REMOVAL OF 17α-ETHYNYLESTRADIOL (EE2) IN AQUEOUS SOLUTION USING ADSORPTION ONTO PALM KERNEL SHELL (PKS) AND ALGAL BIOREMEDIATION

by

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AS	Activated Sludge
BBM	Bold Basal Medium
BET	Braunauer-Emmett-Teller
BPA	Bisphenol A
CAS	Conventional Activated Sludge
CWA	Clean Water Act
D5	decamethylcyclopentasiloxane
E1	Estrone
E2	Estradiol
E3	Estriol
EDCs	Endocrine Disrupting Chemicals
EDX	Energy Dispersive X-ray Spectroscopy
EE2	17α- Ethynylestradiol
EER	Estrogen Related Receptors
EFBs	Empty Fruit Bunches
EPA	Environmental Protection Agency

FCCD	Face Centred Composite Design
FT-IR	Fourier Transform Infrared
GC	Gas Chromatography
GC-MS	Gas Chromatography with Mass Spectrophotometry
GR	Growth Rate
HPLC - MS/MS	High Performance Liquid Chromatography – Tandem Mass Spectrometry
HPLC	High Performance Liquid Chromatography
HRGC/HRMS	High-Resolution Gas Chomatography combined with High-Resolution Mass Spectrometry
HRT	Hydraulic Retention Time
LC	Liquid Chomotography
LC-ESI - MS/MS	Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection

LOQ	Limit of Quantitation
OD	Optical Density
ODi	Oxidation Ditch
PKS	Palm Kernel Shell
POME	Palm Oil Mill Effluent
RO	Reverse Osmosis
RSD	Relative Standard Deviation
RSM	Response Surface Methodology
SEM	Scanning Electron Microscopy
SPE	Solid Phase Extraction
SRT	Solid Retention Time
STD	Standard Deviation
UV	Ultraviolet Light
WWTP	Wastewater Treatment Plant
YES	Yeast Estrogen Screen

LIST OF SYMBOLS

K_F	Freundlich isotherm constant
Q_0	amount of adsorbate adsorbed by unit mass of adsorbent as complete monolayer
K_L	Langmuir constant
%T	percent transmittance
±	plus minus
°C	degree Celsius
µ	experimental growth rate
µg	microgram
µL	microliter
µm	micrometer
μ_{max}	maximum specific growth rate
1/n	adsorption intensity
Abs	absorbance
$\mathbf{C_0}$	initial solute concentration
$\mathbf{C_e}$	concentration of the adsorbate at equilibrium
cm^3	centimeter cube

$\mathbf{C_t}$	solute concentration at any given time, t
d	day
g	gram
h	hour
K	Kelvin
k	rate constant
K_c	equilibrium constant
kg	kilogram
K_p	distribution coefficient
K_s	saturation constant of substrate
L	liter
lm	lumens
m	meter
m^2	meter square
m^3	meter cube
mAU	mass Absorbance Unit
mg	miligram
min	minutes
ml	mililiter

mm Hg	millimeter of mercury
mol	mole
ng	nanogram
nm	nanometer
pg	picogram
q_e	amount of equilibrium uptake
q_t	amount of solute adsorbed at any given time
R	dimensionless constant separation factor
r^2	Coefficient of Determination
rpm	rotation per min
S	the slope of calibration curve
S_m	concentration of limiting carbon source
T	Temperature
t	time
V	volume of the working solution
W	weight of the adsorbent
wt. %	weight percent
$\Delta G°$	standard Gibbs free energy change
$\Delta H°$	standard enthalpy change

$\Delta S°$	standard entropy change
σ	standard deviation of response

PENYINGKIRAN 17α-ETHYNYLESTRADIOL (EE2) DALAM LARUTAN AKUEUS DENGAN MENGGUNAKAN PENJERAPAN KE ATAS CENGKERANG KERNEL KELAPA SAWIT (PKS) DAN BIOREMEDIASI ALGA

ABSTRAK

17α- Ethynylestradiol (EE2), sejenis kompoun estrogen, bukan sahaja dibuktikan hadir dalam air permukaan, tetapi ia juga terbukti berbahaya kepada hidupan akuatik walaupun pada kepekatan yang amat rendah. Secara umum, matlamat kajian ini adalah untuk mengesahkan kaedah mudah menggunakan kromatografi cecair prestasi tinggi (HPLC) untuk mengesan EE2, menjalankan satu kajian komprehensif EE2 mengenai pengoptimuman dan kinetik penjerapannya ke atas cengkerang kernel kelapa sawit (PKS) dan mengkaji keupayaan bakteria /alga untuk merungkai EE2. HPLC dengan kolum C18 yang biasa, disahkan dapat mengesan EE2 menggunakan asetonitril dan air nyahion pada nisbah 45:55, 1 mL/min dan gelombang pada 280 nm. Sementara itu, eksperimen pengoptimuman PKS menunjukkan penyingkiran EE2 lebih daripada 92% dan 70%, masing-masing untuk serbuk PKS dan butiran PKS. Kedua-dua saiz PKS menurut model kinetik pseudo peringkat kedua dan sesuai dengan isoterma Freundlich dengan nilai regresi (R^2) lebih daripada 0.98. Bagi kajian termodinamik pula, perubahan entalpi standard ($\Delta H°$) bagi serbuk PKS menunjukkan nilai positive pada 12231.56 J/mol manakala butiran PKS menunjukan nilai negetif pada -3505.02 J/mol yang menunjukkan sifat penjerapan eksotermik dan endotermik. Di samping kaedah penjerapan, psikoremediasi, iaitu remediasi menggunakan alga, telah dilakukan dengan menggunakan larutan akueus EE2. Spesies alga, *Ankistrodesmus falcatus* telah didapati berkesan untuk merungkai EE2 lebih daripada 98% bagi semua kepekatan yang diuji iaitu 2 μg/mL, 5 μg/mL dan 10 μg/mL dalam hanya 21 hari. Secara keseluruhan, kedua-dua eksperimen ini menunjukkan keupayaan proses penjerapan untuk mengurangkan masa rawatan dan eksperimen biologi yang mampu merungkaikan pencemaran yang berpunca daripada EE2.

REMOVAL OF 17α-ETHYNYLESTRADIOL (EE2) IN AQUEOUS SOLUTION USING ADSORPTION ONTO PALM KERNEL SHELL (PKS) AND ALGAL BIOREMEDIATION

ABSTRACT

An estrogenic compound, 17α- Ethynylestradiol (EE2) is not only proven to be present in surface water, it has also been proven to be very harmful to aquatic life even at very low concentration. In general, the aims of this research are to validate a simple high performance liquid chromatography (HPLC) method for the detection of EE2, to conduct a comprehensive study and analysis on the optimization and kinetics of EE2 adsorption onto raw palm kernel shell (PKS) and to study the degradation ability of EE2 by bacteria/algae. A HPLC with a common C18 column is validated to be able to detect EE2 using acetonitrile and deionised water at a ratio of 45:55, 1 mL/min and wavelength of 280 nm. Meanwhile, optimization runs for PKS showed a removal of more than 92% and 70%, for powder PKS and granular PKS respectively. Both the sizes of PKS follow pseudo-second order kinetic model and fitted well to the Freundlich isotherm with regression value (R^2) of more than 0.98. In thermodynamics study, the standard enthalphy change ($\Delta H°$) for powder PKS shows a positive value at 12231.56 J/mol while granular PKS shows a negative value at -3505.02 J/mol indicating an exothermic and an endothermic nature of adsorption respectively. In addition to the adsorption method of treatment, phycoremediation which is the remediation using algae, has also been performed on the aqueous solution. A microalgae species, *Ankistrodesmus falcatus*, has been found to be effective to remove more than 98% of EE2 at all tested concentrations of 2 µg/mL, 5 µg/mL and 10 µg/mL in just 21 days. Overall results from these two experimental runs showed the abilities of adsorption process to reduce the treatment time and the biological method that can degrade contamination due to EE2.

CHAPTER 1

INTRODUCTION

1.1 Research Background

In general, all chemicals that has the ability to interrupt the normal function of endocrine system in living organism particularly human are known as endocrine disruptors (EDCs). Commonly known EDCs include pesticides, bisphenol A and estrogenic compounds. Natural occurring estrogenic compounds are estrone (E1), estradiol (E2) and estriol (E3), while synthetic form of estrogenic compound is known as 17α- Ethynylestradiol (EE2). The presence of estrogenic compound, 17α-Ethynylestradiol (EE2) in surface water has long been proven (Cargouët et al., 2004, Koyama et al., 2006, Chen et al., 2007a, Lin and Tsai, 2009, Sim et al., 2011). The source of these pollution is mainly from animals, human excrements, hormone replacement therapy and use of contraceptive pills. The amount of estrogen in general, that are released into the sewage system ranged between 1 ng/L to 10000 ng/L (Cargouët et al., 2004, Hutchins et al., 2007).

Although the concentration of estrogenic compounds in rivers or lakes may be at very low concentration in the amount of few ng/L, they have the capability to wipe out the entire fish population (Pelley, 2003). This is because estrogenic compounds in the environment is able to modify the characteristic of aquatic organism, which may result in decrease in fertility, immature fish and fish feminization (Doyle and Lim, 2002, Tashiro et al., 2003, Woodling et al., 2006, Thompson et al., 2009, Ying et al., 2009, Duong et al., 2010). In fact, estrogenic compounds have three to sevenfold greater estrogenic potencies as compared to general EDCs and their maximum concentration that has been studied to cause no effect is merely 1 ng/L for E2, 3 ng/L

to 5 ng/L for E1 and less than 0.1 ng/L for EE2 that is required to interrupt the life cycle of aquatic organism (Racz and Goel, 2010).

The conventional treatment method used to remove estrogenic compound in general is biological treatment and the most common practice is activated sludge system (Baronti et al., 2000, Joss et al., 2004, Hashimoto and Murakami, 2009). Although this method may be effective, the retention time required is usually very long. For example, in a US livestock farm, degradation of estrogen achieved is 99.8% but retention time required is 8 months (Ren et al., 2007a). Meanwhile in the laboratory runs, treatment methods that has been tested for estrogenic compound removal are membrane filtration, coagulation (Bodzek and Dudziak, 2006), chlorination (Chen et al., 2007a) and iron-reducing bacteria (Ivanov et al., 2010) with removal percentage in the range of 16% to 90%.

However, further confirmation research is required as these studies on EE2 treatment are very limited, making comparison and verification of research data a challenge. Hence, this current research work investigates in detail the EE2 treatment method using both physical and biological methods.

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1.2 Problem Statements

Most analytical methods used in past studies to determine the concentration of estrogenic compounds are namely High Liquid Performance Chromatography (HPLC) (Chen et al., 2007a, Chang et al., 2011), Gas Chromatography (GC) (Lee et al., 2005, Zhao et al., 2010) and vitro bioassay (Pawlowski et al., 2004, Gadd et al., 2010, Li et al., 2011a, Pereira et al., 2011). HPLC-based and GC-based analytical techniques are usually used in combination with mass spectrometry and most of these analyses involve a tedious pre-analysis procedure of solid-phase extraction or liquid–liquid

extraction methods. Till date, there is no established simple standard method that can be used widely for estrogen analysis, thus, making estrogenic analysis a challenge. Hence, there is a need to validate a simple and applicable method that can be applied in all common laboratories.

The presence of estrogenic compounds in surface water is alarming to researchers as its presence will alter the natural life cycle of aquatic organisms, leading to extinction. It is this troubling impact that leads researchers to endeavour into the treatment method to reduce and remove the estrogenic compounds in surface water. Of all the reviewed treatment methods, only physical/chemical treatment with manganese oxide (Xu et al., 2008) and sorption with activated carbon (Bodzek and Dudziak, 2006) seems to record a better efficiency of EE2 removal. Highest possible removal of estrogenic compound is vital as there is yet to be any minimal safe limit that has been established by any regulations. Although adsorption method mentioned is undeniably effective and fast, it does not permanently resolve the estrogenic pollution issue as this method merely transfers the estrogenic pollutants from one place to another. For the estrogenic compound to be completely removed, further treatment is required on the secondary waste of adsorbent. In addition, studies on the behaviour of estrogenic compounds in terms of adsorption and its kinetics that take place, are lacking.

As mentioned earlier, adsorption provides a fast and immediate solution, but, it is not able to fully resolve the issue. Thus, biological treatment would be an alternative option that could provide an answer, apart from the physical treatment. Biological treatment has widely been used in the wastewater treatment field, treating many types of wastewater including dye wastewater (Yu et al., 2001), domestic wastewater (Rawat et al., 2011) and pharmaceutical wastewater (Kang and Kondo,

2002, Joss et al., 2005, Yu et al., 2013). This is because biological treatment has the ability to transform contaminants at the molecular structure level and thus degrading it. Common biological treatment that has been employed to treat estrogenic compounds are usually activated sludge systems (Baronti et al., 2000, Joss et al., 2004, Hashimoto and Murakami, 2009). However, the retention time required for the common biological treatment process that is the activated sludge system may usually take up to 100 days. Whereas, the capability of pure biological culture of either bacteria or algae, are rarely being studied in wastewater containing estrogenic compound.

Although the removal of estrogenic compound is vital, there are only a few research publications that discuss the removal of estrogenic compound from water bodies in detail. Research on the technologies using adsorption in terms of physical treatment, and pure culture remediation by bacteria or algae (phycoremediation) has not been thoroughly conducted.

1.3 Research Objectives

The objectives of this research are;

a) to validate a method to detect 17α- Ethynylestradiol (EE2) using a simple High Performance Liquid Chomatography (HPLC) method.

b) to determine the optimum conditions for adsorption of 17α- Ethynylestradiol (EE2) onto raw Palm Kernel Shell (PKS).

c) to determine the kinetics, isotherms and thermodynamics of the adsorption process.

d) to identify suitable bacteria/algae that can degrade 17α- Ethynylestradiol (EE2) and determine their degradation capabilities.

1.4 Scope of Study

The overall scope of study is shown in Figure 1.1. The scope of this study include validation of a simple HPLC method for the analysis of EE2 that uses a commonly available HPLC without mass spectrometry and as a common C18 column. In addition to that, this study also focuses on the removal of EE2 through adsorption which is the physical treatment, and degradation of EE2 by selected bacteria/algae which is the biological treatment. The physical treatment studied here used raw PKS as the adsorbent to remove EE2. A lab scale batch study was conducted to determine the optimum condition for adsorption as well as the kinetics. No further activation was done on the adsorbent as this research investigates the potential of raw biomass in EE2 removal. Whereas, for the biological treatment, batch studies were done for each selected pure culture of bacteria/algae from the initial preliminary runs. Working solution for treatment of EE2 in the whole experimental were done using synthetic solution that ranged within the detection limit of analysis.

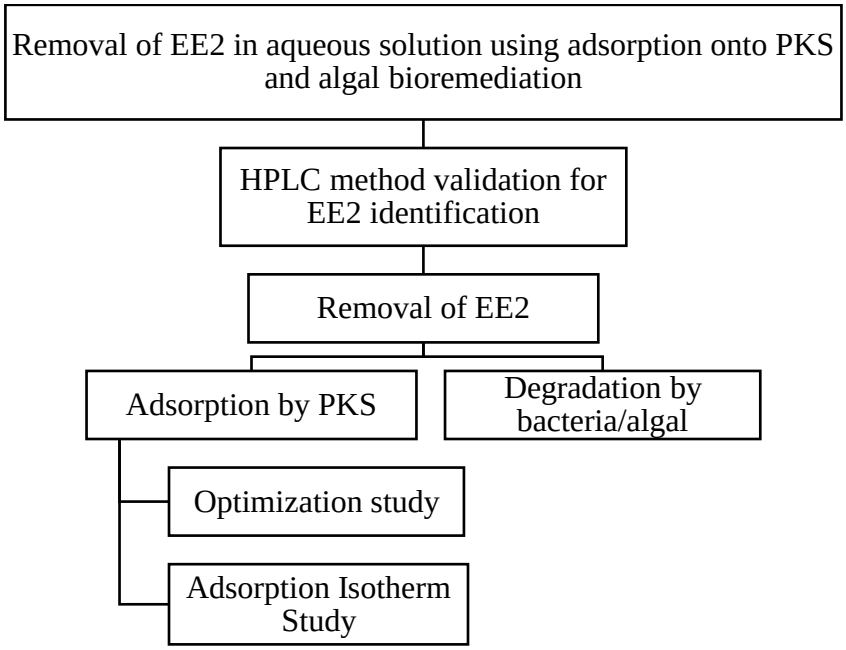


Figure 1.1: Flow diagram for the scope of study.

1.5 Limitation of Study

Concentration used in this experimental work is limited to the detection range of the method developed. Working solution used is also limited to synthetic aqueous solution.

1.6 Thesis Outline

The organization of this thesis is divided into 6 major chapters. Chapter 1 gives an introduction of this research work that includes research background on the pollution due to estrogenic compound, the sources of pollution that caused its presence in surface water and its treatment methods. This chapter is then followed by the problem statement, objective, scope of study as well as its limitation.

Chapter 2 covers the literature review of estrogen hormone as an endocrine disrupter, giving an overall idea on the effect of estrogenic pollutions and its concentration level in water bodies around the world. This is followed by the comparison of methods used to treat estrogenic compound in wastewater and discussion on the availability of regulation for the discharge of estrogenic compounds to surface water. This literature review also discusses on the HPLC method validation, statistical study, kinetic study, equilibrium study and a brief review on bioremediation.

Chapter 3 describes a validation method developed for EE2 determination using HPLC. The HPLC system suitability at different wavelengths, the system sensitivity, including the limit of detection and limit of quantitation, the system accuracy and precision and finally the detection linearity, which is the calibration curve, are all presented in this chapter. This chapter serves as a basis for all analysis on EE2 that follows in this research.

Detailed analysis for the physical treatment of EE2 is outlined in Chapter 4. In addition to the characterization of the adsorbent that is being studied, optimization study, kinetic study, thermodynamic study, isotherm studies and all univariate studies for both the adsorbent sizes used, are presented in this chapter. An overall analysis of this chapter will give a detailed insight on the adsorption process that takes place between the EE2 and the adsorbent.

Chapter 5 on the other hand, outlines the biological aspect of the EE2 degradation. Preliminary studies for several pure culture microorganisms were first to be conducted. This is followed by the studies on the potential of selected algae in degrading EE2. Discussion also covers the growth profile of the selected algae as well as their growth kinetics. Finally, the algae with the highest EE2 degradation is identified.

Finally, chapter 6 gives an overall conclusion with specific recommendations for further work that can be conducted from this current research work.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Interest in surface water pollution by EDCs has significantly increased over the years as the impact of EDCs pollution is undeniably significant. Common EDCs pollutants that are of researcher's interest are pesticides, BPA and estrogenic compounds. More literature evidence on the impact of these pollutant as an exogenous chemical that interferes with hormone action of aquatic life is surfacing (Gore et al., 2014).

Pollution by estrogenic compounds in specific refers to the pollutions caused by natural hormones (E1, E2 and E3), synthetic hormones (EE2 and diethylstilbestrol) and phytoestrogens by plants. Among these estrogenic compounds, EE2 is known to have the highest estrogenic potency.

The outline of this chapter, includes the definition and types of EDCs, further discussion into estrogen hormone in specific, methods of estrogen determination and their concentration that are found present in the surface water worldwide. Comparison of treatment methodologies for EE2 removal and its discharge regulation were also discussed.

Apart from EDCs, this chapter also gives a general background of algae, which is used as the biological treatment for this research. This background discussion includes the algae characteristic and their usage in different type of wastewater. Identification methods for the photosynthetic pigment of algae, which is the cell count, chlorophyll content and optical density is also included in this chapter.

Lastly, a comprehensive discussion of the research gaps in this field of study were done in the last section of this chapter.

2.2 Endocrine Disruptors (EDCs)

Estrogenic Compound is considered as an EDC, which means it has the ability to interfere with the functions of endocrine system of all living organism at a certain concentration (Li et al., 2011a). Natural EDCs are defined as an exogenous agent that interfere with the production, release, transport, metabolism, binding, act, or elimination of the natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes (Kavlock, 1999, Sarmah et al., 2006) such as estrogens and androgens. Apart from that, other EDCs that are present are of anthropogenic origin such as pesticides and detergents (Lucas and Jones, 2006). EDCs have the potential to disrupt the internal mechanism of an organism. Compound of such, could mimic the presence of the natural hormones and thus causing a wrong message transmission. They too block the hormone binding site which is also known as hormone receptor (Palanza et al., 1999), causing communication breakdown within the organism's body system. Having such substance that mimics the hormone in an organism's body will thus prevent the system from producing natural hormone that is required by the organism for sustainability.

The presence of EDCs has already been detected in surface waters and river water (Chen et al., 2007a, Duong et al., 2010). EDCs that have been identified are alkyl phenols, polychlorinated biphenyls, phenolic estrogen mimic such as Bisphenol A and phenolic estrogens (Lin et al., 2008). The adverse health effects do not only affect the organism itself but it also affects its generation to come. The vital estrogenic endocrine disruptors that are found in natural environment are E1, 17β-E2 and EE2. Besides that, nonylphenol, octylphenol, bisphenol A, and phytoestrogen which come under phenolic group is also classified as EDCs (Duong et al., 2010). In fact, the common household

used product such as plastic cups and container, cosmetics and toys contains styrene, phthalates and lead are also classified as an EDC.

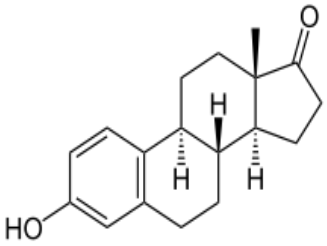
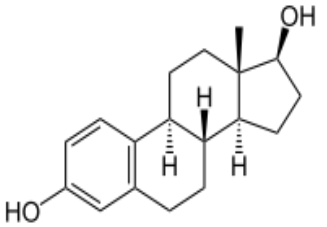
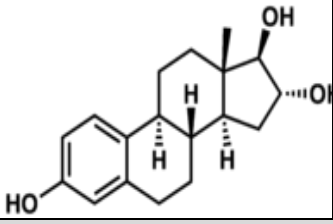
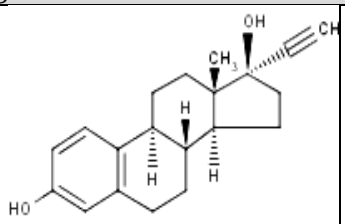
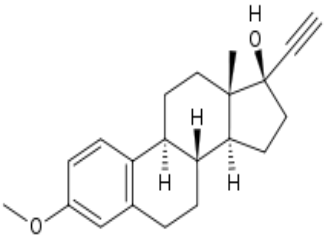
Research attention has focused on the endocrine disruption of the estrogen receptors and estrogen related receptors (EER). It has been reported that a number of artificial chemicals such as bisphenol A, diethylstilbestrol, 4-nonylphenol and some phytoestrogen are able to bind with human EER interfering with our endocrine system (Li et al., 2011a). Estrogenicity of a compound is evaluated by its capability in binding the estrogen receptor and blocks the activity of natural estrogen. Its capability however depends on its size and degree of the alkyl group branching and also their position in the phenol group (Racz and Goel, 2010). Estrogenic compound itself can also be classified to several other classes such as free estrogen, conjugated estrogens, progestogens and phytoestrogens (Kuster et al., 2009).

2.2.1 Estrogen Hormone

The dominating natural hormones in females are E1, E2 and E3 (Snyder et al., 2007). Together they are called estrogen. Estrogen is essential in the development and maintenance of female reproduction system and female physical characteristic. In nature, they may present in conjugated or unconjugated form. Natural occurring hormone such as E1 has a ketone group attached to the D ring, E2 with a hydroxyl group on the D ring while E3 had two hydroxyl groups on the same D ring. The estrogen that are reported to be responsible for the estrogenic activities in effluent and runoff from agricultural activities are usually E1, 17β-E2 and EE2 (Chang et al., 2011).

All steroid hormones, whether naturally or synthetically produced, share a common structure of cyclopentanoperhydrophenanthrene skeleton (Thomas and Colby, 1997). Table 2.1 shows the properties of both natural and synthetic estrogenic

Table 2.1: Properties of estrogenic compounds

Name	Structure	Molecular Weight	Solubi-lity (mg/L at 20°C)	Vapour Pressure (mm Hg)	In vivo vitellogenin response in trout, E2 equivalent*a
Natural Estrogens					
Estrone (E1) $C_{18}H_{22}O_2$		270.4	13	2.3×10^{-10}	0.5
17β-Estradiol (E2) $C_{18}H_{24}O_2$		272.4	13	2.3×10^{-10}	1
Estriol (E3) $C_{18}H_{24}O_3$		288.4	13	6.7×10^{-15}	-
Synthetic Estrogens					
17α – Ethynylestradiol (EE2) $C_{20}H_{24}O_2$		296.4	4.8	4.5×10^{-11}	25
Mestranol (MeEE2) $C_{21}H_{26}O_2$		310.4	0.3	7.5×10^{-10}	-

(Ying et al., 2002, Combalbert and Hernandez-Raquet, 2010) *a(Ying et al., 2009)

compounds including their vitellogenin response, i.e the formation of egg yolk protein, in trout. Natural occurring estrogens are classified under C-18, a group with only 18

carbons. E1, E2 and E3 are all natural estrogen. E1 is an oxidized form of 17β-E2 and is usually present in urine. In the past, intake of estrogens are used to decrease secretion of estrogens in ovary, dermatitis, eye diseases, rheumatoid arthritis, neurosis, prostatic cancer and gastric ulcer (Gorog and Szasz, 1978).

Estrogens are final secretion of ovaries. Beginning with cholesterol and coenzyme-A in blood, progesterone and testosterone are synthesized before being converted to estrogens. Testosterone is converted to β-E2 through aromatase. While some β-E2 is being secreted by the ovarian follicle, some are converted to E3. β-E2 is said to be 10-12 times more potent than E1 and 80 times as potent as E3. Estrogen hormones have very low concentration in plasma with only approximately 10^{-11}-10^{-7} mol/L (Thomas and Colby, 1997).

Estrogen is categorized under steroid hormone. Steroid hormone has five major groups of binding receptors which are glucocorticoids, mineralocorticoids, androgens, estrogens, and progestogens. Steroid hormone works by binding to a receptor on a plasma membrane like a Lock-and-Key Model of hormone receptor to further trigger other biochemical activity (Pétra et al., 2000).

Among the most popularly used synthetic estrogen derivatives in medical are those of C-17 group such as EE2, mestranol and quinestrol. Among the synthetic estrogenic hormones that were synthesized in the past were diethylstilbestrol and hexestrol (Dorfman, 1969). They can be consumed orally and they are an extremely active estrogens (Gorog and Szasz, 1978).

All types of estrogen including the natural or synthetic estrogen are listed as moderately toxic, with 0.5 – 5 g/kg as the probable oral lethal dose for humans. Absorption may take place via oral, percutaneous and respiratory routes. Effect of

estrogen overdose includes headache, nausea, vomiting and vaginal bleeding (Thomas and Colby, 1997).

2.2.2 Sources of Estrogenic Pollution.

The presence of estrogenic compounds in our aquatic systems has been proven by several studies (Sarmah et al., 2006, Snyder et al., 2007, Kuster et al., 2009, Lin and Tsai, 2009, Duong et al., 2010). The concentrations of estrogen released in the sewage system range between 1 ng/L to 10000 ng/L (Cargouët et al., 2004, Hutchins et al., 2007), whereas the efficiency of their removal from sewage treatment only range from 50% to 95%, before being discharged into the river line (Ivanov et al., 2010).

Sources of estrogenic compounds are from contraceptive pills used for birth control, hormone treatments, such as growth promoter, induced abortions, muscle building, estrous cycle of farm animals, and discharges of humans and animals that end up in sewage treatment plants (Ying et al., 2002, Duong et al., 2010, Ivanov et al., 2010). Thus, wastewater treatment plants have become the cumulative center for estrogenic compounds which are subsequently released in water bodies after treatment (Sim et al., 2011).

Discharges from farm animals, such as cow, sheep, swine, and goat, have steroid hormones with a concentration range of 14 ng/g to 533 ng/g dry waste, whereas a typical range of 44 ng/g was reported for E2 (Ying et al., 2002). Excretion of farm animals according to their groups is shown in Table 2.2. The amount and proportion of estrogen excreted by each individual animal varies. Majority of estrogen discharged from cattle are in faeces (58%), whereas that in swine and poultry, the discharge is mostly in urine with 96% and 69%, respectively (Lucas and Jones, 2006, Sarmah et al., 2006). Discharge of estrogenic compounds in all animals also varies at different

stages of their maturity, pregnancy, and lactation. The use of manure fertilizer can also contribute to estrogenic activity in surface water (Thompson et al., 2009) because the half-life of estrogenic activity in manure fertilizer in soils takes up to 5 to 25 days, whereas sheep and cattle manure of different ages will take 7 days to 2 years.

Table 2.2: Excretions of estrogenic hormones by each farm animals per day.

Group	Total estrogen, µg/d
Cattle	
Calves	45
Cycling cows	299
Bulls	540
Swine	
Cycling sows	120
Boars	2300
Sheep	
Cycling ewes	23
Rams	25
Chickens	
Female broilers	0.93
Male broilers	0.19
Laying hens	19.45
Cocks	3.29

(Lange et al., 2002)

Water runoff and leaching also cause contamination of freshwater supply (Lucas and Jones, 2006). In the US, the overall hormone discharge has been estimated at more than 330 tons per year. According to Zhao et. al., 2010, only 0.003% of the total amount of estrogen excreted will eventually end up in rivers. Although animal wastes are often applied in agricultural plantations, the high manure to land ratio often results in their disposal because the waste produced are way above the needs of the plantations. Meanwhile, Table 2.3 shows the amount of estrogenic hormones excreted

by each individual per day, with the pregnant woman producing the highest contribution of estrogen compound to wastewater. The males and menopausal females have the lowest excretion. On average, 10.5 µg/d of E1, 6.6 µg/d of E2, 3.3 µg/d transformation of E1 to E2, and 1 µg/d of EE2 are excreted by humans per individual (Braga et al., 2005).

Table 2.3: Excretions of estrogenic hormones by each person per day are as followed.

Group	E1 (µg)	βE2 (µg)	E3 (µg)	References
Male	3.9	1.6	1.5	(Johnson et al., 2000)
Women	20	5	64	(Racz and Goel, 2010)
Menstruating Females	8	3.5	4.8	(Johnson et al., 2000)
Menopausal Female	4	2.3	1	(Johnson et al., 2000)
Pre-menopausal women	2.66	1.09	5.68	(Chen et al., 2007a, Lei et al., 2009)
Pregnant Women	600	259	6000	(Johnson et al., 2000)

2.2.3 Effects of Estrogenic Pollution.

Several studies on the effects of estrogen to aquatic organisms have been conducted. Estrogens in the environment cause the adaptation of aquatic organisms to the exposure by modifying their characteristics, causing female gonadal phenotype, decrease in fertility, and fish feminization (Doyle and Lim, 2002, Tashiro et al., 2003, Woodling et al., 2006, Thompson et al., 2009, Ying et al., 2009, Duong et al., 2010).

One of the most drastic examples on the effect of estrogen was reported in a study conducted over a three-year period on fathead minnow fish by Pelley, 2003. The study started out with 7000 fish before the addition of EE2; the fish community was

almost completely wiped out after 3 years of study. This phenomenon was due to kidney failure, tissue death in the testes, immature fish with little or no sperm for male fish, and immature egg for female fish (Pelley, 2003). Compared with other EDCs found in wastewater, estrogens have three to sevenfold greater estrogenic potencies. The general prediction of maximum concentration that causes no effect is 1 ng/L for E2 and 3 ng/L to 5 ng/L for E1 (Racz and Goel, 2010).

For humans, the increasing rate of breast cancer and certain anomalies in the reproductive system have been attributed to estrogenic exposure, even at small concentrations (Naz, 1999, Pereira et al., 2011)

2.2.4 Methods in Determining Estrogenic Concentration

Currently, there is no particular worldwide accepted standard to determine the estrogenic compounds concentration in water bodies (Boyd et al., 2003). Most analytical methods used in past studies include HPLC-based (Chen et al., 2007a, Chang et al., 2011), GC-based (Lee et al., 2005, Zhao et al., 2010) and vitro bioassay (Pawlowski et al., 2004, Gadd et al., 2010, Li et al., 2011a, Pereira et al., 2011). HPLC-based and GC-based analytical techniques are used in combination with mass spectrometry. Most of these analyses involve a pre-analysis procedure of solid-phase extraction or liquid–liquid extraction methods. Water sample is extracted into a medium, and then eluted for analysis.

According to the Environmental Protection Agency (EPA), GC-based is the standard procedure for hormone identification under the Clean Water Act (CWA) (U.S. Environmental Protection Agency, 2007). This standard is however, not employed by most researchers because the use of high-resolution GC combined with high-resolution mass spectrometry (HRGC/HRMS) which are required in the standard, is not available

in most research labs. Furthermore, GC is not preferable as most of the pharmaceutical metabolites are thermolabile (Robinson et al., 2007) and application of GC require additional steps where analytes of interest must be first extracted before injecting into the GC (Murtagh et al., 2013).

Thus, LC-MS and bioassay are the most frequently used methods. LC however, has a broader range of metabolites detection as compared to bioassay, where detection is specified only for target analyte (Murtagh et al., 2013). In addition to these conventional analysis methods, complementary methods such as liquid-chromatography, electrospray, and atmospheric pressure photoionization have been developed to analyze estrogenic compounds (Chen et al., 2009). Table 2.4 shows the estrogenic pollution levels and the methods used for analysis.

Guidelines were established for the validation of the analytical method used for pharmaceutical drugs detection (ICH Expert Working Group, 2005). However, method validation was rarely done as plentiful analytical data is required to fulfil various guideline. Meanwhile, up-to-date, computerised instrument with validated similar results gives an impression of good reliable results (Görög, 2007) ignoring the accuracy of data collected from possible different manufacturer of instrument and parts.

Table 2.4: Levels of E1, E2, E3 and EE2 at different water bodies according to continent.

Asia							
Country	Sample Type	E1, ng/L	E2, ng/L	E3, ng/L	EE2, ng/L	Analysis Method	References
Taiwan	River water	22.4 - 66.2	1.40 – 33.9	12.4 - 73.6	7.53 – 27.4	LC-MS/MS-negative electrospray ionization	(Chen et al., 2007a)
Taiwan	WWTP Effluent	10.2 – 48.6	4.5 – 44.5	ND – 39.1	2.25 – 37.9	LC-MS/MS-negative electrospray ionization	(Chen et al., 2007a)
Taiwan	Hospital Effluent	415	230	-	432	SPE / HPLC – MS/MS – positive electrospray ionization	(Lin and Tsai, 2009)
Taiwan	Pharmaceutical Production Facilities Effluent	115	112	-	-	SPE / HPLC – MS/MS – positive electrospray ionization	(Lin and Tsai, 2009)
Japan	Sewage Treatment Work	0.39-10.49	1.35 - 9.05	-	0.59 - 6.56	SPE/ HPLC-MS – negative electrospray ionization	(Song et al., 2009)
Malaysia (Kuala Selangor)	Urban and Recreation Areas	2.4	0.2	-	-	SPE/ Assay	(Koyama et al., 2006)
Malaysia (Kapar)	Adjecent to Coal-fired Power Plant	16.1	5.9	-	-	SPE/ Assay	(Koyama et al., 2006)

Table 2.4: Continued

Country	Sample Type	E1, ng/L	E2, ng/L	E3, ng/L	EE2, ng/L	Analysis Method	References
Malaysia (Sg. Buluh)	Fishing Village	58	3.7	-	-	SPE/ Assay	(Koyama et al., 2006)
Malaysia (Selat Kelang)	Urban	57.3	5.8	-	-	SPE/ Assay	(Koyama et al., 2006)
Malaysia (Sg. Sepang Kecil)	Agricultural and Fishing	10.5	4	-	-	SPE/ Assay	(Koyama et al., 2006)
Malaysia (Sg. Sepang Besar)	Agricultural and Fishing	3.9	2	-	-	SPE/ Assay	(Koyama et al., 2006)
Malaysia (Kuala Lukut)	Agricultural	2.8	2	-	-	SPE/ Assay	(Koyama et al., 2006)
Malaysia (Kuala Linggi)	Agricultural and Aquacultural	6.9	2.1	-	-	SPE/ Assay	(Koyama et al., 2006)
Malaysia (Sabah)	Surface water	6.5	2.3	-	8.6	LLE/ GC-MS	(Duong et al., 2010)
Republic of Korea	Influent Municipal WWTPs	29	17	379	-	SPE/ LC-MS/MS - negative electrospray ionization	(Sim et al., 2011)
Republic of Korea	Effluent Municipal WWTPs	19	-	206	-	SPE/ LC-MS/MS - negative electrospray ionization	(Sim et al., 2011)

Table 2.4: Continued

Country	Sample Type	E1, ng/L	E2, ng/L	E3, ng/L	EE2, ng/L	Analysis Method	References
Republic of Korea	Influent Livestock WWTPs	3650	237	656	-	SPE/ LC-MS/MS - negative electrospray ionization	(Sim et al., 2011)
Republic of Korea	Effluent Livestock WWTPs	164	-	200	-	SPE/ LC-MS/MS - negative electrospray ionization	(Sim et al., 2011)
China	Influent WWTPs	8.7 ± 7.5	1.5 ± 1.5	-	-	SPE/ LC-ESI-MS/MS	(Chang et al., 2011)
China (Beitang River	River Water	23.4	8.69	10.3	10.0	SPE/ GC-MS	(Lei et al., 2009)
China (Dagu River)	River Water	19.7	10.3	12.4	9.45	SPE/ GC-MS	(Lei et al., 2009)
China (Yongding New River)	River Water	10.5	7.26	5.76	3.54	SPE/ GC-MS	(Lei et al., 2009)
Japan (Manko Tidal Flat)	Wetlands	9.2	<1	-	-	SPE/ LC-MS/MS	(Tashiro et al., 2003)
Europe							
Country	Sample Type	E1, ng/L	E2, ng/L	E3, ng/L	EE2, ng/L	Analysis Method	References
France	WWTP Influent	78.8	23.7	313	-	SPE/ LC-MS/MS	(Gabet-Giraud et al., 2010)

Table 2.4: Continued

Country	Sample Type	E1, ng/L	E2, ng/L	E3, ng/L	EE2, ng/L	Analysis Method	References
France (Rhône-Alpes)	Surface Water	0.3	-	-	-	SPE/ LC-MS/MS - electrospray ionization	(Vulliet et al., 2008)
France	WWTP Effluent	8.2	4.2	33.5	-	SPE/ LC-MS/MS	(Gabet-Giraud et al., 2010)
France (Rhône-Alpes)	Ground Water	3.5	1.3	-	3	SPE/ LC-MS/MS - electrospray ionization	(Vulliet et al., 2008)
France (Eysines)	STP	57.8 ± 2.8	4.4 ± 0.8	2.9 ± 0.1	<2.0	SPE/ GC-MS	(Labadie and Budzinski, 2005)
France (Upstream Acheres)	Surface Water	1.1 ± 0.3	1.4 ± 0.6	1.5 ± 0.5	1.5 ± 0.5	SPE/ GC-MS	(Cargouët et al., 2004)
France (Downstream Acheres)	Surface Water	3.0±0.9	3.0±0.6	2.5±0.6	2.9±0.6	SPE/ GC-MS	(Cargouët et al., 2004)
Germany (Bayreuth)	WWTP	2100±1000	2100±900	-	-	SPE/ GC-MS	(Beck and Radke, 2006)

Table 2.4: Continued

Country	Sample Type	E1, ng/L	E2, ng/L	E3, ng/L	EE2, ng/L	Analysis Method	References
Germany (River Neckar)	Effluent of STP	19	5.6	-	1.5	SPE/ YES assay	(Pawlowski et al., 2004)
Germany (River Rhine)	Effluent of STP	1.2	1	-	<1	YES assay	(Pawlowski et al., 2004)
Italy	Condominium Collecting Tank	58	9	62	-	SPE/ LC-MS	(D'Ascenzo et al., 2003)
Italy	Influent STP	44	11	72	-	SPE/ LC-MS	(D'Ascenzo et al., 2003)
Italy	Effluent STP	17	1.6	2.3	-	SPE/ LC-MS	(D'Ascenzo et al., 2003)
Oceania							
Country	Sample Type	E1, ng/L	E2, ng/L	E3, ng/L	EE2, ng/L	Analysis Method	References
New Zealand	Farm Dairy Shed Effluent	100 (10-580)	24 (3-310)	-	85	SPE/ GC-MS	(Gadd et al., 2010)
Australia (South East Queensland)	WWTP Effluent in Ipswich City	29.12±0.54	5.69±0.51	-	1.14±0.32	SPE/ Assay	(Ying et al., 2009)

Table 2.4: Continued

Country	Sample Type	E1, ng/L	E2, ng/L	E3, ng/L	EE2, ng/L	Analysis Method	References
Australia (South East Queensland)	WWTP Effluent in Logan City	21.33±2.06	3.73±0.11	-	0.57±0.02	SPE/ Assay	(Ying et al., 2009)
Australia (South East Queensland)	WWTP Effluent in Brisbane City	25.77±0.41	6.35±0.14	-	1.20±0.04	SPE/ Assay	(Ying et al., 2009)
Australia (South East Queensland)	WWTP Effluent in Beaudesert Shire	17.64±0.58	3.60±0.35	-	0.75±0.03	SPE/ Assay	(Ying et al., 2009)
Australia (South East Queensland)	WWTP Effluent in Gatton Shire	32.17±3.89	4.71±0.09	-	0.71±0.01	SPE/ Assay	(Ying et al., 2009)
Australia (Malabar, Sydney)	STP	54	14	-	<5	SPE/ GC-MS	(Braga et al., 2005)

Table 2.4: Continued

North America							
Country	Sample Type	E1, ng/L	E2, ng/L	E3, ng/L	EE2, ng/L	Analysis Method	References
United State (Oklahomam)	Swine lagoon	9940	194	6290	-	SPE/ GC-MS/MS	(Hutchins et al., 2007)
Canada (Thames River)	WWTP	29.5	8.3	-	-	SPE/ GC-MS	(Lishman et al., 2006)
South America							
Country	Sample Type	E1, ng/L	E2, ng/L	E3, ng/L	EE2, ng/L	Analysis Method	References
Brazil (Rio de Janeiro)	River Water	-	-	3.68	-	SPE/ LC-MS/MS	(Kuster et al., 2009)