and stored in an amber bottle at 4°C; this could be used for six months (Yan *et al.*, 2009). The mix stock solution of two estrogens was prepared at a concentration of 100 mg L^{-1} by dilution from the individual stock solution with acetonitrile and stored in an amber bottle at 4°C.

The working standard solutions for the optimization and performance studies were freshly prepared by series dilution with acetonitrile from the stock standard solution.

2.4 Methods

2.4.1 Optimization of HPLC-UV system

In order to obtain the highest response with good chromatogram peak shape, short analysis time and the best separation, some related conditions in HPLC-UV (Figure 2.1) (Töppne *et al.*, 2015) such as mobile phase composition (isocratic), mobile phase flow rate, and detection wavelength were optimized. These parameters were optimized by injecting the working standard of 2.50 mg L^{-1} estrogens mixture

for five replications into the system while one parameter was verified and kept others constant. The optimum of one parameter was used for the other parameters optimization.

The initial conditions used for the optimization are shown in Table 2.1.

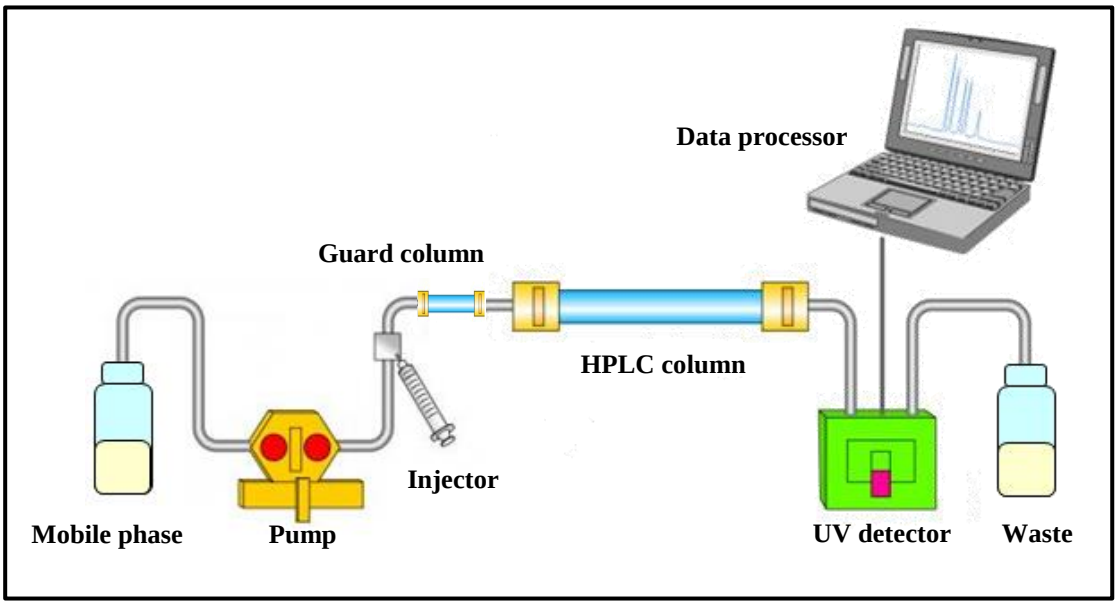


Figure 2.1 The typical setup of high performance liquid chromatography-ultraviolet detector (HPLC-UV) (modified from Töppne *et al.*, 2015).

Table 2.1 The initial condition of HPLC-UV system (Zheng *et al.*, 2011)

Parameters	**Conditions**
Composition of mobile phase (ACN: H_2O, v/v)	60:40
Mobile phase flow rate (mL min^{-1})	0.50
Wavelength (nm)	198

2.4.1.1 Mobile phase composition (ACN: H_2O, v/v)

Mobile phase composition (acetonitrile:water, v/v) was verified in order to obtain the suitable interaction between the analytes and the stationary phase which help to improve the HPLC performance (Moldoveanu and David, 2013a). The compositions of the mixture solvents were investigated at 50:50, 60:40, 63:37, 65:35 and 70:30 (ACN:H_2O, v/v). The optimum was the percentage of acetonitrile and water that provide a good separation, short analysis time and also good peak shape.

2.4.1.2 Mobile phase flow rate

The mobile phase flow rate is also one of the most important parameters that involves with the band broadening and the column efficiency for the analyte (Moldoveanu and David, 2013a). Therefore, to increase the column efficiency and to decrease the band broadening of the analytes, flow rate of the mobile phase was studied from 0.50, 0.60, 0.70, 0.80 and 0.90 mL min^{-1}. The optimum flow rate was selected at the minimum height equivalent to a theoretical plate (HETP) considering by the van Deemter plot; the plot between HETP and flow rate of the mobile phase.

2.4.1.3 Detection wavelength

Absorption wavelength is one of the parameters that have to optimize for the UV detector. It was studied from 193, 194, 195, 196, 197, 198, 200, 202, 205, 206 and 210 nm. The optimum wavelength was the wavelength which gave the highest peak area.

2.4.2 HPLC-UV system performance

The HPLC-UV system performance was evaluated by studying the linearity, limit of detection (LOD), limit of quantitation (LOQ) and precision.

2.4.2.1 Linearity

The linearity range of estrogens was studied by injecting a series of mixture estrogens standard concentration from 0.020 mg L^{-1} to 75.0 mg L^{-1} into the

HPLC-UV system under the optimum conditions, five replications for each concentration. Then, the calibration curve was plotted between the average values of the peak areas *versus* the corresponded concentrations. The linearity was determined by considering the coefficient of determination (R^2).

2.4.2.2 Limit of detection (LOD)

Limit of detection (LOD) is the minimum quantity (concentration) of the analytes that can be measured but not certainly quantified under the optimized condition of the test (Araujo, 2009). There are several ways to determine the LOD, but in this research LOD was determined by the IUPAC (the International Union of Pure and Applied Chemistry) method. In this method, 20 blank responses were measured and the standard deviation of these responses was used for the calculation (Long and Winefordner, 1983).

2.4.2.3 Limit of quantification (LOQ)

Limit of quantification (LOQ) is the smallest concentration of analyte which is possible to determine with a specified degree of accuracy and precision (Araujo, 2009). It was also calculated based on the IUPAC methods.

2.4.2.4 Instrument precision

The instrument precision was indicated based on the %RSD of the peak area and retention time of the injected 2.50 mg L^{-1} mix standard estrogens for six replications (n = 6). The acceptable RSD is 1% for retention time and 4% for the peak area (Snyder and Kirkland, 1979).

2.4.3 Synthesis of polypyrrole (PPY)

PPY was synthesized by chemical oxidation of pyrrole in nonaqueous solution using a previously reported method (Ahmadi *et al.*, 2008). 1.4 mL of distilled pyrrole (0.02 mol) dissolved in 10 mL ACN was slowly added to 50 mL of ACN containing 6.48 g of $FeCl_3$ with continuous stirring, the black precipitate of PPY was immediately formed. After half an hour, the solid product was filtered and washed with 50.0 mL, ultrapure water, EtOAc, ACN, and MeOH, consecutively. The polymer

was dried under vacuum and subsequently placed in 200 mL MeOH: EtOH (50:50) overnight to extract and remove any impurities from the solid polymer. The synthesized polymer was then filtered, washed with ultrapure water and MeOH several times. Finally, it was dried in an oven at 60°C and stored in a desiccator in the dark. Chemical structure of the polymer is shown in Figure 2.2. The size of the particles were controlled using the 40/60 mesh sieves in order to prevent the particles coming out of the tea bag (Pelden *et al.*, 2014).

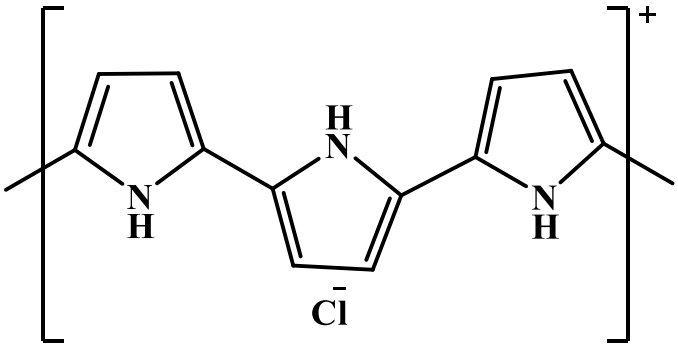


Figure 2.2 Chemical structure of synthesized polypyrrole (PPY) (modified from Bagheri and Mohammadi, 2003).

2.4.4 µ-solid phase extractor preparation

µ-Solid phase extractor is a tea bag filter paper envelope packed with polypyrrole (Figure 2.3). It was prepared by following the previous report (Pelden *et al.*, 2014). The tea bag paper was fold along the longer edge to a width of 1.0 cm, and then heat-sealed along the length using an electrical sealer. The fold-over section was cut from the main sheet and cut into the small pieces with the length of 1.2 cm to get the final dimension of 1.0 cm×1.2 cm. Then, one of the two open ends of each piece was heat-sealed. A suitable amount of polypyrrole was introduced *via* the remaining open end of the envelope which was then also heat-sealed to secure the contents.

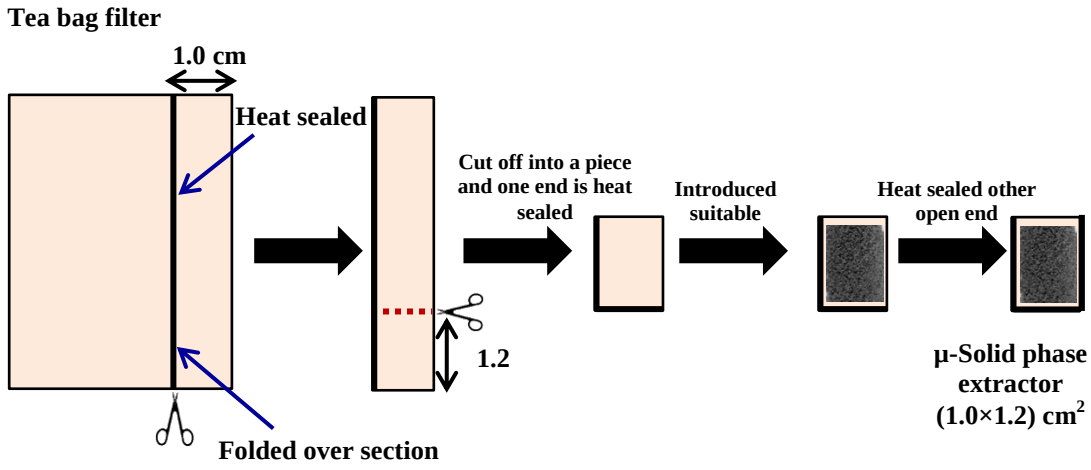


Figure 2.3 The preparation of μ-solid phase extractor.

2.4.5 Extraction of estrogens by μ-SPE procedure

μ-Solid phase extraction method using tea bag filter paper filled with polypyrrole sorbent was applied to extract the estrogens from pre-treated water sample before analysis. The μ-solid phase extractor was prepared by packing 15.0 mg of the synthesized polypyrrole inside the tea bag filter paper sachet (1.0×1.2) cm^2 and kept in methanol to avoid other contamination and also to increase the wettability of the sorbent.

Each μ-solid phase extractor was previously conditioned for 5 min with 7.5 mL of acetonitrile and then with 7.5 mL of ultrapure water for another 5 min by ultrasonication. It was then dried using filter paper and placed in 10 mL of water sample with a magnetic bar. The sample was stirred at 1000 rpm for a period of time to enhance the extraction, during which time the device moved freely in the whole sample. After the extraction, the device was removed, dried with filter paper then immersed in a vial of an appropriate solvent for desorption using ultrasonication with a proper period of time. The desorbed analytes was transferred into a 2.0 mL vial to evaporate at 60°C, and was re-dissolved with 1.0 mL acetonitrile. The solution was filtered with a nylon syringe filter before injected to the HPLC-UV system (Figure 2.4).

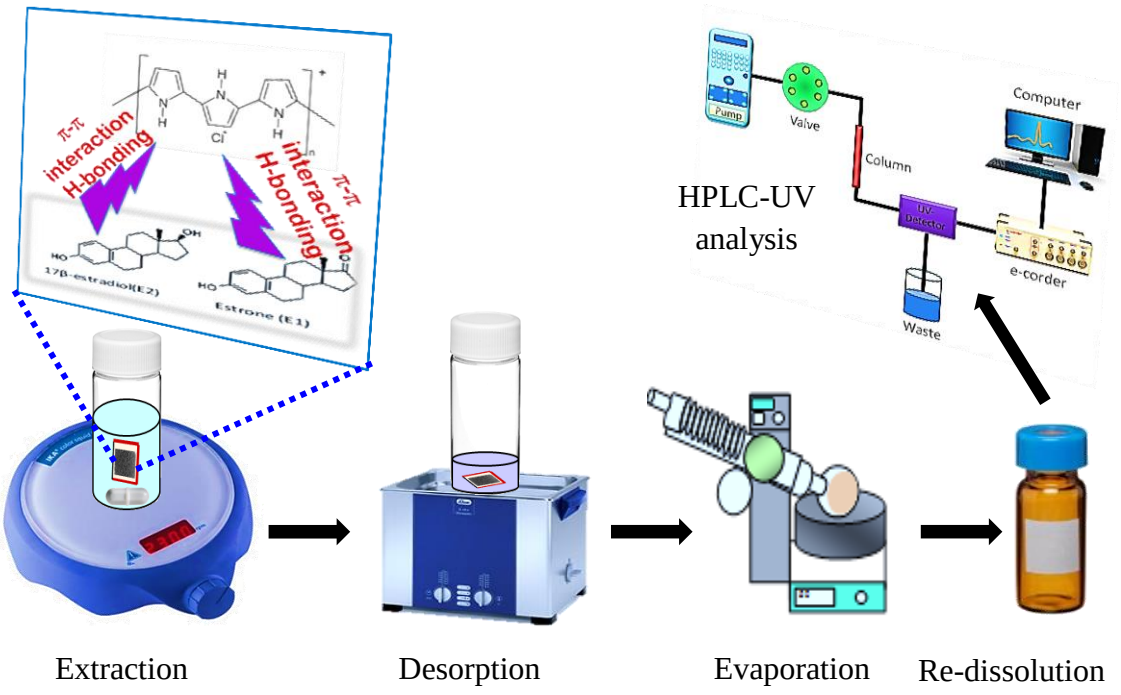


Figure 2.4 The process of µ-SPE and analysis system for estrogens determination.

2.4.6 Optimization of µ-SPE

The optimization was studied in 10.0 mL ultrapure water with a spiked mixture estrone and 17β-estradiol at a concentration of 0.050 mg L^{-1} (50.0 µg L^{-1}), five replicates were run in all cases. The extraction using µ-SPE technique depends on the equilibrium between the extraction solution and the sorbent (Huang *et al.*, 2012). Thus, several parameters influencing the extraction efficiency were investigated including the amount of sorbent (PPY), extraction time, type of desorption solvent, volume of desorption solvent and desorption time. The extraction procedure was optimized in order to achieve the highest response, short extraction time and good recovery. One variable at a time optimization was used to obtain the most favorable conditions for µ-SPE and the initial conditions used for the extraction are reported in Table 2.2. The extraction recovery in the optimization was observed by comparing the response of HPLC-UV after µ-SPE injection and that from the direct injection of standard solution.

Table 2.2 The initial condition for micro-solid phase extraction

No.	Parameters	Initial conditions
1	Amount of sorbent	20 (mg)
2	Extraction time	60 (min)
3	Type of desorption solvent	Acetone
4	Desorption solvent volume	1.0 (mL)
5	Desorption time	30 (min)

2.4.6.1 Amount of sorbent

To examine the effect of mass of sorbent on the extraction efficiency, 5.0, 10.0, 15.0 and 20.0 mg of sorbents were studied. The lowest mass of sorbent which provided the highest response (peak area) of estrogens was chosen.

2.4.6.2 Extraction time

Since µ-SPE is an equilibrium extraction mode, the maximum amount of analyte that can be extracted by the µ-SPE device is achieved at equilibrium time. Therefore, in this study, the extraction time was examined from 20.0, 30.0, 40.0, 50.0 and 60.0 min in order to get the highest extraction efficiency. The optimum extraction time was selected where the highest response was obtained.

2.4.6.3 Type of desorption solvent

After extraction, the analytes were desorbed from the absorbing µ-SPE device by ultrasonication with a suitable organic solvent. Solvent desorption capabilities were evaluated by four solvents including acetonitrile (ACN), methanol (MeOH), acetone and ACN: H_2O (9:1). These solvents were studied based on the polarity of the analytes. The best eluting is one that was able to provide the highest response.

2.4.6.4 Desorption solvent volume

The volume of desorption solvent on the extraction efficiency of estrogens was investigated at 1.0, 1.5, 2.0 and 2.5 mL and the volume which could immerse the absorbing μ-SPE device during ultrasonication and gave the best response would be selected.

2.4.6.5 Desorption time

The influence of desorption time in the range of 15.0-35.0 min with an interval of 5.0 min was explored. A short desorption time with the maximum recovery would be chosen.

2.4.7 Sampling locations and sample pre-treatment

2.4.7.1 Sampling locations

Samples were collected from the surface of four locations around Thale Sap Songkhla , *i.e.*, Leam Chai (site 1), Muang Songkhla (site 2), Kok Yor (site 3) and Klong U-Tapao (site 4) (Figures 2.5A and 2.5B). These areas are the possible contaminated areas owing to agricultural pollution, pollution from boats, from Songkhla urban area, fish wastes from Songkhla harbour, near shore drainage and municipal waste from Hat Yai city (Kanatharana *et al.*, 1994; Sirinawin *et al.*, 1998). The sampling was conducted during the wet season in November and December 2014 and during the dry season in February and March 2015. All four sampling locations were recorded by a global positioning system (GPS) and the physical characteristics of the samples are shown in Table 2.3.

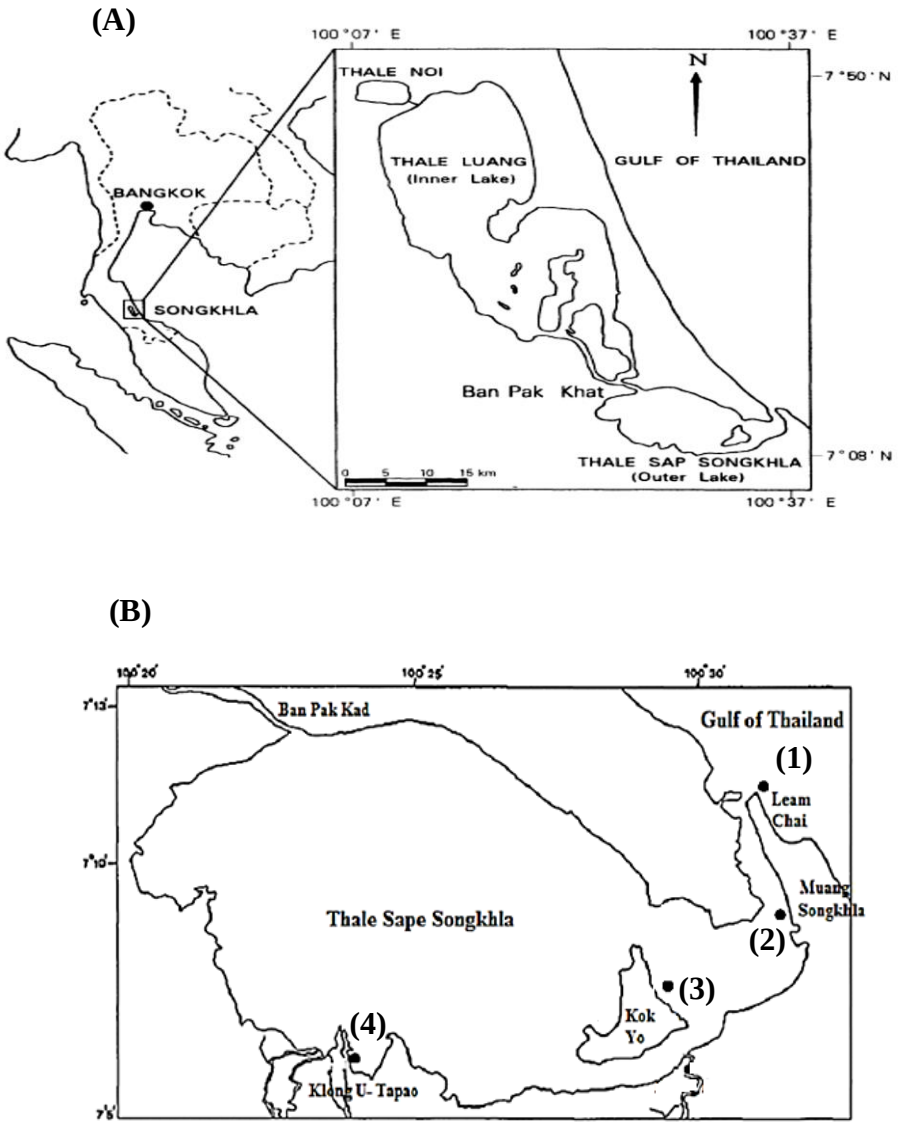


Figure 2.5 Location of (A) Thale Sap Songkhla (Angsupanich and Rakkheaw, 1997) and (B) four sampling sites in Thale Sap Songkhla (modified from Kanatharana *et al.*, 1994).

Table 2.3 The location and physical characteristic of the sample in each site for wet and dry season

Wet season							
Sampling site	**Location (GPS)**	**November 2014**			**December 2014**		
		T (°C)	**Color**	**pH**	**T (°C)**	**Color**	**pH**
1	N: 07°12’39.3” E: 100°34’57.6”	29.3	Light yellow	7.60	31.5	Light yellow	8.10
2	N: 07°10’48.7” E: 100°35’40.5”	28.3	Light yellow	8.10	28.9	Light yellow	7.64
3	N: 07°09’21.6” E: 100°31’57.0”	28.9	Light yellow	7.10	30.4	Light yellow	8.04
4	N: 07°08’05.5” E: 100°27’12.7”	29.0	Light yellow	6.60	31.0	Light yellow	6.95
Dry season							
Sampling site	**Location (GPS)**	**February 2015**			**March 2015**		
		T (°C)	**Color**	**pH**	**T (°C)**	**Color**	**pH**
1	N: 07°12’39.3” E: 100°34’57.7”	28.2	Light yellow	8.02	30.0	Light yellow	8.50
2	N: 07°10’48.7” E: 100°35’40.3”	28.3	Transparent	7.80	31.5	Light yellow	7.63
3	N: 07°09’21.5” E: 100°31’56.6”	27.5	Light yellow	7.82	30.3	Light yellow	8.05
4	N: 07°08’05.4” E: 100°27’12.6”	29.4	Yellow	7.23	31.7	Yellow	7.80

2.4.7.2 Sample pre-treatment

Surface water samples were collected and stored in pre-cleaned glass bottles with Teflon cap. They were immediately preserved with 1.0% formalin to prevent bacterial degradation of natural estrogens and kept in the dark on ice in a container while transporting to the laboratory at Prince of Songkla University, Hat Yai until the extraction. With these conditions, it is able to keep the samples for 24 days without the loss of the estrogens (Baronti *et al.*, 2000). The samples were filtered through Whatman filter paper No. 42 (pore size 2.5 µm), with glass microfiber filter GF/F (pore size 0.70 µm) and finally nylon membrane filters (pore size 0.20 µm) to remove any particulates prior to extraction (follows the procedure in 2.4.5).

2.4.8 Validation of method performances

The analytical characteristics of the optimized µ-SPE with HPLC-UV procedure were determined in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy in order to estimate the efficiency and the feasibility of the method for its application to the analysis of water sample. Such related parameters were evaluated under the optimum condition of µ-SPE and HPLC-UV with spiked water samples.

2.4.8.1 Linearity, LOD and LOQ

Under the optimal conditions of µ-SPE and HPLC-UV, a series of estrogens mixture concentrations (5.0 µg L^{-1} to 1000 µg L^{-1}) spiked in deionized water (DI) were evaluated. The linearity was obtain by considering to the coefficients of the determination (R^2) which must be greater than 0.99.

Limit of detection (LOD) was determined based on the concentration of the analytes which provided the signal-to-noise ratio equal or greater than 3 ($S/N \geq 3$) and the limit of quantification (LOQ) by a signal-to-noise ratio equal or greater than 10 ($S/N \geq 10$) (Wisconsin, 1996).

2.4.8.2 Precision

Precision of an analytical procedure is the closeness degree of replication measurement of the sample under the same condition. The precision was confirmed by the percentage relative standard deviation (%RSD) of the response from three different spiked concentrations at 25.0 µg L^{-1}, 100 µg L^{-1} and 1000 µg L^{-1} in all real samples with five replications for each concentration. The relative standard deviation will be calculated by the following equation (Long and Winefordner, 1983):

$$\%\text{RSD} = \frac{\text{SD}}{\bar{x}} \times 100 \qquad (2.1)$$

Where, SD = standard deviation

$\bar{x}$ = mean of n measurement (n=5)

2.4.8.3 Accuracy

Accuracy is the degree of the closeness of the experimental result to the true or accepted value. Accuracy of the method was studied in term of recovery which was investigated by spiked three concentrations of the analytes, 25.0 µg L^{-1}, 100 µg L^{-1} and 500 µg L^{-1} into all real samples (for five replicates) and was calculated by the following equation (AOAC, 2012):

$$\%\text{Recovery} = \frac{\text{CF} - \text{CU}}{\text{CA}} \times 100 \qquad (2.2)$$

Where, CF = the concentration of analyte measured in fortified sample (spkied sample)

CU = the concentration of analyte measured unfortified sample (blank sample)

CA = the concentration of analyte added in the sample

2.4.9 Matrix effect

Before the analysis of estrone and 17β-estradiol in real samples, the matrix effect of each sample was studied by comparing the slopes between the matrix matched calibration curve and standard calibration curve of each analyte with two-way ANOVA. The matrix in the sample shows the effect if these two slopes are significant different ($P < 0.05$), so the matrix matched calibration curve must be used for the calculation. However, if there is no significant different between these two slopes ($P > 0.05$) the matrix has no effect and the standard calibration curve can be used. The matrix matched calibration curve and standard calibration curve were acquired by spiking standard solution into the sample and DI water, respectively. The final spiked concentrations were 25, 50, 100, 250 and 500 μg L^{-1} in 10.0 mL. They were extracted by μ-SPE and analyzed under the same conditions. The calibration curves were the plots of peak area *versus* spiked concentration.

2.4.10 Qualitative and quantitative analysis of estrogens in water sample

2.4.10.1 Qualitative analysis

Qualitative analysis was studied by comparing the retention time of estrone and 17β-estradiol obtained from the chromatogram of each sample with the retention time of estrone and 17β-estradiol from the chromatogram of the standard solution under the same operating conditions (Snyder and Kirkland, 1979).

2.4.10.2 Quantitative analysis

Quantitative analysis was studied by extracting the samples with the μ-SPE technique. The obtained response of the anlytes was used to calculate the concentration from the linear equation of the matrix calibration curve or standard calibration curve as stated in section 2.4.9.

CHAPTER 3

Results and Discussion

The monitoring of estrogens in Songkhla Lake was studied based on the extraction by μ-SPE technique and analysis by HPLC-UV. Synthesized polypyrrole packed inside a tea bag filter paper sachet was used as the sorbent for the extraction of two kinds of estrogens (estrone and 17β-estradiol) in water samples. The HPLC-UV analysis used acetonitrile and water as the mobile phase and a C18 column, 4.6 × 150 mm, 5 μm for the separation. The chromatographic conditions and extraction conditions were optimized to obtain the best performance.

3.1 Optimization of HPLC-UV system

3.1.1 Mobile phase composition (ACN: H_2O, v/v)

The selection of mobile phase composition is an important variable that control the separation. The choice of a mobile phase is governed by the physical properties of the solvent including polarity, miscibility with other solvents, chemical inertness (no reaction with stationary phase), UV cut off wavelength and toxicity. In the reversed phase chromatography the common polar solvent is water with acetonitrile or methanol as an organic modifier (Moldoveanu and David, 2013b). However, methanol has the disadvantage of producing viscous solution (Table 3.1) when mixed with water, giving rise to much higher pressure than other mobile phases which can result in a fluctuated chromatogram (FDA, 1996). Thus, in this study the mixture of acetonitrile and water was used for the mobile phase. The composition of acetonitrile: water (v/v) was verified from 50:50, 60:40, 63:37, 65:35 and 70:30.

Table 3.1 Properties of common HPLC solvent for reversed phase chromatography (FDA, 1996)

Solvent	**UV cut-off (nm)**	**Boiling point (°C)**	**Viscosity (cP), 25°C**	**Polarity index (P')**
Water	-	100	0.89	10.2
acetonitrile	190	82	0.34	5.8
Methanol	205	65	0.54	5.1

(-) = Not available

The general way to choose the mobile phase composition is to find the correct strength of the solvent which provides the retention factor or capacity factor (k) between 2 and 10 (Moldoveanu and David, 2013a). The retention factor is the parameter that describes the migration of the analytes on a column. It can be calculated by the following equation:

$$k = \frac{t_R - t_0}{t_0} \qquad (3.1)$$

Where, k = Capacity factor or retention factor

t_R = Retention time of analyte

t_0 = Retention time of solvent or dead time

(**Note:** The notation for retention factor k may vary in the literature regarding chromatographic parameters, the notation k' being sometimes used.)

When the retention factor is less than 2 it indicates poor retention of the analyte on the column and sometime acceptable, but care must be taken to make sure that the analyte separates from the other matrix compound. The retention factor which is higher than 10 show the strong retention and the obtained peak is wider when it elutes in longer

time. However, some studies which are in a complex matrix the retention factor of 20 is also acceptable (Moldoveanu and David, 2013a).

The compromising parameters to consider for the mobile phase composition selecting are short retention time and best separation which depend on the value of resolution (R_s). The resolution is the parameter that characterizes by the peak separation and can be calculated based on the following equation (Moldoveanu and David, 2013a):

$$R_s = \frac{2[t_R(X) - t_R(Y)]}{W_X - W_Y} \qquad (3.2)$$

Where, t_R (X) = Retention time of analyte X

t_R (Y) = Retention time of analyte Y

W_X = Peak width of anlyte X

W_Y = Peak width of analyte Y

The acceptable value of R_s is equal or greater than 1.5. W_X and W_Y can be measured as shows in Figure 3.1.

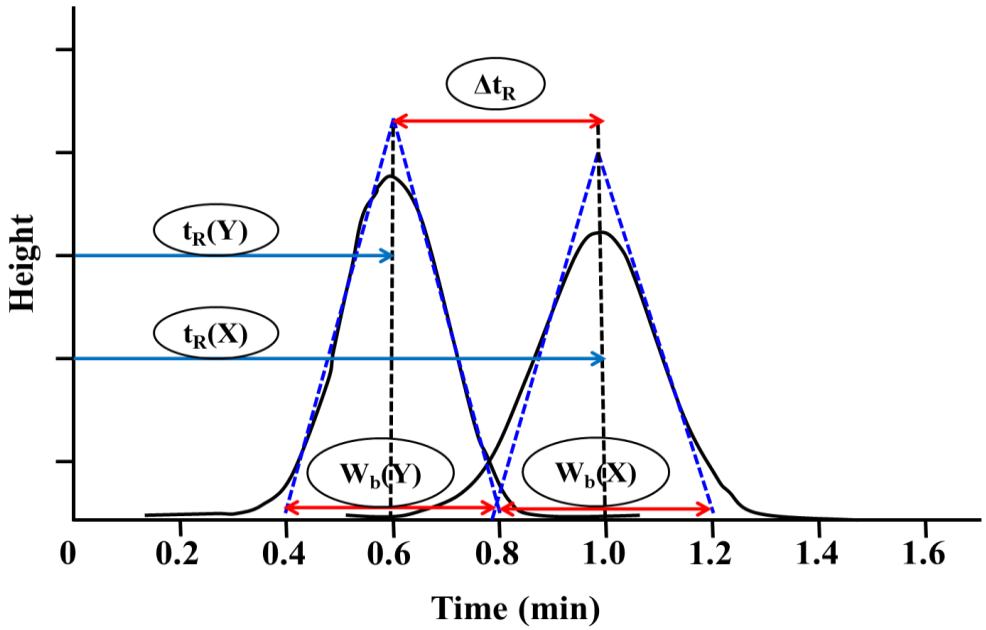


Figure 3.1 Chromatogram which shows the measureable parameters for resolution calculation.

From the results of the optimization (Tables 3.2A and 3.2B), the composition of 60:40 (ACN: H_2O) was chosen as it provided the acceptable capacity factor between 2 and 10, resolution and also suitable analysis time comparing to 50: 50 which provided broad peak and also long analysis time.

Table 3.2A The obtained capacity factor, resolution of the mobile phase composition optimization (n = 5)

ACN: H_2O (v/v)	k (E1) ± SD	k (E2) ± SD	R_s (E1, E2) ± SD
50: 50	6.547 ± 0.012	4.153 ± 0.021	5.918 ± 0.016
60: 40	3.563 ± 0.025	2.450 ± 0.017	3.837 ± 0.015
63: 37	2.420 ± 0.010	1.643 ± 0.012	3.367 ± 0.015
65: 35	2.467 ± 0.012	1.747 ± 0.015	3.263 ± 0.012
70: 30	1.933 ± 0.015	1.3767 ± 0.0058	3.037 ± 0.012

Table 3.2B The retention time of analytes for mobile phase composition optimization

ACN: H_2O (v/v)	t_R (E1) ± SD (min)	t_R (E2) ± SD (min)
50: 50	18.883 ± 0.012	12.920 ± 0.010
60: 40	10.6433 ± 0.0058	7.980 ± 0.010
63: 37	9.3440 ± 0.0036	7.2173 ± 0.0067
65: 35	8.560 ± 0.020	6.7533 ± 0.0058
70: 30	7.250 ± 0.010	5.9333 ± 0.0058

3.1.2 Mobile phase flow rate

The mobile phase flow rate is also one of the most important parameters that involves with the band broadening. However, the widening of the substance band is dependent on the column efficiency for the analyte, which can be expressed in term of number of theoretical plate or plate number (N). The relationship between the column efficiency and the peak width is described below:

$$N = 16\left(\frac{t_R}{W}\right)^2 \qquad (3.3)$$

Where, t_R = Retention time of the peak

W = Baseline peak width

(**Note:** t_R and W must have the same unit, which are the distance measured with the ruler which show in Figure 3.1)

Therefore, the better separation and narrower peak in the chromatogram are achieved when the value of N is larger (Snyder *et al.*, 2010).

However, the baseline peak width is difficult to measure accurately while the peak is broaden, thus the width of a chromatographic peak is typically measured at a point half way between the baseline and the top of the peak which defines as the half-height peak width ($W_{1/2}$). N can be calculated using equation 3.4 while $W_{1/2}$ is used instead of W.

$$N = 5.54\left(\frac{t_R}{W_{1/2}}\right)^2 \qquad (3.4)$$

Where, $W_{1/2}$ can be measured as show in Figure 3.2.

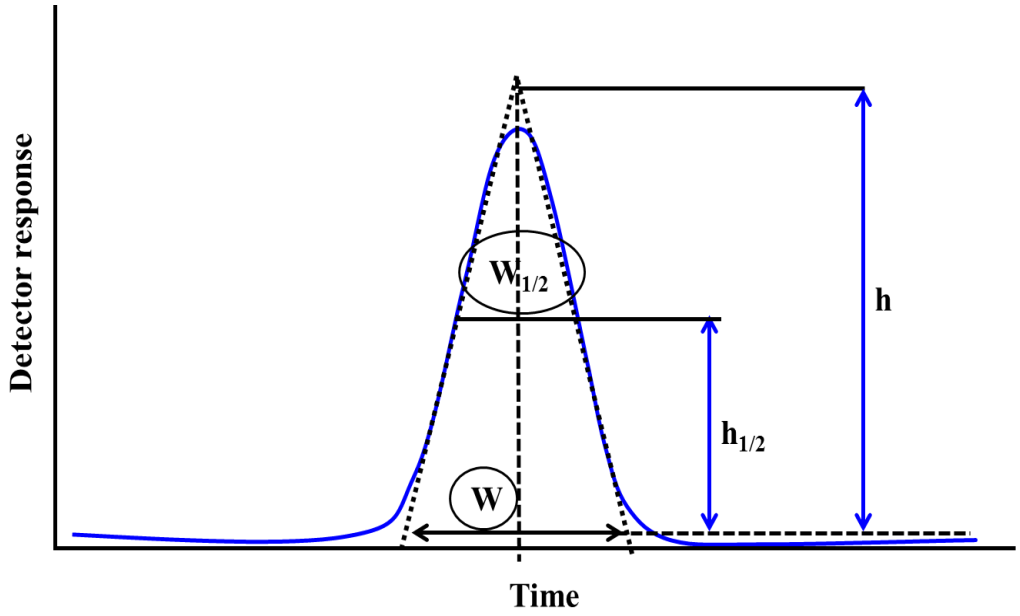


Figure 3.2 The measurement of half-height peak width ($W_{1/2}$).

The other relative parameter which commonly uses to describe the column efficiency is plate height (H) or height equivalent to a theoretical plate (HETP) (Figure 3.3). The column efficiency is expressed in term of HETP by the following equation (Snyder *et al.*, 2010):

$$\text{HETP (H)} = \frac{L}{N} \qquad (3.5)$$

Where, L = Length of the column

(**Note:** L and H must be in the same unit)

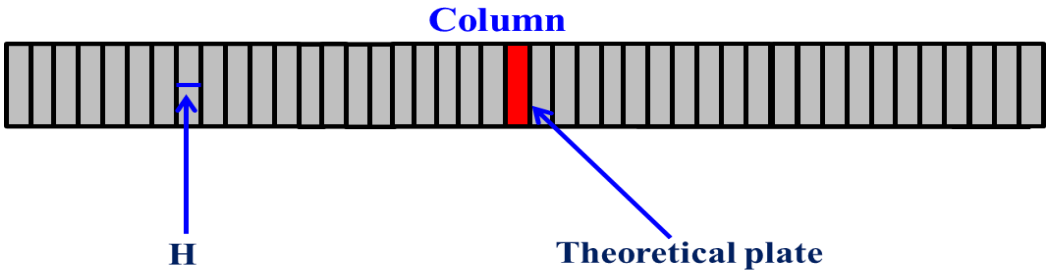


Figure 3.3 Height equivalents to a theoretical plate in a column.

The above equation shows that to increase the column efficiency which leads to decrease the band broadening, longer column or small plate height must be used. Hence, with a fixed column length the variable that can effect to the efficiency of the column and band broadening is the plate height.

The van Deemter derived an equation which describes the phenomena that related with the plate height as following (Snyder *et al.*, 2010):

$$\text{HETP (H)} = A + \frac{B}{u} + Cu \qquad (3.6)$$

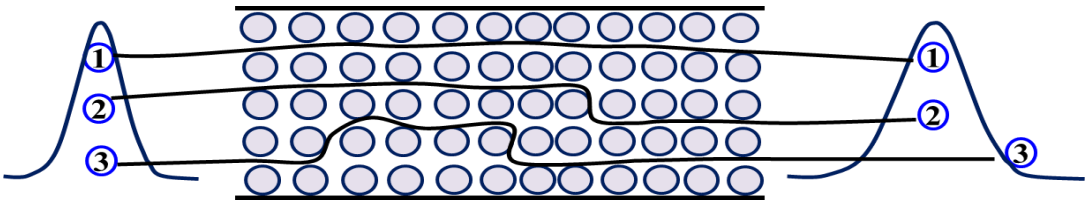
Where, A = Eddy diffusion (multiple path effect)

B = Longitudinal diffusion (random molecule diffusion)

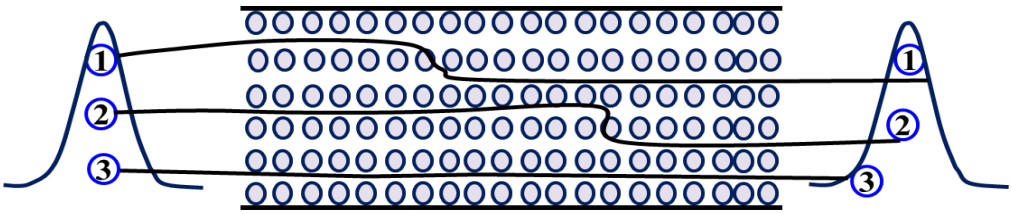
C = Mass transfer within particle cause by mobile phase

u = Mobile phase velocity

Eddy diffusion is used to define the analyte flow path within the chromatographic column. This phenomenon results from multiple flow paths in the column due to the in homogeneities in column packing and small variations in particle size of the packing materials and is independent of mobile phase flow rate. As molecules of the analyte move through the column, they take many different paths around the packed particles. Some of these paths are undoubtedly longer than others so as the molecules move through the column, they tend to spread out which make the band of analyte broader (Figure 3.4A). Smaller packing particles offer smaller differences in path length, thus reducing peak broadening (Figure 3.4B).



(A) Large particles size of packing material



(B) Small particles size of packing material

Figure 3.4 Band broadening due to the eddy diffusion in column with (A) large particles size of packing material and (B) small particles size of packing material.

Longitudinal diffusion depends on the diffusion coefficient of the analyte molecules in the mobile phase. Faster mobile phase flow rates reduce resident time, and shorter resident time of the analyte molecules in the column reduces the effects of longitudinal diffusion (Figure 3.5). This reduction contributes to better separation efficiencies since the analyte molecules have less opportunity to spread out through diffusion, thus explaining the 1/u factor.

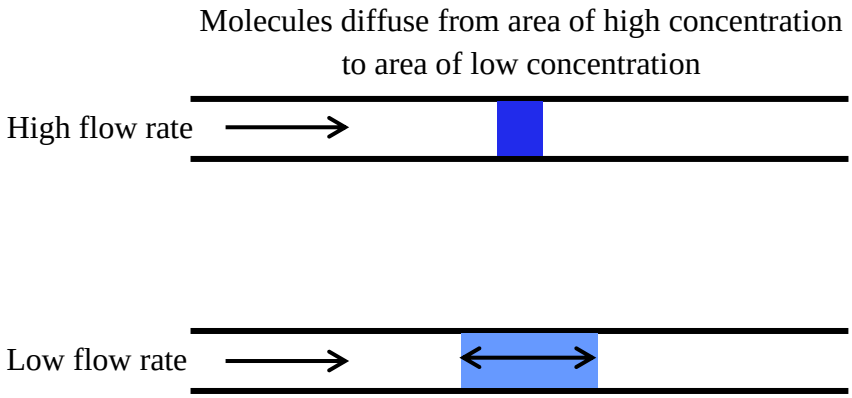


Figure 3.5 Longitudinal diffusion of the analyte with high flow rate and low flow rate of mobile phase.

Mass transfer term related to the equilibrium between the mobile and stationary phases, this process is based on the time. If the equilibration is too slow, then some of the analyte molecules which already equilibrated to the mobile phase and did not have enough time to bond to the stationary phase will flow down the column with the mobile phase, whereas, the other molecules which did not equilibrated to the mobile phase and still have time to interact with the stationary phase are left behind (Figure 3.6). Therefore, higher mobile phase flow rates will contribute to the spreading out of the analyte molecules as explain by the u factor.

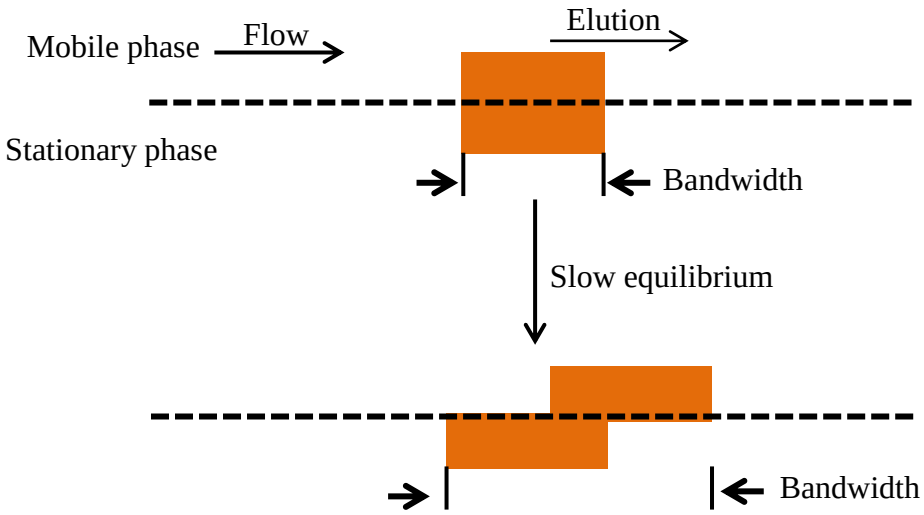


Figure 3.6 The effect of mass transfer to band broadening.

Thus, to minimize the band broadening flow rate of the mobile phase has to optimize to get the small value of plate height. The optimum flow rate can be selected by using the van Deemter plot (Figure 3.7). This plot is the composition curve which related to the plate height and the mobile phase velocity (flow rate).

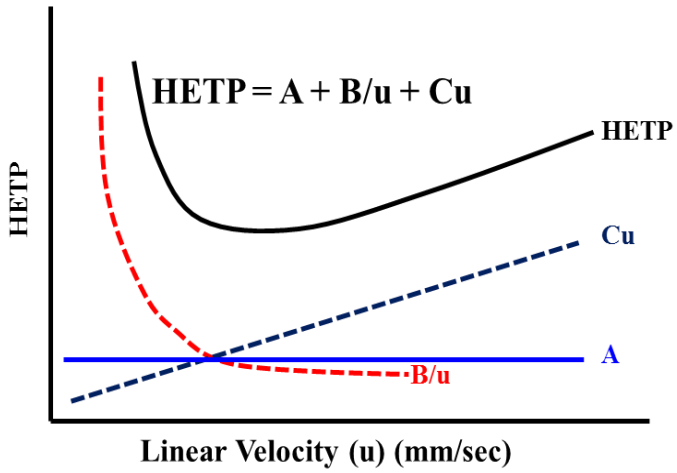


Figure 3.7 The van Deemter plot.

The result of flow rate optimization is shown in Table 3.3 and from the van Deemter plot (Figure 3.8), the optimum flow rate which provided the minimum plate height was 0.70 mL min^{-1}.

Table 3.3 Plate height of estrone and 17β-estradiol at various flow rates of mobile phase (n = 5)

Flow rate (mL min^{-1})	HETP (E1) ± SD (mm)	HETP (E2) ± SD (mm)
0.50	2.943 ± 0.012	2.7133 ± 0.0058
0.60	2.2440 ± 0.0089	2.508 ± 0.016
0.70	1.9260 ± 0.0055	1.8880 ± 0.0045
0.80	2.5180 ± 0.0045	2.476 ± 0.021
0.90	3.1433 ± 0.0058	3.100 ± 0.017

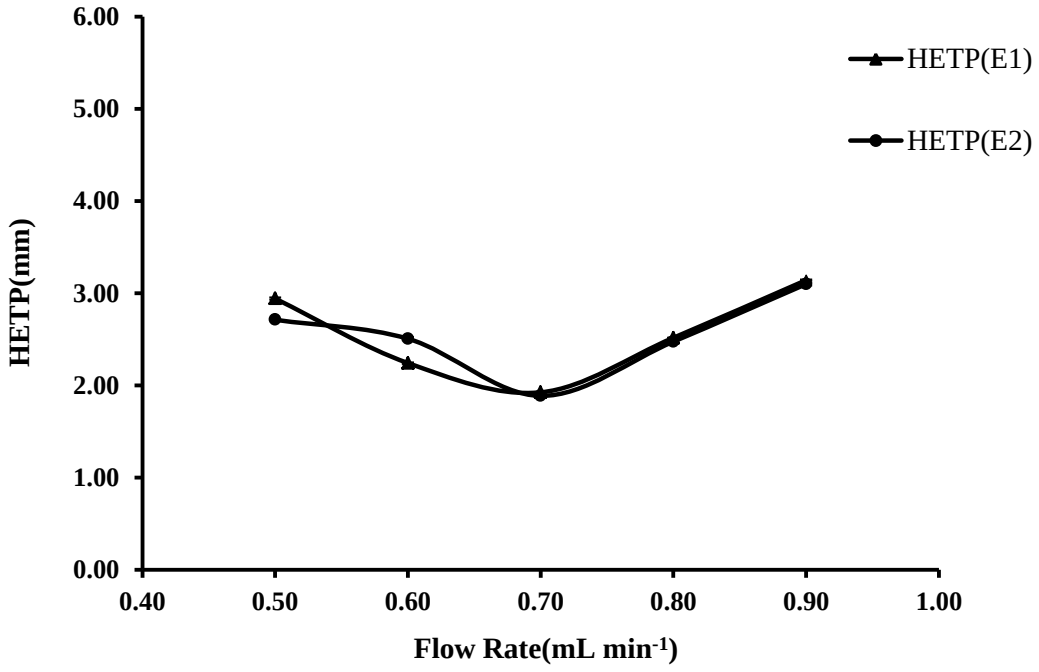


Figure 3.8 Van Deemter plots of estrone (E1) and 17β-estradiol (E2)

3.1.3 Detection wavelength

The UV detector absorbs light in the UV region (190-400 nm). For the absorption of the two estrogens (Table 3.4 and Figure 3.9) the highest response was at 197 nm. Therefore, this wavelength was selected for further study.

Table 3.4 The responses of 2.50 mg L^{-1} of estrone and 17β-estradiol at different wavelengths (n = 5)

Wavelength (nm)	Peak area (E1) ± SD (V.s)	Peak area (E2) ± SD (V.s)
193	0.6739 ± 0.0035	0.6419 ± 0.0070
194	0.6914 ± 0.0052	0.6547 ± 0.0029
195	0.6989 ± 0.0038	0.6608 ± 0.0038
196	0.7088 ± 0.0058	0.67720 ± 0.00078
197	0.7117 ± 0.0087	0.672 ± 0.027
198	0.6711 ± 0.0081	0.645 ± 0.014
200	0.6366 ± 0.0053	0.6145 ± 0.0061
202	0.5707 ± 0.0087	0.5521 ± 0.0070
205	0.4471 ± 0.0068	0.4450 ± 0.0036
206	0.4041 ± 0.0062	0.4032 ± 0.0077
210	0.2778 ± 0.0020	0.2706 ± 0.0030

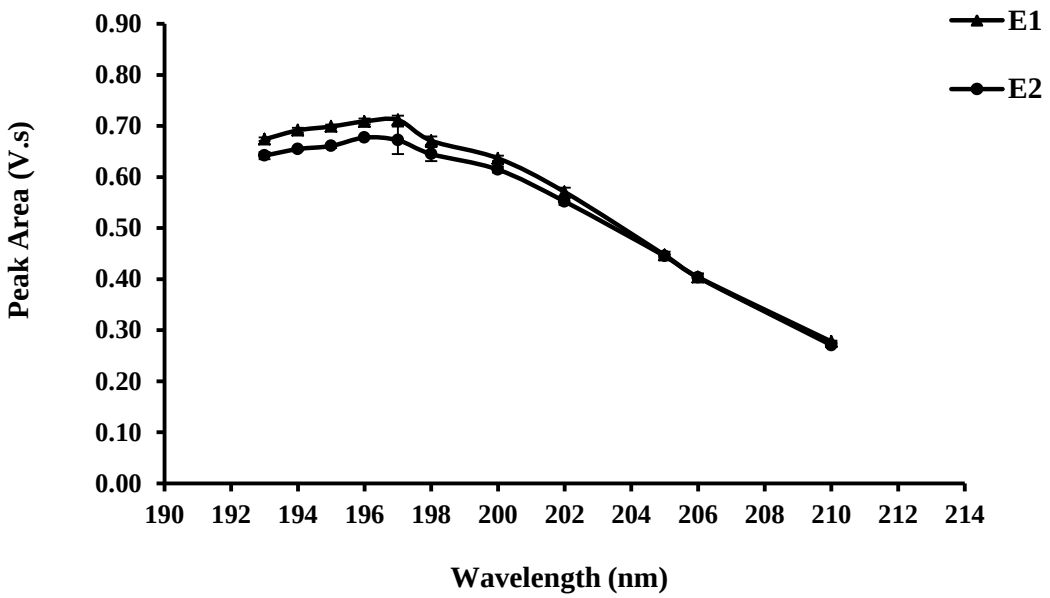


Figure 3.9 Response of 2.50 mg L^{-1} of estrone and 17β-estradiol at different wavelength.

3.1.4 Summary of the optimized HPLC-UV conditions

The optimized parameters of the HPLC-UV system were summarized in Table 3.5 and under the optimum conditions the chromatogram was obtained as shown in Figure 3.10.

Table 3.5 Optimum conditions of HPLC-UV for estrone and 17β-estradiol

Parameters	Studied values	Optimum values
Mobile phase composition % (v/v)	50: 50, 60: 40, 63: 37, 65: 35, 70: 30	60: 40
Mobile phase flow rate (mL min^{-1})	0.50, 0.60, 0.70, 0.80, 0.90	0.70
Detection wavelength (nm)	193, 194, 195, 196, 197, 198, 200, 202, 205, 206, 210	197

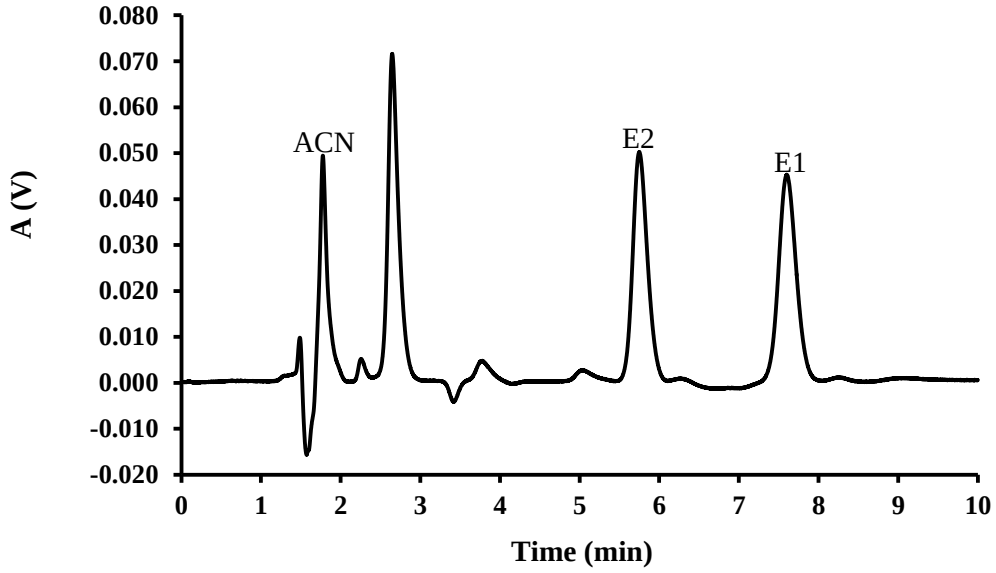


Figure 3.10 Chromatogram of 2.50 mg L^{-1} of estrone (E1) and 17β-estradiol (E2) under the optimum conditions.

3.2 HPLC-UV system performance

3.2.1 Linearity

The linearity can be achieved if the coefficient of determination (R^2) equal or greater than 0.99. Under the optimum conditions of HPLC-UV, the linearity of the instrument was performed from 0.020 mg L^{-1} to 75.0 mg L^{-1}. The calibration curve (Figure 3.11) shows the linear range were from 0.050 mg L^{-1} to 50.0 mg L^{-1} for both estrone and 17β-estradiol with the correlation of determination (R^2) of 0.9997 and 0.9994, respectively and relative standard deviations (RSD) of peak area lower than 4%.

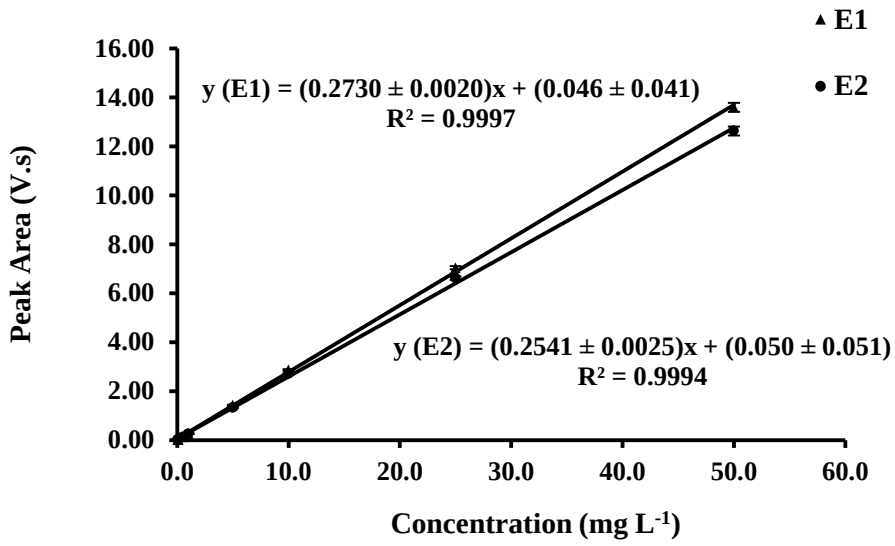


Figure 3.11 Calibration curves of estrone and 17β-estradiol in the range of 0.050-50.0 mg L^{-1}.

3.2.2 Limit of detection (LOD) and limit of quatification (LOQ) of HPLC-UV

The limit of detection and limit of quantification were determined using IUPAC method from the peak area of 20 blank injections (Table 3.6), the peak which is stably appeared in the chromatogram of the blank. Both of LOD and LOQ can be calculated by the following equations (Long and Winefordner, 1983):

$$\text{LOD or LOQ} = \frac{kS_B}{m} \qquad (3.7)$$

Where, k = 3 and 10 for LOD and LOQ, respectively

S_B = SD of blank

m = slope of the calibration curve of each analyte

For this method, SD of LOD and LOQ were also calculated by:

$$\text{SD of LOD} = f_A \times \text{LOD} \qquad (3.8)$$

$$\text{SD of LOQ} = f_A \times \text{LOQ} \qquad (3.9)$$

$$f_A = \frac{\text{SD of slope}}{\text{slope}} \qquad (3.10)$$

The acquired results are reported in Table 3.7.

Table 3.6 The response of 20 blank injections

Injection times	**Peak area (V.s)**	**Injection times**	**Peak area (V.s)**
1	0.0244	16	0.0212
2	0.0222	17	0.0213
3	0.0222	18	0.0223
4	0.0204	19	0.0239
5	0.0220	20	0.0216
6	0.0209	**Average**	**0.0220**
7	0.0216	**SD**	**0.0015**
8	0.0216		
9	0.0247		
10	0.0226		
11	0.0253		
12	0.0204		
13	0.0209		
14	0.0200		
15	0.0211		

Table 3.7 Limit of detection and limit of quantification of estrone and 17β-estradiol

Analytes	**Limit of detection (LOD) μg L^{-1}**	**Limit of quatification (LOQ) μg L^{-1}**
Estrone (E1)	16.00 ± 0.12	55.00 ± 0.40
17β-estradiol (E2)	18.00 ± 0.17	59.00 ± 0.58

3.2.3 Instrument precision

To evaluate the consistency of HPLC-UV system, the precision was studied. %RSD of the retention time and peak area of 2.50 mg L^{-1} mix standard of estrone and 17β-estradiol were used to examine the system and it can be calculated by equation 2.1. The results (Table 3.8) were within the acceptable %RSD, i.e., less than 1% for retention time (%RSD = 0.095 for E1 and 0.063 for E2) and less than 4% for the peak area (%RSD = 1.2 for E1 and 2.4 for E2) (Snyder and Kirkland, 1979).

Table 3.8 %RSD of the retention time and peak area of 2.50 mg L^{-1} of estrone and 17β-estradiol (n = 6)

Injection time	**t_R (E1) (min)**	**t_R (E2) (min)**	**Peak area (E1) (V.s)**	**Peak area (E2) (V.s)**
1	7.609	5.762	0.7000	0.6706
2	7.597	5.754	0.7088	0.6586
3	7.602	5.752	0.7237	0.6657
4	7.611	5.756	0.7106	0.6902
5	7.617	5.759	0.7154	0.6983
6	7.603	5.755	0.7186	0.6899
Mean	**7.6065**	**5.7563**	**0.7129**	**0.679**
SD	**0.0072**	**0.0036**	**0.0083**	**0.016**
%RSD	**0.095**	**0.063**	**1.2**	**2.4**

3.3 Optimization of μ-SPE

3.3.1 Amount of sorbent

The effect of polypyrrole amount was investigated by observing the peak area of each analyte (Table 3.9 and Figure 3.12). As expected, the extraction efficiency increased with the amount of sorbent. Since the responses of 15.0 and 20.0 mg were not much different and to reduce the amount of sorbent used, 15.0 mg was selected.

Table 3.9 The peak area corresponded with the different amount of polypyrrole (n =5)

Amount of sorbent (mg)	peak area (E1) ± SD (mV.s)	peak area (E2) ± SD (mV.s)	%RSD (E1)	%RSD (E2)
5.0	72.3 ± 6.2	77.2 ± 1.2	8.5	1.6
10.0	94.8 ± 2.6	88.9 ± 4.8	2.7	5.3
15.0	107.0 ± 1.2	103.77 ± 0.15	1.1	0.15
20.0	108.3 ± 3.0	108.6 ± 1.2	2.8	1.1

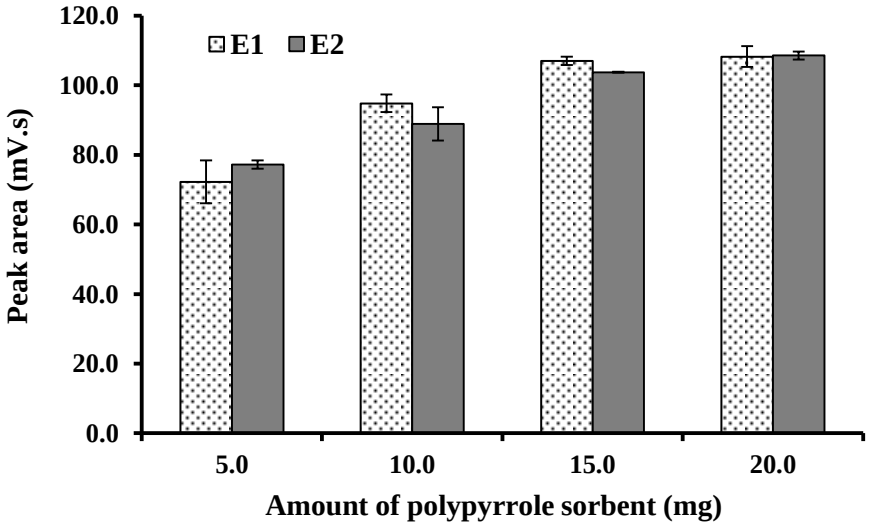


Figure 3.12 The effect of sorbent amount on the obtained peak area of E1 and E2.

3.3.2 Extraction time

The obtained results (Table 3.10 and Figure 3.13) showed that, when the extraction time increased from 20 min to 40 min, the response of the analytes also increased. This is because extraction is based on the transfer of the analytes from the sample solution to the sorbent, which is a time-dependent process. Therefore, when the time increases more analytes can be transferred to the sorbent. However, when the time was more than 40 min, there was no increase of the responses indicating that the diffusion of the analytes from the sample solution to the sorbent was already in the equilibrium state. Hence, 40 min was chosen as the optimum extraction time.

Table 3.10 Effect of the extraction time of µ-SPE on the response (n =5)

Extraction time (min)	**Peak area (E1) ± SD (mV.s)**	**Peak area (E2) ± SD (mV.s)**	**%RSD (E1)**	**%RSD (E2)**
20.0	85.73 ± 0.45	77.3 ± 2.4	0.53	3.1
30.0	96.5 ± 2.2	81.9 ± 5.8	2.2	7.0
40.0	101.7 ± 1.6	96.2 ± 1.2	1.6	1.2
50.0	102.7 ± 8.6	96.3 ± 3.7	8.4	3.8
60.0	103.8 ± 4.0	96.6 ± 3.4	3.8	3.6

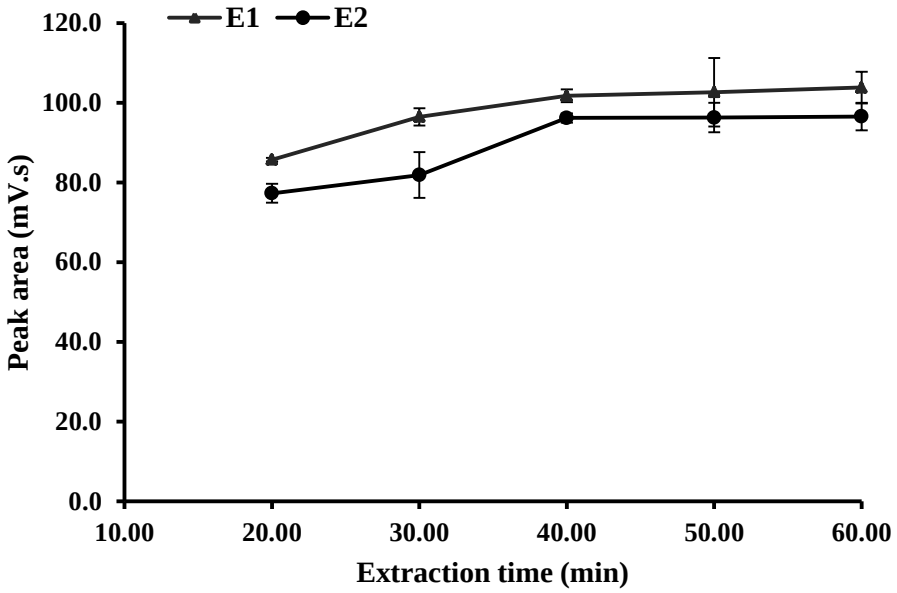


Figure 3.13 Effect of extraction time on the obtained peak area of E1 and E2.

3.3.3 Type of desorption solvents

By considering the polarity of both the analytes (slightly polar compounds) and the solvents, four desorption solvents were investigated *i.e.*, acetonitrile, acetonitrile: H_2O (9:1), methanol and acetone. The highest responses were obtained with acetone (Table 3.11 and Figure 3.14). This is because acetone is less polar, having a lower polarity index (5.1) than others, *i.e.* 5.8 and 9.0 for acetonitrile and H_2O, respectively (FDA, 1996). Although, methanol and acetone have the same polarity index but the dielectric constant (express the polarity index) of methanol is 33 and acetone is 21 (Anslyn and Dougherty, 2006), therefore acetone is less polar. Hence, acetone is more suitable for desorbing the slightly polar analytes from the sorbent.

Table 3.11 The peak area of estrone and 17β-estradiol with the different types of desorption solvent (n = 5)

Type of desorption solvents	Peak area E1 ± SD (mV.s)	Peak area E2 ± SD (mV.s)	%RSD (E2)	%RSD (E1)
ACN	91.23 ± 0.70	67.5 ± 2.6	3.9	0.77
ACN:H_2O(9:1)	96.9 ± 1.2	78.0 ± 1.0	1.3	1.2
MeOH	95.8 ± 1.3	85.10 ± 0.92	1.1	1.4
Acetone	116.0 ± 3.5	104.7 ± 2.2	2.1	3.0

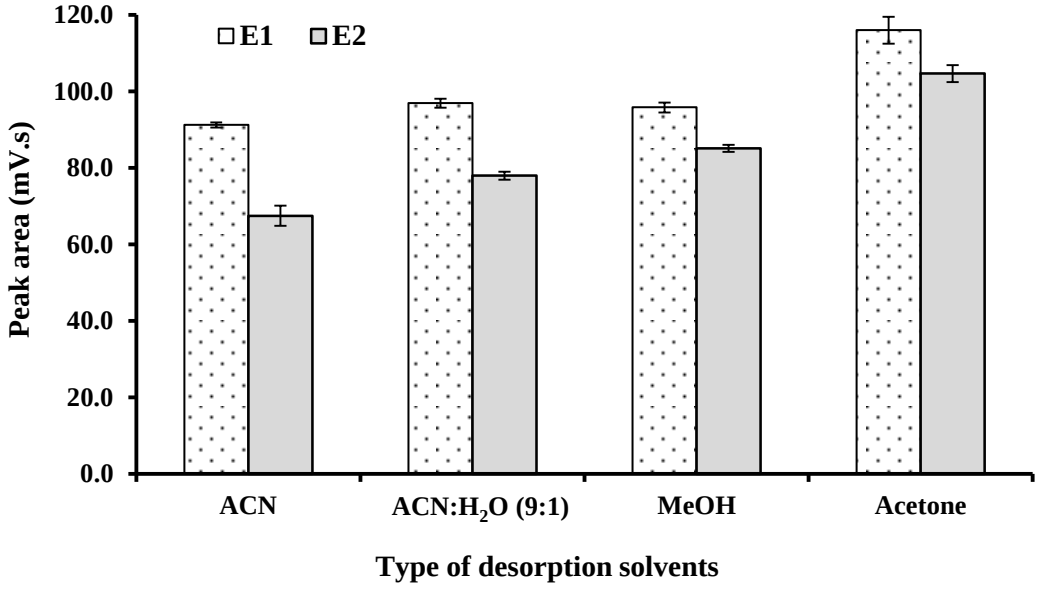


Figure 3.14 Effect of type of desorption solvents on the obtained peak area of E1 and E2.

3.3.4 Volume of desorption solvent

The desorption volume was studied between 1.0 and 2.5 mL (Table 3.12). An increase in the volume up to 1.5 mL resulted in an increase of the peak area (Figure 3.15), since a larger volume could desorb more analytes from the sorbent.

However, further increase did not provide a larger peak area. This might be because the amount of the desorbed analyte had already reached its maximum. Therefore, 1.5 mL was chosen.

Table 3.12 The peak area of estrone and 17β-estradiol with the different volume of desorption solvent (n = 5)

Desorption solvent volume (mL)	**Peak area E1 ± SD (mV.s)**	**Peak area E2 ± SD (mV.s)**	**%RSD (E1)**	**%RSD (E2)**
1.0	125.6 ± 2.3	106.27 ± 0.21	1.8	0.20
1.5	133.03 ± 0.67	113.1 ± 1.5	0.50	1.4
2.0	133.07 ± 0.46	113.40 ± 0.87	0.35	0.76
2.5	133.3 ± 1.7	113.6 ± 1.1	1.3	0.97

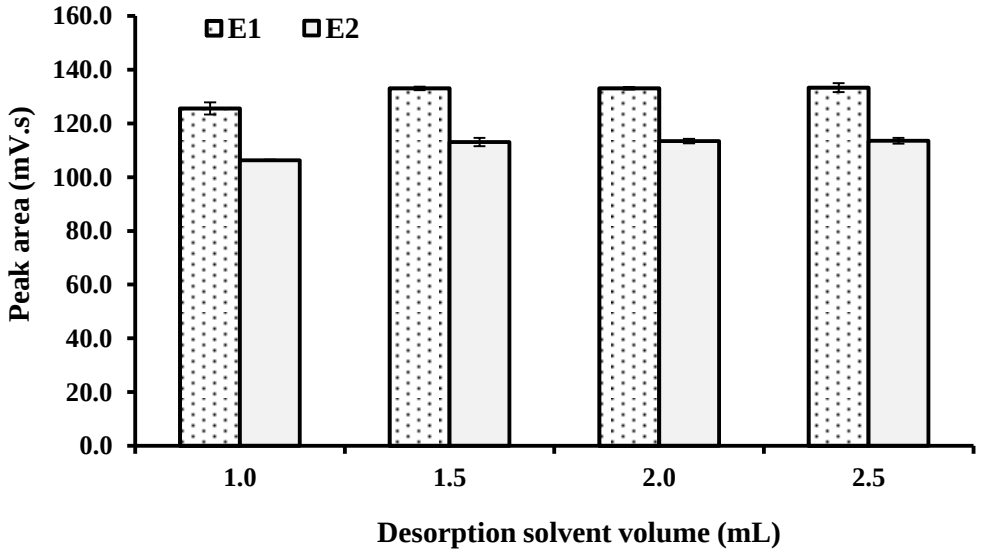


Figure 3.15 Effect of desorption solvent volume on the obtained peak area of E1 and E2.

3.3.5 Desorption time

Desorption time was investigated and the results (Table 3.13 and Figure 3.16) showed that when the time was increased from 15.0 min to 25.0 min there was an increase in the recovery from 81.55% ± 0.34 to 85.1% ± 1.0 and 82.6% ± 1.2 to 86.86% ± 0.43 for E1 and E2, respectively. After 25.0 min the recovery decreased, this would be because the prolonged desorption time could lead to re-adsorption of the analyte onto the sorbent. So, 25.0 min was selected for desorption.

Table 3.13 The obtained recovery of estrone and 17β-estradion with the different of desorption time (n = 5)

Desorption time (min)	**%Recovery E1 ± SD**	**%Recovery E2 ± SD**	**%RSD (E1)**	**%RSD (E2)**
15.0	81.55 ± 0.34	82.6 ± 1.2	0.41	1.4
20.0	83.14 ± 0.85	83.0 ± 1.5	1.0	1.8
25.0	85.1 ± 1.0	86.86 ± 0.43	1.1	0.50
30.0	78.15 ± 0.78	82.9 ± 1.9	1.0	2.3
35.0	74.07 ± 0.27	79.99 ± 0.59	0.37	0.74

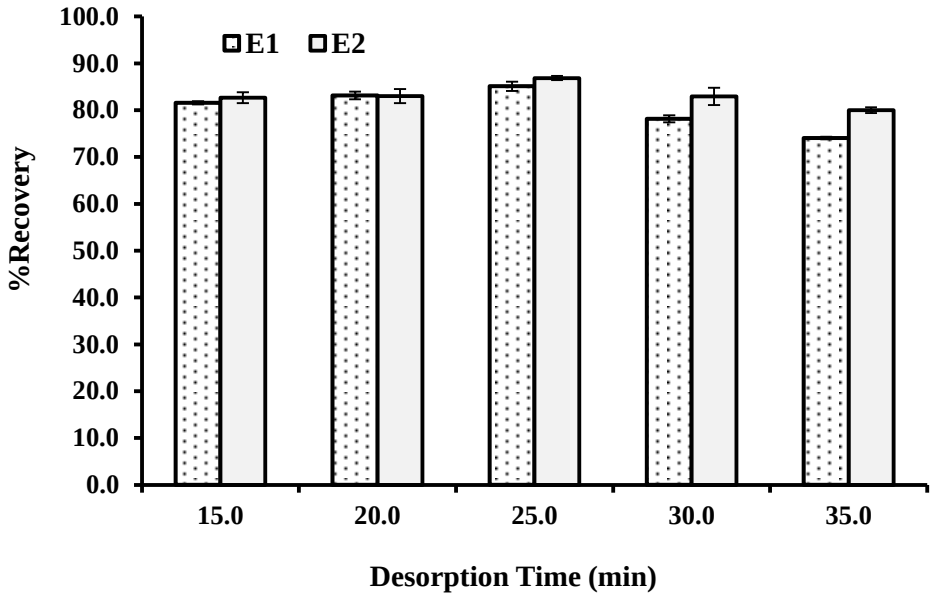


Figure 3.16 Effect of desorption time on the extraction recovery.

The optimum conditions of µ-SPE for estrone and 17β-estradiol with HPLC-UV analysis are summarized in Table 3.14. The chromatogram of direct injection of 50.0 ppb estrogens standard and the chromatogram of 50.0 ppb estrogens standard after extracting with µ-SPE are shown in Figure 3.17A and 3.17B, respectively. The peak areas of the analytes after etraction were increased about 8 times comparing with the peak areas of the direct injection of anlytes standard.

Table 3.14 The optimum conditions of µ-SPE for estrone and 17β-estradiol in water sample

No.	Parameters	Studied range	Optimum conditions
1	Amount of sorbent (mg)	5.0, 10.0, 15.0 and 20.0	15.0
2	Extraction time (min)	20.0, 30.0, 40.0, 50.0 and 60.0	40.0
3	Type of desorption solvent	ACN, MeOH, Acetone and ACN: H_2O (9:1)	Acetone
4	Desorption solvent volume (mL)	1.0, 1.5, 2.0 and 2.5	1.5
5	Desorption time (min)	15.0, 20.0, 25.0, 30.0 and 35.0	25.0

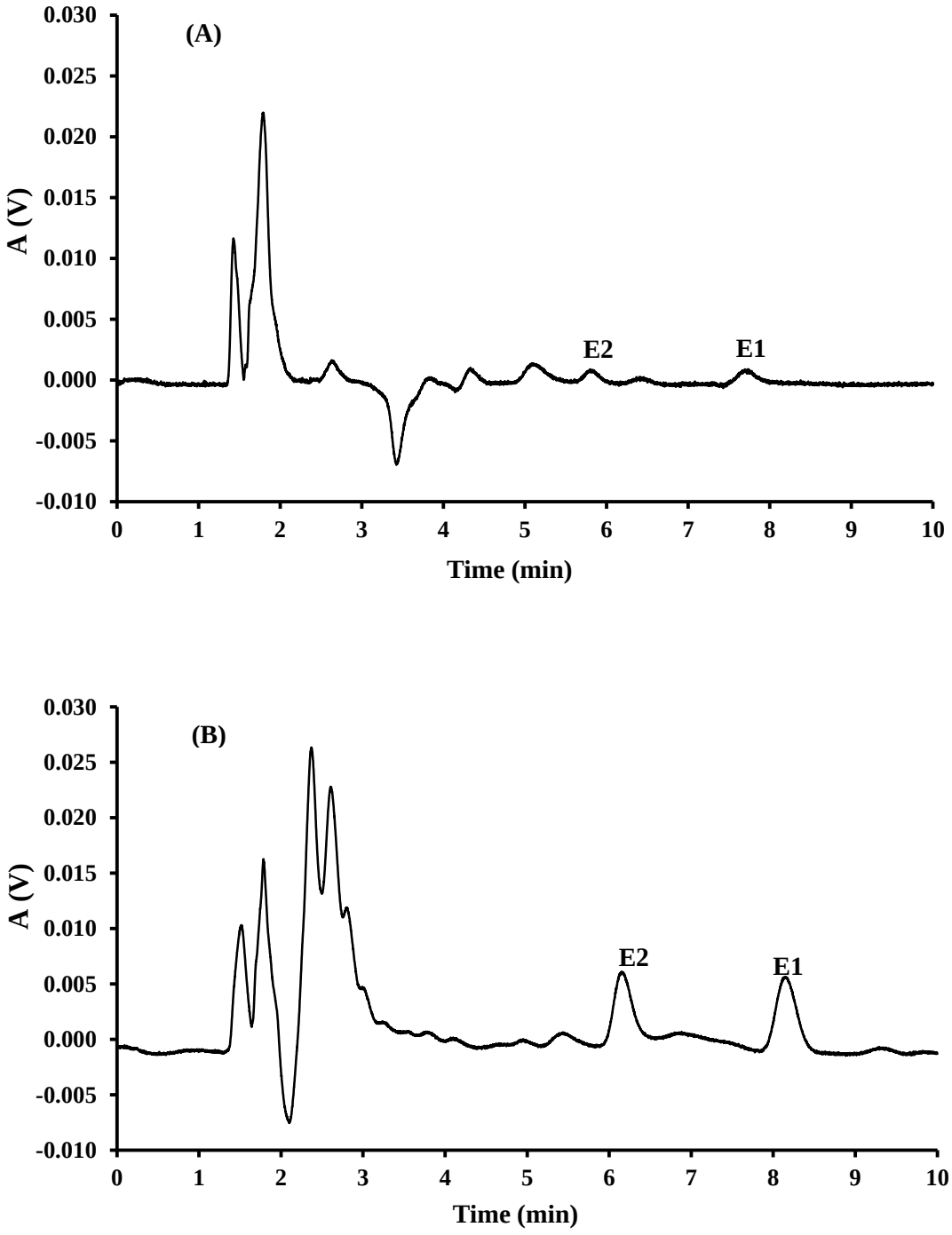


Figure 3.17 Chromatograms of 50.0 ppb estrone and 17β-estradiol (A) without μ-SPE and (B) with μ-SPE.

3.4 Validation of method performance

3.4.1 Linearity

Spiked mixture of estrone and 17β-estradiol in DI water in the range of 5.000 µg L^{-1} – 1.000 mg L^{-1} were extracted by the µ-SPE technique and analyzed by HPLC-UV under the optimum conditions. Five µ-SPEs (n = 5) were used for the extraction of each concentration level. The calibration curve was plotted between the average values of the corresponding peak area as the function of analytes concentration.

The method provided the linearity in the range of 0.025 – 1.000 mg L^{-1} (25 – 1000 µg L^{-1}) for both analytes (Figures 3.18A and 3.18B).

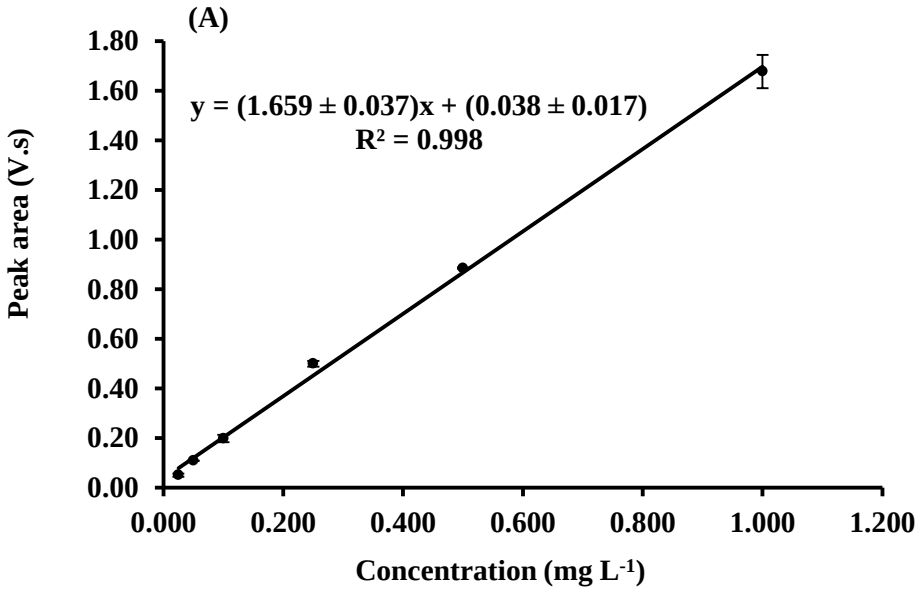


Figure 3.18 (A) Linearity of estrone (E1) from 0.025 – 1.000 mg L^{-1} by µ-SPE technique.

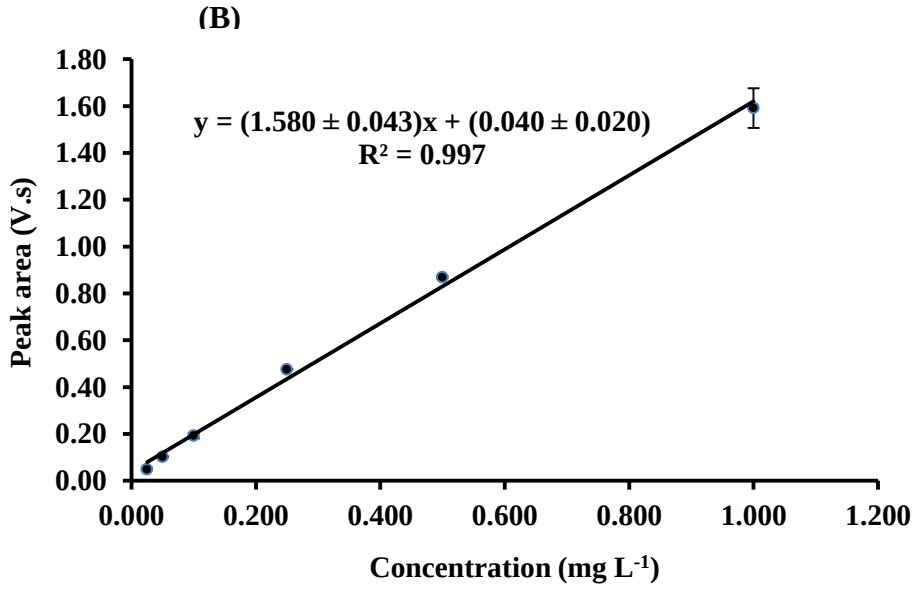


Figure 3.18 (B) Linearity of 17β-estradiol (E2) from 0.025 – 1.000 mg L^{-1} by µ-SPE technique.

3.4.2 Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined using the signal-to-noise ratio (S/N). The LOD (S/N ≥ 3) and LOQ (S/N≥ 10) for both of E1 and E2 were 10 µg L^{-1} and 25 µg L^{-1}, respectively.

However, the LOD and LOQ of this technique are higher than other researches, *i.e.*, 0.10 µg L^{-1} and 0.05 µg L^{-1} (Wang *et al.*, 2013a), 0.014 µg L^{-1} and 0.022 µg L^{-1} (Kanimozhi *et al.*, 2011) for LOD of E1 and E2, respectively. LOQ were 0.042 µg L^{-1} and 0.065 µg L^{-1} for E1 and E2, respectively (Kanimozhi *et al.*, 2011). This is most likely because of the analytical methods, the UPLC-MS-MS (Wang *et al.*, 2013a) and GC-MS (Kanimozhi *et al.*, 2011) that have been used are more sensitive than the HPLC-UV employed in this work. Still, this proposed method can apply for the extraction of estrogens.

3.4.3 Precision

For evaluation the µ-SPE method, precision of the method was investigated every month during the monitoring period to make sure that this method is reliable. This was carried all by spiking the real water sample of each site with three level concentrations, 0.025, 0.100 and 0.500 mg L^{-1}. Each concentration was extracted with five µ-SPE devices (n = 5), then analyzed with HPLC-UV. The %RSD obtained from each concentration in the sample was used to confirm the precision of the method. The results are reported in Tables 3.15 A, B, C, D, E, F, G and H. The %RSD for both of estrone and 17β-estradiol were in the range of 0.78 - 9.3%, 0.10 - 6.7% and 0.11 – 6.3% for 0.025, 0.100 and 0.500 mg L^{-1}, respectively. These values were in the acceptable ranges which are 14.0% for 0.025 mg L^{-1}, 11.0% for 0.100 mg L^{-1} and 9.0 % for 0.500 mg L^{-1} (AOAC, 2012).

Table 3.15 A Precision of the method for estrone at three concentration levels in November 2014 (n = 5)

Sampling site	**Spiked concentration (mg L^{-1})**	**Peak Area of E1 ± SD (V.s)**	**%RSD**
1	0.025	0.0468 ± 0.0038	**8.0**
	0.100	0.197 ± 0.010	**5.1**
	0.500	0.8290 ± 0.055	**0.67**
2	0.025	0.0532 ± 0.0021	**3.9**
	0.100	0.2154 ± 0.0037	**1.7**
	0.500	0.8356 ± 0.0037	**0.45**
3	0.025	0.0524 ± 0.0037	**7.0**
	0.100	0.2013 ± 0.0057	**2.8**
	0.500	0.8387 ± 0.0083	**0.99**
4	0.025	0.0543 ± 0.0022	**4.1**
	0.100	0.20550 ± 0.00020	**0.10**
	0.500	0.841 ± 0.011	**1.3**

Table 3.15 B Precision of the method for 17β-estradiol at three concentration levels in November 2014 (n = 5)

Sampling site	**Spiked concentration (mg L^{-1})**	**Peak Area of E2 ± SD (V.s)**	**%RSD**
	0.025	0.0473 ± 0.0023	**4.8**
1	0.100	0.195 ± 0.012	**6.4**
	0.500	0.7890 ± 0.0071	**0.90**
	0.025	0.0485 ± 0.0045	**9.3**
2	0.100	0.2004 ± 0.0069	**3.4**
	0.500	0.8032 ± 0.0018	**0.22**
	0.025	0.0443 ± 0.0015	**3.3**
3	0.100	0.1962 ± 0.0021	**1.0**
	0.500	0.7987 ± 0.0041	**0.51**
	0.025	0.0471 ± 0.0042	**9.0**
4	0.100	0.2027 ± 0.0026	**1.3**
	0.500	0.817 ± 0.020	**2.5**

Table 3.15 C Precision of the method for estrone at three concentration levels in December 2014 (n = 5)

Sampling site	**Spiked concentration (mg L^{-1})**	**Peak Area of E1 ± SD (V.s)**	**%RSD**
1	0.025	0.0554 ± 0.0032	**5.8**
	0.100	0.2208 ± 0.0071	**3.2**
	0.500	1.230 ± 0.032	**2.6**
2	0.025	0.0518 ± 0.0015	**3.0**
	0.100	0.2219 ± 0.0092	**4.2**
	0.500	1.2306 ± 0.0068	**0.55**
3	0.025	0.0665 ± 0.0032	**4.7**
	0.100	0.215 ± 0.014	**6.7**
	0.500	1.2306 ± 0.0077	**0.63**
4	0.025	0.0571 ± 0.0049	**8.5**
	0.100	0.2162 ± 0.0014	**0.65**
	0.500	1.2343 ± 0.0041	**0.33**

Table 3.15 D Precision of the method for 17β-estradiol at three concentration levels in December 2014 (n = 5)

Sampling site	**Spiked concentration (mg L^{-1})**	**Peak Area of E2 ± SD (V.s)**	**%RSD**
	0.025	0.0543 ± 0.0021	**3.8**
1	0.100	0.2145 ± 0.0011	**0.51**
	0.500	1.113 ± 0.012	**1.1**
	0.025	0.0557 ± 0.0032	**5.8**
2	0.100	0.2186 ± 0.0018	**0.80**
	0.500	1.1154 ± 0.0050	**0.45**
	0.025	0.06067 ± 0.00047	**0.78**
3	0.100	0.2030 ± 0.0067	**3.3**
	0.500	1.1123 ± 0.0043	**0.39**
	0.025	0.0535 ± 0.0038	**7.1**
4	0.100	0.2122 ± 0.0083	**3.9**
	0.500	1.1108 ± 0.0040	**0.36**

Table 3.15 E Precision of the method for estrone at three concentration levels in February 2015 (n = 5)

Sampling site	**Spiked concentration (mg L^{-1})**	**Peak Area of E1 ± SD (V.s)**	**%RSD**
	0.025	0.0472 ± 0.0016	**3.4**
1	0.100	0.2022 ± 0.0048	**2.4**
	0.500	1.1950 ± 0.0054	**0.45**
	0.025	0.0454 ± 0.0035	**7.6**
2	0.100	0.2036 ± 0.0036	**1.8**
	0.500	1.1856 ± 0.0050	**0.42**
	0.025	0.0473 ± 0.0016	**3.3**
3	0.100	0.2066 ± 0.0028	**1.4**
	0.500	1.188 ± 0.023	**1.9**
	0.025	0.04843 ± 0.00070	**1.5**
4	0.100	0.2021 ± 0.0042	**2.1**
	0.500	1.1908 ± 0.0071	**0.59**

Table 3.15 F Precision of the method for 17β-estradiol at three concentration levels in February 2015 (n = 5)

Sampling site	**Spiked concentration (mg L^{-1})**	**Peak Area of E2 ± SD (V.s)**	**%RSD**
	0.025	0.0446 ± 0.0012	**2.7**
1	0.100	0.1939 ± 0.0031	**1.6**
	0.500	1.0374 ± 0.0235	**2.3**
	0.025	0.0441 ± 0.0012	**2.6**
2	0.100	0.1933 ± 0.0043	**2.2**
	0.500	1.0204 ± 0.0011	**0.11**
	0.025	0.0438 ± 0.0016	**3.7**
3	0.100	0.1907 ± 0.0012	**0.61**
	0.500	1.0278 ± 0.0014	**0.13**
	0.025	0.0461 ± 0.0012	**2.5**
4	0.100	0.1933 ± 0.0030	**1.5**
	0.500	1.0372 ± 0.0051	**0.50**

Table 3.15 G Precision of the method for estrone at three concentration levels in March 2015 (n = 5)

Sampling site	**Spiked concentration (mg L^{-1})**	**Peak Area of E1 ± SD (V.s)**	**%RSD**
	0.025	0.0467 ± 0.0023	**4.8**
1	0.100	0.2018 ± 0.0054	**2.7**
	0.500	1.1950 ± 0.0054	**0.45**
	0.025	0.0441 ± 0.0021	**4.7**
2	0.100	0.2039 ± 0.0031	**1.5**
	0.500	1.204 ± 0.026	**2.2**
	0.025	0.04650 ± 0.00070	**1.5**
3	0.100	0.2066 ± 0.0028	**1.4**
	0.500	1.199 ± 0.042	**3.5**
	0.025	0.0481 ± 0.0021	**2.6**
4	0.100	0.2021 ± 0.0042	**2.1**
	0.500	1.2032 ± 0.0044	**0.37**

Table 3.15 H Precision of the method for 17β-estradiol at three concentration levels in March 2015 (n = 5)

Sampling site	**Spiked concentration (mg L^{-1})**	**Peak Area of E2 ± SD (V.s)**	**%RSD**
	0.025	0.04530 ± 0.00070	**1.5**
1	0.100	0.1939 ± 0.0032	**1.6**
	0.500	1.070 ± 0.011	**1.0**
	0.025	0.0445 ± 0.0014	**3.1**
2	0.100	0.1932 ± 0.0044	**2.3**
	0.500	1.0645 ± 0.0074	**0.69**
	0.025	0.0444 ± 0.0010	**2.3**
3	0.100	0.1992 ± 0.0065	**3.2**
	0.500	1.068 ± 0.068	**6.3**
	0.025	0.0460 ± 0.0015	**3.2**
4	0.100	0.1980 ± 0.0053	**2.7**
	0.500	1.071 ± 0.024	**2.2**

3.4.4 Accuracy

The accuracy of the method was investigated by considering the %recoveries from the spiked mixture of estrone and 17β-estradiol standard into four real samples. It was studied every month during the monitoring period. The spiked concentrations were in three levels, 0.025, 0.100 and 0.500 mg L^{-1}. For each concentration, five μ-SPE devices (n = 5) were used. Recoveries were calculated based on the equation 2.2.

The obtained %recoveries for both analytes (Tables 3.16 A, B, C and D) were in the range of 84.4 ± 3.1 % to 116.16 ± 0.90 % which were in the acceptable range of 70% to 120% for the concentration range from 0.025 – 0.500 mg L^{-1} (AOAC, 2012).

Table 3.16 A Recoveries of estrone and 17β-estradiol from spiked water samples with three levels of concentration in November 2014 (n =5)

Sampling site	**Spiked concentration (mg L^{-1})**	**%Recovery of E1 ± SD**	**%Recovery of E2 ± SD**
1	0.025	85.8 ± 6.9	99.0 ± 4.7
	0.100	101.7 ± 5.2	101.8 ± 6.5
	0.500	98.04 ± 0.66	97.75 ± 0.88
2	0.025	97.5 ± 3.8	101.6 ± 9.5
	0.100	110.1 ± 1.9	100.7 ± 3.6
	0.500	98.82 ± 0.44	99.50 ± 0.22
3	0.025	96.0 ± 6.7	92.7 ± 3.1
	0.100	104.0 ± 3.0	102.5 ± 1.1
	0.500	99.18 ± 0.98	98.95 ± 0.50
4	0.025	99.4 ± 4.0	98.7 ± 8.9
	0.100	106.20 ± 0.10	105.9 ± 1.4
	0.500	99.5 ± 1.3	101.2 ± 2.5

Table 3.16 B Recoveries of estrone and 17β-estradiol from spiked water samples with three levels of concentration in December 2014 (n =5)

Sampling site	**Spiked concentration (mg L^{-1})**	**%Recovery of E1 ± SD**	**%Recovery of E2 ± SD**
1	0.025	103.0 ± 6.0	104 ± 3.9
	0.100	95.5 ± 3.1	97.10 ± 0.50
	0.500	100.3 ± 2.6	99.4 ± 1.1
2	0.025	115.9 ± 3.4	106.7 ± 6.1
	0.100	95.9 ± 4.0	98.96 ± 0.79
	0.500	100.33 ± 0.55	99.54 ± 0.45
3	0.025	115.0 ± 5.5	116.16 ± 0.90
	0.100	93.1 ± 6.2	91.9 ± 3.0
	0.500	100.33 ± 0.63	99.27 ± 0.39
4	0.025	106.2 ± 9.1	102.4 ± 7.3
	0.100	93.47 ± 0.61	96.1 ± 3.7
	0.500	100.64 ± 0.33	99.13 ± 0.35

Table 3.16 C Recoveries of estrone and 17β-estradiol from spiked water samples with three levels of concentration in February 2015 (n =5)

Sampling site	**Spiked concentration (mg L^{-1})**	**%Recovery of E1 ± SD**	**%Recovery of E2 ± SD**
1	0.025	104.0 ± 3.5	86.0 ± 2.3
	0.100	89.0 ± 2.1	92.8 ± 1.5
	0.500	99.85 ± 0.45	99.1 ± 2.2
2	0.025	100.1 ± 7.6	85.1 ± 2.2
	0.100	89.6 ± 1.6	92.5 ± 2.0
	0.500	99.06 ± 0.42	97.50 ± 0.11
3	0.025	104.2 ± 3.4	84.4 ± 3.1
	0.100	90.9 ± 1.2	91.30 ± 0.56
	0.500	99.2 ± 1.9	98.21 ± 0.13
4	0.025	107 ± 1.5	89.0 ± 2.3
	0.100	80.0 ± 1.8	92.5 ± 1.4
	0.500	99.50 ± 0.59	99.11 ± 0.49

Table 3.16 D Recoveries of estrone and 17β-estradiol from spiked water samples with three levels of concentration in March 2015 (n =5)

Sampling site	**Spiked concentration (mg L^{-1})**	**%Recovery of E1 ± SD**	**%Recovery of E2 ± SD**
1	0.025	103.8 ± 5.0	88.5 ± 1.4
	0.100	88.5 ± 2.4	91.0 ± 1.5
	0.500	99.21 ± 0.45	99.4 ± 1.0
2	0.025	98.0 ± 4.6	87.0 ± 2.7
	0.100	89.4 ± 1.4	90.6 ± 2.1
	0.500	99.9 ± 2.2	98.87 ± 0.68
3	0.025	103.3 ± 1.6	86.8 ± 2.0
	0.100	90.6 ± 1.2	93.5 ± 3.0
	0.500	99.6 ± 3.5	99.2 ± 6.3
4	0.025	106.8 ± 2.8	89.8 ± 2.8
	0.100	88.6 ± 1.8	92.9 ± 2.5
	0.500	99.89 ± 0.37	99.5 ± 2.2

3.5 Matrix effect

To investigate the matrix effect, the slope of the matrix match calibration curve (spiked estrogens in water sample) was compared with the slope of the standard calibration curved (spiked estrogens in DI water) in the concentration range of 0.025 to 0.500 mg L^{-1} through the statistic test, two-way ANOVA. The result indicated that there was no effect of the interference for both E1and E2 in every sample for each month of the monitoring (P>0.05). Therefore, the standard calibration curved was used for the quantitative analysis. The matrix match calibration curve and standard calibration equation for each month are reported in Tables 3.17 A, B, C and D.

3.6 Qualitative and quantitative analysis of estrogens in water sample

Typical chromatograms of each unspiked sample for the wet and dry season are shown in figure 3.19. Qualitative analysis of estrone and 17β-estradiol was conducted by comparing the retention time (t_R) obtained from the chromatogram of the blank sample to the retention time of the standard solution under the same performance conditions. The results are summarized in Tables 3.18 A and B. As can be seen, none of the two target estrogens was found in the water sample for all sites both wet season and dry season. From the chromatograms for the wet and dry seasons (Figures 3.20 and 3.21), no suspicious signals were obtained in all real samples while an obvious increase in signals were observed in the spiked samples, which were associated with the increasing spiked concentration levels.

The method can be verified for the accuracy and precision by the analysis of the spiked sample at three different concentration levels (0.025, 0.10 and 0.50 mg L^{-1}). The recoveries were in the range of 80.0- 116.16% and the precision (%RSD) was in the range of 0.10-9.0%. According to the results, the applied method under the optimal conditions could be applied with accuracy and precision.

Table 3.17 A The matrix match calibration and standard calibration curve equations of the E1 and E2 in November 2014

Analyte	Spiked sample	Calibration curve equation	R^2
	DI water	y = (1.653 ± 0.016)x + (0.0212 ± 0.0041)	0.9997
	Site 1	y = (1.632 ± 0.031)x + (0.0166 ± 0.0079)	0.9989
E1	Site 2	y = (1.630 ± 0.048)x + (0.028 ± 0.012)	0.9974
	Site 3	y = (1.632 ± 0.028)x + (0.0237 ± 0.0071)	0.9991
	Site 4	y = (1.634 ± 0.032)x + (0.0242 ± 0.0082)	0.9989
	DI water	y = (1.579 ± 0.029)x + (0.0221 ± 0.0073)	0.9990
	Site 1	y = (1.545 ± 0.037)x + (0.0224 ± 0.0094)	0.9983
E2	Site 2	y = (1.559 ± 0.041)x + (0.022 ± 0.010)	0.9980
	Site 3	y = (1.559 ± 0.039)x + (0.025 ± 0.010)	0.9981
	Site 4	y = (1.593 ± 0.039)x + (0.0220 ± 0.0099)	0.9982

Note: Unit of y is V.s and x is mg L^{-1}

Table 3.17 B The matrix match calibration and standard calibration curve equations of the E1 and E2 in December 2014

Analyte	Spiked sample	Calibration curve equation	R^2
	DI water	y = (2.488 ± 0.033)x - (0.0175 ± 0.0083)	0.9995
	Site 1	y = (2.489 ± 0.029)x - (0.0124 ± 0.0073)	0.9996
E1	Site 2	y = (2.482 ± 0.029)x - (0.0109 ± 0.0073)	0.9996
	Site 3	y = (2.487 ± 0.041)x - (0.012 ± 0.010)	0.9992
	Site 4	y = (2.488 ± 0.037)x - (0.0172 ± 0.0094)	0.9993
	DI water	y = (2.249 ± 0.042)x - (0.004 ± 0.011)	0.9990
	Site 1	y = (2.247 ± 0.045)x - (0.001 ± 0.012)	0.9988
E2	Site 2	y = (2.245 ± 0.054)x - (0.006 ± 0.014)	0.9982
	Site 3	y = (2.245 ± 0.052)x - (0.001 ± 0.013)	0.9984
	Site 4	y = (2.247 ± 0.039)x - (0.0038 ± 0.0099)	0.9991

Note: Unit of y is V.s and x is mg L^{-1}

Table 3.17 C The matrix match calibration and standard calibration curve equations of the E1 and E2 in February 2015

Analyte	Spiked sample	Calibration curve equation	R^2
	DI water	y = (2.424 ± 0.031)x - (0.0152 ± 0.0078)	0.9995
	Site 1	y = (2.427 ± 0.039)x - (0.018 ± 0.010)	0.9992
E1	Site 2	y = (2.401 ± 0.047)x - (0.013 ± 0.012)	0.9989
	Site 3	y = (2.412 ± 0.042)x - (0.013 ± 0.011)	0.9991
	Site 4	y = (2.416 ± 0.038)x - (0.0171 ± 0.0098)	0.9992
	DI water	y = (2.094 ± 0.037)x - (0.0005 ± 0.0093)	0.9991
	Site 1	y = (2.093 ± 0.038)x - (0.002 ± 0.010)	0.9990
E2	Site 2	y = (2.051 ± 0.047)x + (0.003 ± 0.012)	0.9984
	Site 3	y = (2.077 ± 0.044)x - (0.002 ± 0.011)	0.9985
	Site 4	y = (2.098 ± 0.036)x - (0.0044 ± 0.0091)	0.9991

Note: Unit of y is V.s and x is mg L^{-1}

Table 3.17 D The matrix match calibration and standard calibration curve equations of the E1 and E2 in March 2015

Analyte	Spiked sample	Calibration curve equation	R^2
	DI water	y = (2.441 ± 0.034)x - (0.0160 ± 0.0086)	0.9994
	Site 1	y = (2.430 ± 0.043)x - (0.017 ± 0.011)	0.9991
E1	Site 2	y = (2.447 ± 0.038)x - (0.019 ± 0.010)	0.9993
	Site 3	y = (2.432 ± 0.035)x - (0.0166 ± 0.0089)	0.9994
	Site 4	y = (2.441 ± 0.039)x - (0.019 ± 0.010)	0.9992
	DI water	y = (2.159 ± 0.016)x - (0.0028 ± 0.0041)	0.9998
	Site 1	y = (2.159 ± 0.028)x - (0.0080 ± 0.0070)	0.9995
E2	Site 2	y = (2.146 ± 0.043)x - (0.003 ± 0.011)	0.9988
	Site 3	y = (2.154 ± 0.023)x - (0.0067 ± 0.0059)	0.9997
	Site 4	y = (2.164 ± 0.019)x - (0.0089 ± 0.0048)	0.9998

Note: Unit of y is V.s and x is mg L^{-1}

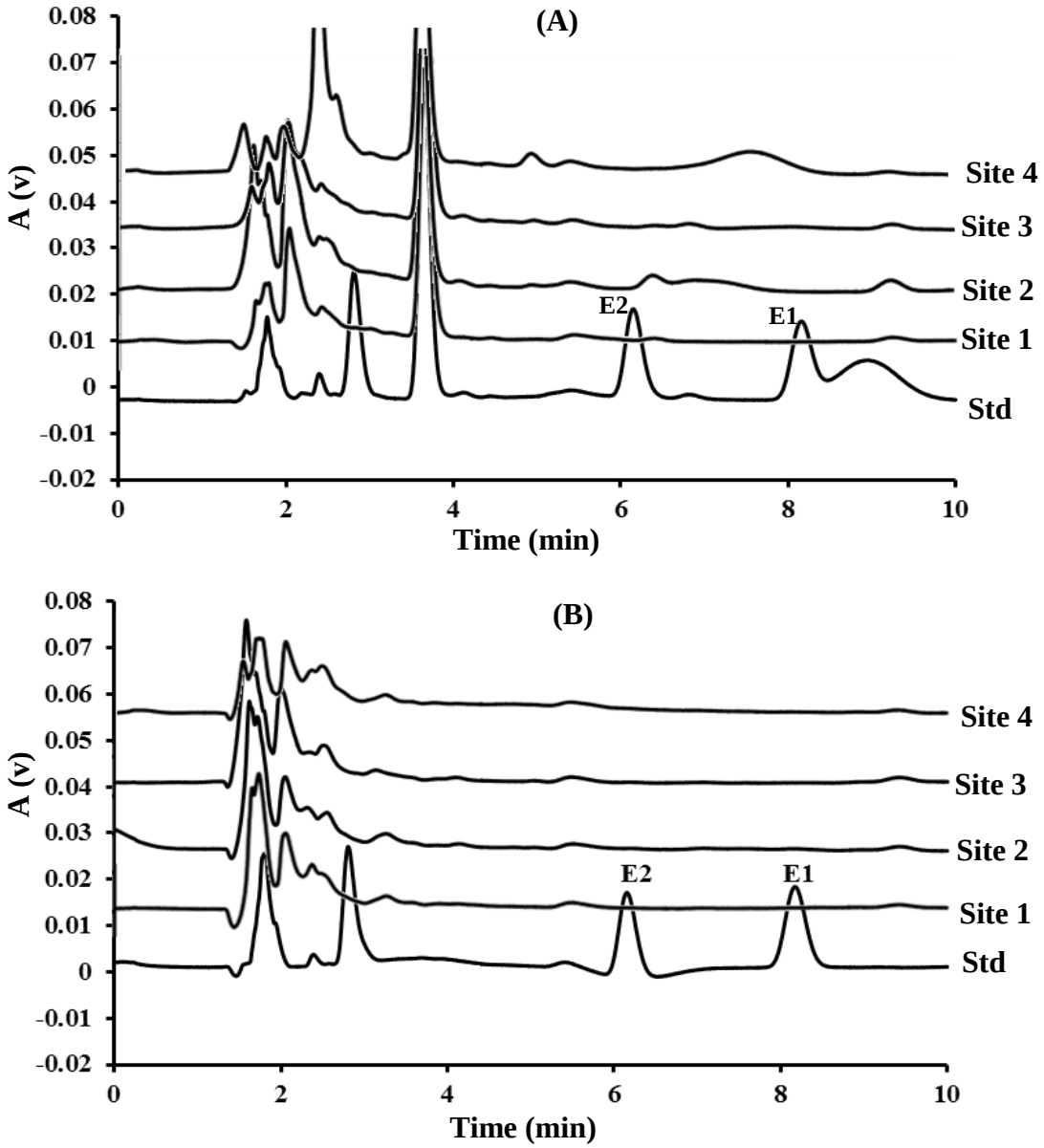


Figure 3.19 Chromatograms of unspiked sample in site 1, 2, 3, 4 and chromatogram of standard estrogens at 1.0 mg L^{-1} in (A) wet season and (B) dry season.

Table 3.18 A The concentration of estrogens in all samples for wet season

Wet season	Sample	Analyte	Found concentration	%Recovery		
				0.025 mg L^{-1}	**0.10 mg L^{-1}**	**0.50 mg L^{-1}**
Nov, 2014	site 1	E1	ND	85.8 ± 6.9	101.7 ± 5.2	98.04 ± 0.66
		E2		99.0 ± 4.7	101.8 ± 6.5	97.75 ± 0.88
	site 2	E1	ND	97.5 ± 3.8	110.1 ± 1.9	98.82 ± 0.44
		E2		101.6 ± 9.5	100.7 ± 3.6	99.50 ± 0.22
	site 3	E1	ND	96.0 ± 6.7	104.0 ± 3.0	99.18 ± 0.98
		E2		92.7 ± 3.1	102.5 ± 1.1	98.95 ± 0.50
	site 4	E1	ND	99.4 ± 4.0	106.20 ± 0.10	99.5 ± 1.3
		E2		98.7 ± 8.9	105.9 ± 1.4	101.2 ± 2.5
Dec, 2014	site 1	E1	ND	103.0 ± 6.0	95.5 ± 3.1	100.3 ± 2.6
		E2		104 ± 3.9	97.10 ± 0.50	99.4 ± 1.1
	site 2	E1	ND	115.9 ± 3.4	95.9 ± 4.0	100.33 ± 0.55
		E2		106.7 ± 6.1	98.96 ± 0.79	99.54 ± 0.45
	site 3	E1	ND	115.0 ± 5.5	93.1 ± 6.2	100.33 ± 0.63
		E2		116.16 ± 0.90	91.9 ± 3.0	99.27 ± 0.39
	site 4	E1	ND	106.2 ± 9.1	93.47 ± 0.61	100.64 ± 0.33
		E2		102.4 ± 7.3	96.1 ± 3.7	99.13 ± 0.35

Note: ND = non detectable

Table 3.18 B The concentration of estrogens in all samples for dry season

Dry season	Sample	Analyte	Found concentration	%Recovery		
				0.025 mg L^{-1}	**0.10 mg L^{-1}**	**0.50 mg L^{-1}**
Feb, 2015	site 1	E1	ND	104.0 ± 3.5	89.0 ± 2.1	99.85 ± 0.45
		E2		86.0 ± 2.3	92.8 ± 1.5	99.1 ± 2.2
	site 2	E1	ND	100.1 ± 7.6	89.6 ± 1.6	99.06 ± 0.42
		E2		85.1 ± 2.2	92.5 ± 2.0	97.50 ± 0.11
	site 3	E1	ND	104.2 ± 3.4	90.9 ± 1.2	99.2 ± 1.9
		E2		84.4 ± 3.1	91.30 ± 0.56	98.21 ± 0.13
	site 4	E1	ND	107 ± 1.5	80.0 ± 1.8	99.50 ± 0.59
		E2		89.0 ± 2.3	92.5 ± 1.4	99.11 ± 0.49
Mar, 2015	site 1	E1	ND	103.8 ± 5.0	88.5 ± 2.4	99.21 ± 0.45
		E2		88.5 ± 1.4	91.0 ± 1.5	99.4 ± 1.0
	site 2	E1	ND	98.0 ± 4.6	89.4 ± 1.4	99.9 ± 2.2
		E2		87.0 ± 2.7	90.6 ± 2.1	98.87 ± 0.68
	site 3	E1	ND	103.3 ± 1.6	90.6 ± 1.2	99.6 ± 3.5
		E2		86.8 ± 2.0	93.5 ± 3.0	99.2 ± 6.3
	site 4	E1	ND	106.8 ± 2.8	88.6 ± 1.8	99.89 ± 0.37
		E2		89.8 ± 2.8	92.9 ± 2.5	99.5 ± 2.2

Note: ND = non detectable

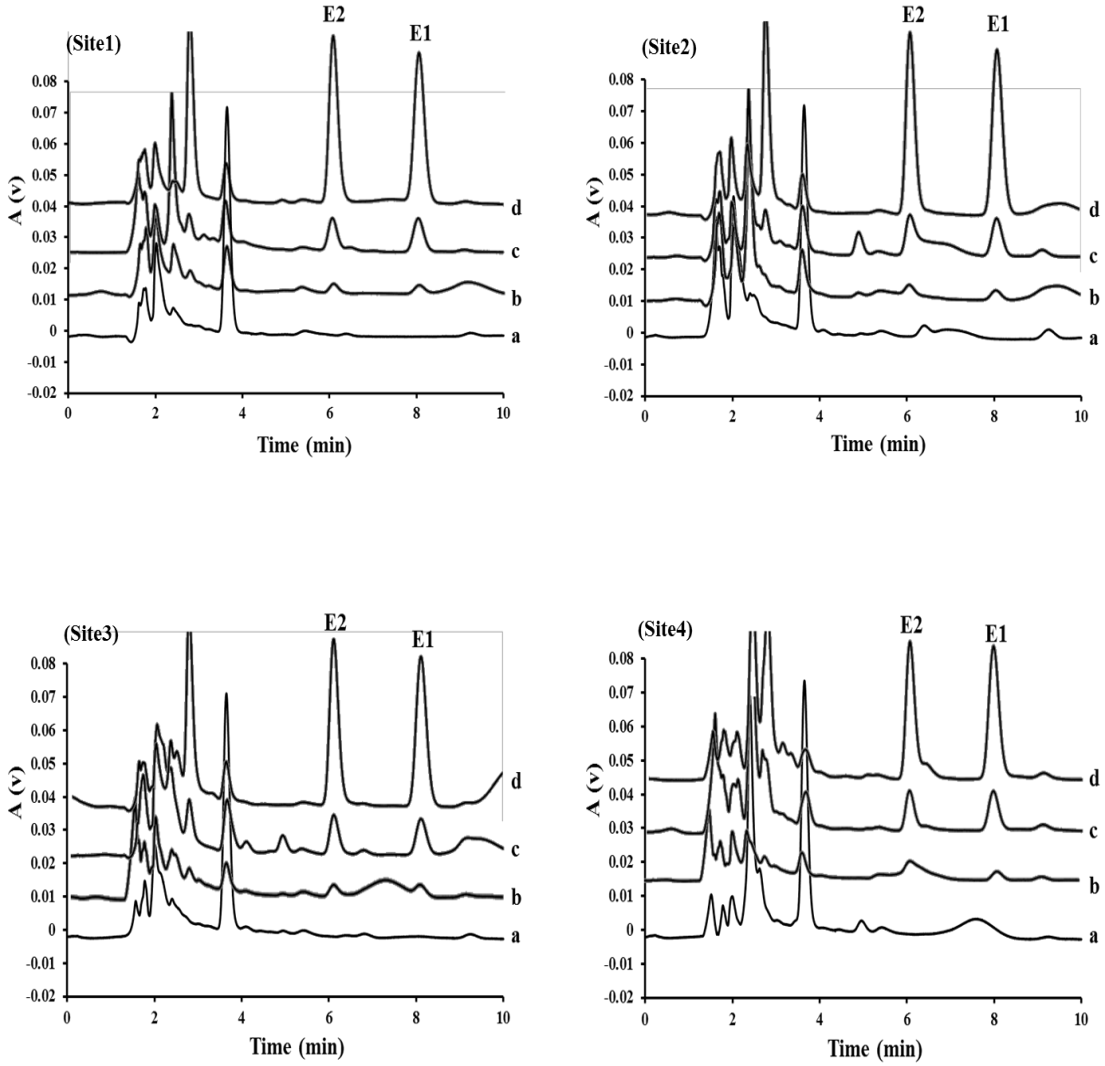


Figure 3.20 Chromatogram of all four sites samples for wet season: (a), (b), (c), and (d) are blank, spiked sample in the concentration of 0.025, 0.10, and 0.50 mg L^{-1}, respectively.

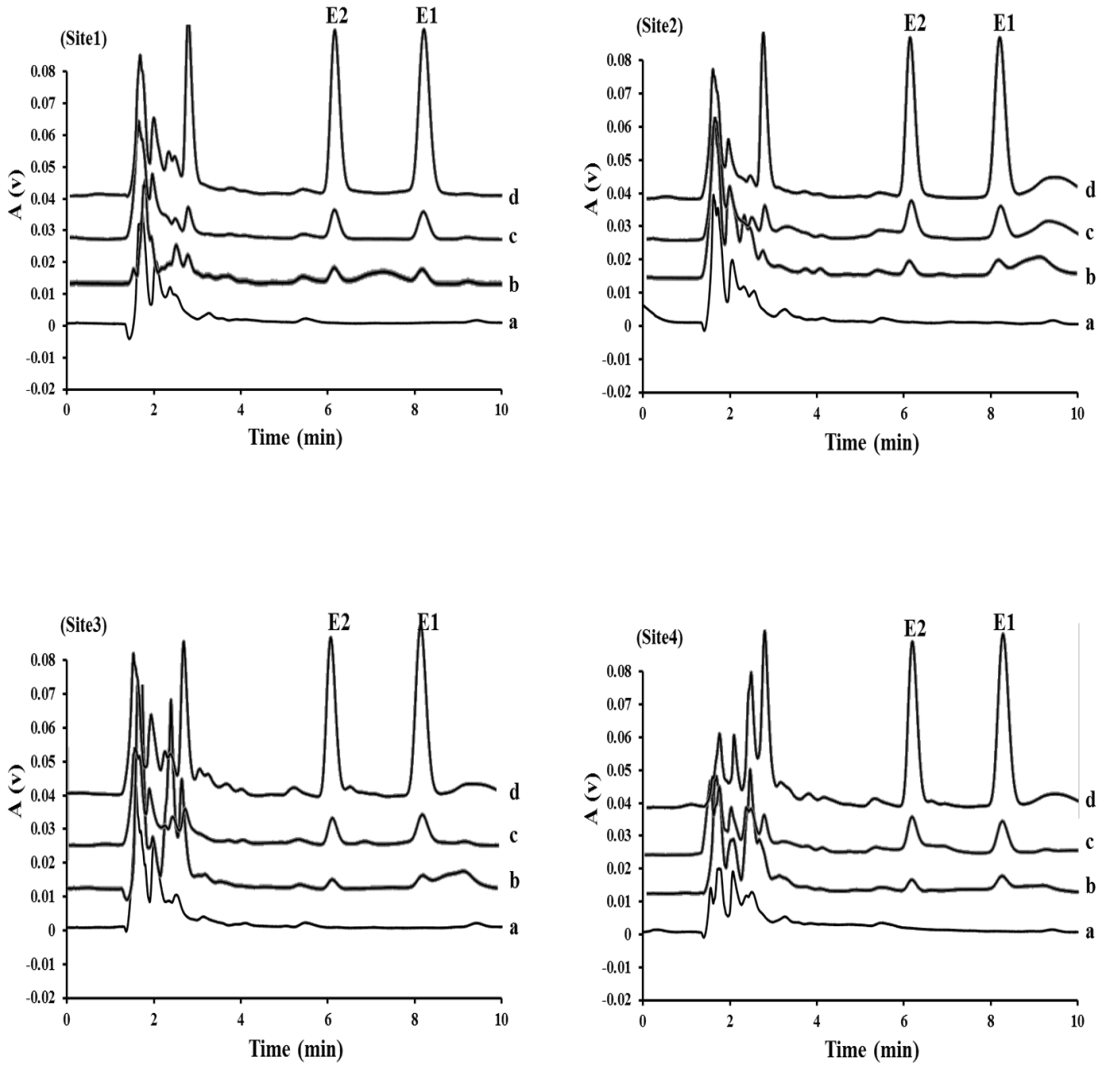


Figure 3.21 Chromatogram of all four sites samples for dry season: (a), (b), (c), and (d) are blank, spiked sample in the concentration of 0.025, 0.10, and 0.50 mg L^{-1}, respectively.

CHAPTER 4

Conclusions

This is the first time a miniaturized polypyrrole filled tea bag was employed for µ-SPE to extract two types of estrogens from water sample. Due to its hydrophobicity, polypyrrole was demonstrated to be a suitable sorbent which could extract the hydrophobic analytes. This method provided the linearity in the range of 0.025 - 1.000 mg L^{-1} (25 – 1000 µg L^{-1}) with R^2 = 0.998 for E1 and R^2 = 0.997 for E2, the limit of detection was 0.010 mg L^{-1} (10.0 µg L^{-1}) and limit of quantification was 0.025 mg L^{-1} (25.0 µg L^{-1}) for both of the analytes. However, LOD and LOQ of this technique are higher than other researches. This is most likely because of the analytical methods (UPLC-MS-MS, GC-MS) that have been used which are more sensitive than the HPLC-UV employed in this work. Still, this proposed method can apply for the extraction of estrogens with good accuracy and precision, the recoveries of three spiked levels were in the range of 80.0 ± 1.8 % to 116.16 ± 0.90 % with RSD of 0.10- 9.0 %. Therefore, this method is hopeful to be utilised as a routine sample preparation way.

For the monitoring of Songkhla Lake, both estrogens (E1 and E2) were not detected either on wet or dry season, the possible reasons could be the absent of estrogens in the lake or they might be present in the lake but with a lower level than the LOD of the method. Therefore, for better monitoring, further improvement could be to couple this extraction method with a more sensitive detector (MS or MS-MS). This may allow a much wider application for the analysis of ultra-trace substances. Sampling sites and sampling time should also be increase to get the representative data.

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