DEVELOPMENT OF METHODS FOR DETERMINATION OF CONTAMINATED ESTROGEN AND HEAVY METALS IN THE SAEN SAEP CANAL AND INVESTIGATION THEIR BIOLOGICAL EFFECTS ON HUMAN AND THE ENVIRONMENT

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BY

LALITA SRICHAIMOON

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The objectives of this study are to determine the contamination of 17b-estradiol (E2) and heavy metals in water samples from the Saen Seep canal collected from five sampling areas, including Panfa Leelard Pier, Prasanmit Pier Phatu Nam Pier, Watklang Pier, and Wat Sriboonreung Pier. A pre-treatment methodology of analysis based on High-Performance Liquid Chromatography with ultraviolet absorption detection (HPLC-UV) and Flame Atomic Absorption Spectrophotometry (FAAS) were developed for the determination of E2 and the analysis of heavy metals trace elements, respectively. Then, mutagenic and estrogenic activities of the water samples were investigated with an Ames test and Yeast estrogen screen assay (YES assay), respectively. The determination of the E2 content with HPLC-UV indicated that concentrations of E2 in the water samples collected from Saen Saep canal were in the range of 0.63-11.89 mg/L. Heavy metals (Cd, Cu, Fe, Ni, Pb, and Zn) were determined by FAAS utilizing two methods: the external standard and the standard addition technique. In the first method, there was contamination of Cd, Cu, Fe, and Zn in all sampling areas, while the quantities of Ni and Pb could not be detected. The results of the Ames test demonstrated that E2 in the concentration levels found in the Saen Saep canal did not induce the frame-shift mutations and base-pair substitutions in *Salmonella typhimurium* TA98 and TA100, respectively. For the YES bioassay, the water extracts containing E2 in the concentration range of 4.22-13,347.6 ng/L and raw water in the concentration of 67 ng/L were used for estrogenic activity tests. The results revealed that the estradiol equivalent (EEQ) of E2 extract equals to 4.12 ng/L while the EEQ of the raw water could not be determined. The bioassays demonstrated that the E2 level found in the water collected from the Saen Saep canal did not express the mutagenic and estrogenic activities.

Keyword : Saen Saep canal, Estrogen, 17beta-estradiol, HPLC-UV, Heavy metals, FAAS, Risk assessments, Mutagenicity, Estrogenicity, Ames test, YES assay

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CHAPTER 1
INTRODUCTION

1.1 Background

Pollution from industrial, agricultural, and urban activities has been found to contaminate into natural water sources. This pollution might increase the risk of toxicity to human and organisms in the aquatic environment. Pollutants contaminating in aquatic ecosystems such as estrogens (Adeel, Song, Wang, Francis, & Yang, 2017), polycyclic aromatic hydrocarbons (White, 2002), pesticides (De Souza, da Silva, & Machado, 2006), and heavy metals (Vargas et al., 2001; Wetzel, Wahrendorf, & Peter, 2013) are known to have damaging toxic, mutagenic and genotoxic effects on aquatic biota and human. In this research, estrogens and heavy metals are selected for determination of a risk to human health.

Estrogens are hormones that are biologically active and are synthesized from cholesterol. They help regulate reproduction, cognitive behavior, bone strength, successful pregnancy, cardiovascular function, and gastrointestinal systems. In humans and animals, estrogens are released by the placenta, ovary, testes, and adrenal cortex (Adeel et al., 2017). Steroid estrogen can be classified into two groups. The first one is natural estrogens, for example, estrone (E1), 17β-estradiol (E2), and estriol (E3). The other is synthetic hormones such as diethylstilbestrol (DES) and 17α-ethinylestradiol (EE2). Both natural and synthetic steroid estrogens can be released from wastewater treatment plants, hospital effluent, and effluent from livestock feedlots, which can contaminate natural water sources. They can be detected in surface waters, soil water, groundwater, and runoff water from agricultural sites (Adeel et al., 2017). Many prior studies have found evidence that estrogen-polluted waters cause fish to develop abnormally. If concentrations of natural and synthetic estrogens increase, they will affect male fish by reducing testes size (Arnold, Brown, Ankley, & Sumpter, 2014; Tetreault et al., 2011), impact reproductive fitness (Rose, Paczolt, & Jones, 2013), lowering the number of sperms, and supporting the production of vitellogenin (Kidd et al., 2007).

Furthermore, EE2 caused a significantly reduced number of fish in the water resources and disrupted the aquatic food chain (Hallgren et al., 2014). Estrogens that accumulate in the environment and enter the food chain can raise the risk of cancer and cause cardiovascular disease in human (Wocławek-Potocka et al., 2013). For example, incidences of breast cancer (Moore et al., 2016) and prostate cancer (Nelles, Hu, & Prins, 2011) are related to estrogen concentration levels.

The other pollutants contaminated in water sources and representing human biotoxic effects is heavy metals. Heavy metal is classified as an element with 4-5 time specific gravity to water at the same temperature and pressure (Duruibe & Egwurugwu, 2007; Meade, 1995) and is generally dangerous even at low concentration (Lenntech, 2004). Heavy metals comprise lead (Pb), cadmium (Cd), zinc (Zn), mercury (Hg), arsenic (As), silver (Ag), chromium (Cr), copper (Cu), iron (Fe), and platinum group elements. Heavy metals can be released into the environment through both natural and anthropogenic sources. The anthropogenic sources are major causes of heavy metals emission such as mining operations (Battarbee et al., 1988; Hutton & Symon, 1986; Nriagu, 1989). The released heavy metals are still contained in the environment, even long after mining operations have terminated. Moreover, surface water can receive heavy metals from municipal waste, industry, and non-point sources from both agricultural and urban areas, which could enter the water resources via leaching (Hussain, Husain, Arif, & Gupta, 2017). Heavy metals easily enter and accumulate in the food chain, accumulating until hazardous amounts are reached. These might potentially kill fish, birds, and mammals (Abah, Ubwa, Onyejefu, & Nomor, 2013). In living organisms, heavy metals can impact organelles and components of cells, such as the lysosome, mitochondria, endoplasmic reticulum, cell membrane and nuclei. Heavy metals have also been reported to damage some enzymes presented in metabolism, detoxification, and damage repair (Wang & Shi, 2001). Moreover, there are many evidences showing estrogenicity (biological responses in exposed organisms similar to those produces by estrogen) of heavy metals such as interact with steroid receptors, replace DNA binding domain (Predki & Sarkar, 1992). Many countries in the world have

to experience metal contamination in water bodies and affecting a huge number of people.

In this research, the Saen Saep canal was used as a model for human and environmental risk assessments in water source. This is because the Saen Saep canal is one of Bangkok's important drainage arteries surrounded by hospitals, restaurants, markets, hotels, condominiums, department stores, and homes. Therefore, this canal has been heavily polluted. However, determination of pollutants in the Saen Saep canal has not been reported. Additionally, there was no study on the adverse biological effects of pollutants contaminated in the Saen Saep canal. To perform human and environmental risk assessments, chemical analyses and biological tests are utilized for the analytical detection of pollutants in the environment. In this study, water samples were collected from 5 points along the canal (Panfa Leelard Pier, Prasanmit Pier, Phatu Nam Pier, Watklang Pier and Wat sriboonreung Pier) which have collected waste waters from city including hospitals, restaurants, markets, hotels, condominiums, department stores, and homes. A sample preparation method was developed by optimization the pre-concentration and extraction procedures applicable for E2 and heavy metal determination. The amount of E2 and heavy metals (Cd, Cu, Fe, Ni, Pb, and Zn) were determined by using High-Performance Liquid Chromatography (HPLC) and Flame Atomic Absorption Spectrophotometry (FAAS), respectively. Then, one heavy metal inducing mutation and found in the water samples was selected for the Ames test to investigate the mutagenic activity of such heavy metal. For the E2, mutagenic and estrogenic activities were investigated using Ames test and Yeast estrogen screen assay (YES assay), respectively. The results from chemical analyses and biological tests were employed to diagnose the relation between the concentration of pollutes and their adverse biological effects.

1.2 Objectives of the study

1. To optimize the determination method and determine the concentration of E2 and heavy metals contaminating in the Saen Saep canal.

2. To determine the overall mutagenicity and estrogenic activity of E2 and heavy metals of water samples in Saen Saep canal by Ames test and YES assay.

3. To interpret the relationship between biological effects (mutagenic and estrogenic activities) and concentration of pollutants (E2 and heavy metal).

1.3 Significance of the study

The Saen Saep canal is a canal in central Bangkok, the capital city in Thailand, which has been polluted by wastewaters from buildings and residences beside the canal. Therefore, water samples from the Saen Saep canal were employed as a study model representing water samples from any natural water sources located in the same environment. Among water pollutants, estrogens and heavy metals are increasingly contaminated in the environments, especially in natural water sources. They can be exposed and affected on human health and the environment. However, there is no clearly evidence demonstrating the relation between number of pollutants (E2 and heavy metals) and adverse biological (mutagenic and estrogenic) activities in the water samples. In this work, the amount of E2 and heavy metals in the water samples from the Saen Saep canal as well as their biological effects were investigated. This study will provide the environmental information including (i) quantities of E2 and heavy metals (Cd, Cu, Fe, Ni, Pb, and Zn) contaminated in the water samples, (ii) mutagenic and estrogenic potency of E2, and (iii) estrogenicity of a selected heavy metal. Moreover, these results can be utilized for interpretation of the relation between amount of the pollutants and their biological effects in order to perform the risk assessment on their release into the aquatic environment.

1.4 Scope of the study

In the first part, percent recoveries from various digestion methods were compared in order to obtain an appropriated digestion method used in the determination of heavy metals. Likewise, extraction methods of E2 were compared between solid phase and liquid-liquid extraction techniques. The technique providing

more percent recovery was employed in E2 determination. After that, water samples were collected from various sites of the Saen Saep canal and were prepared using those appropriated digestion and extraction methods. Determination of freely available concentration of E2 was carried out by using HPLC technique. For the heavy metals, concentrations of Cd, Cu, Fe, Ni, Pb, and Zn in sample waters were evaluated by using FAAS.

In the second part, mutagenicity of water samples was detected with the Ames test which is a widely used method for determination of mutagenic activity of the environmental water samples, while estrogenic activity was investigated using the YES assay. Finally, relationship between results from biological tests and chemical analyses was analyzed.

CHAPTER 2
REVIEW AND LITERATURES

2.1 Estrogens

There are many kinds of chemicals that have a negative effect on animals and human health such as food additives, pesticides, and contaminants in the environment. In the last few decade, estrogenic and androgenic compounds have been widely detected in water bodies (Kolok & Sellin, 2008; Matthiessen et al., 2006), and included in a group of endocrine disrupting chemicals (EDCs) (Adeel et al., 2017). In cells, EDCs bind to the estrogenic receptors resulting in abnormal hormone secretion and subsequent diseases (Masuda et al., 2006).

Estrogens, a group of sex hormones, are biological active hormones relating to female attributes in the human body in terms of development and maintenance of woman characteristics. In human and animals, cholesterol is used to constructed the estrogens and mainly found in adrenal cortex, testes, ovary and placenta (Adeel et al., 2017). Steroid estrogens can be classified into two groups, natural and synthetic estrogens. Steroidal estrogens are one of the C-18 steroidal group that have four rings with different configuration of the D-ring (Figure 1) at C-16 and C-17 positions. Figure 1 shows the difference of chemical structures of natural (estrone; E1, estradiol; E2, and estriol; E3) and synthetic (ethynylestradiol; EE2) steroidal estrogens. For example, at C-17 position, E1 has a carbonyl group while E2 a hydroxyl group. E3 has two hydroxyl groups on C-16 and C-17. The OH group at C-17 of E2 can either arrange downward and upward on the structure plane of molecule, resulting in either the α- or β-compound as shown in Figure 2. For the synthetic steroidal estrogens, chemical structure of EE2 derived from the natural estrogen E2 has the hydroxyl and ethinyl groups on C-17 as shown in Figure 1. Diethylstilbestrol (DES), another synthetic estrogen, is rarely used. It is also associated with increased risk of cancer in women, including reproductive tract abnormalities, poor pregnancy outcomes, and infertility (Conlon, 2017).

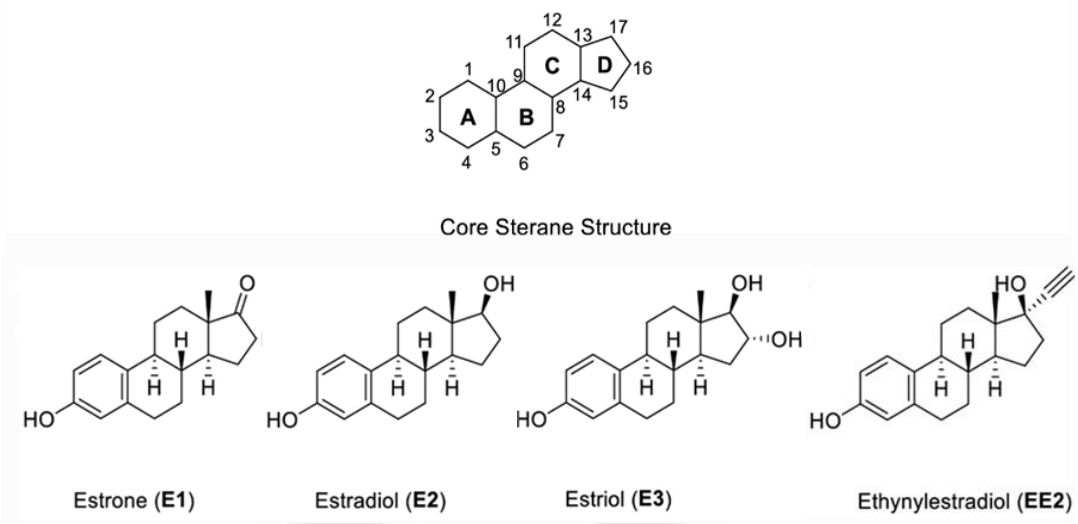

Figure 1 The core sterane structure and structures of estrogens

Source: Silva, Otero, & Esteves. (2012) Processes for the elimination of estrogenic steroid hormones from water: a review p. 38.

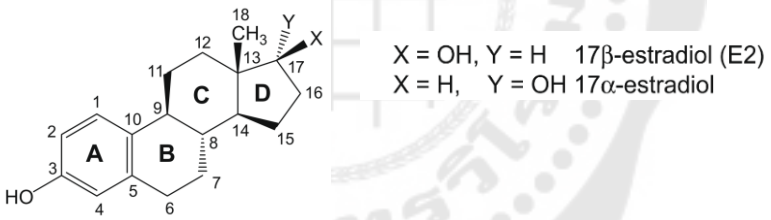

Figure 2 Structure of 17β-estradiol (E2) and 17α-estradiol

Source: Guo, Duclos Jr, Vemuri, & Makriyannis. (2010). The conformations of 17β-estradiol (E2) and 17α-estradiol as determined by solution NMR p. 3465.

2.1.1 Estrogen contamination in the environment

Several studies have shown that both natural and synthetic steroid estrogens can be detected in surface waters (Gall, Sassman, Lee, & Jafvert, 2011), effluents from wastewater treatment plants (WWTPs) (Desbrow, Routledge, Brighty, Sumpter, & Waldock, 1998; Lee & Peart, 1998), hospital waste, and livestock activity (Ying, Kookana, & Ru, 2002). The source of the natural estrogen hormones is the urine of

Figure 3 Major sources of estrogen discharge to the environment. SEs stands for "steroidal estrogens"

Source: Adeel, Song, Wang, Francis, & Yang. (2017). Environmental impact of estrogens on human, animal and plant life: A critical review p. 107.

respectively. In Bangkok, Thailand, steroidal estrogens were detected in influents and effluents of seven WWTPs including Chongnonsri (CNS), Chatuchak (CTC), Sripraya (SPY), Thungkru (TK), Nongkham (NK), Dindang (DD), and Rattanakosin (RKS). The extremely high concentration of the estrogen E2 was observed in all influent samples in the rage of n.d.–12.38 µg/L while E1, E3, and EE2 concentrations were reported in the range of n.d.–2.21, n.d.–2.08, and n.d.–3.76 µg/L, respectively (Ruchiraset & Chinwetkitvanich, 2014). For effluent samples, main estrogen hormones found were E1 and E2 which range from n.d.–1.61 and 0.42–3.96 µg/L, respectively. This indicates that steroidal estrogens cannot be totally eliminated in biological wastewater treatment plants. Likewise, samples collected from surface waters near WWTPs displayed high level of E2. Steroidal estrogen determination in surface water from Bang-sue canal, which receive water from CTC-WWTP, found the high level of E2 at concentrations of 1.40 and 1.86 µg/L in upstream and downstream, respectively. Moreover, from Chaopraya River (receiving water from CNS-WWTP), E2 concentration in upstream was also lower than that in downstream samples and higher than E1, E3, and EE2. However,

researchers did not conclude that increase of downstream E2 concentrations caused by WWTPs because the increase was too small.

2.1.2 Effects of estrogen on domestic animals and human health

Evidences showing the effects of steroidal estrogens on aquatic ecosystems at concentrations detected in the environment have increased, especially for E2 and EE2 frequently found in WWTP effluents (Kunz, Kienle, Carere, Homazava, & Kase, 2015). Several studies demonstrated that natural and synthetic estrogens contaminated in water can feminize male fish. This feminization can be caused even concentration as low as 0.1 ng/L of steroidal estrogens (Purdom et al., 1994). Study in the Grand River watershed in Ontario, Canada, receiving water which is a mixture of domestic and industrial wastes showed that sewage exposed male fish resulted in reducing the capacity to produce testosterone (Tetreault et al., 2011). The research also demonstrated the occurrence of intersex of male fish at the downstream agricultural region. Investigation of relationship between EE2 concentrations and reproduction found that low concentrations (2.0 ng/L) of EE2 exposed in Gulf pipefish enhanced female reproduction but higher EE2 concentrations (5.0 ng/L) caused Gulf pipefish males failed in complete reproduction (Rose et al., 2013). Municipal wastewaters are known as a complex mixture containing estrogens and estrogen compounds. Kidd et al. studied the reproductive health of wild fishes by monitoring vitellogenin (VTG, a protein synthesized during oocyte maturation in females) (Kidd et al., 2007). Results showed that male fishes inhabit at downstream of municipal wastewater outfalls produce VTG. This feminization has been attributed to the presence of E2 and EE2 in the water. However, there was microsatellite field study on fish which revealed that fish can eliminate the EE2 effects by observing the increase of fish population (Blanchfield et al., 2015). This indicates that the steroidal estrogen E2 is the major cause of estrogenic effects and reduction in population in fish found in wastewater receiving waters.

In biology and physiology, estrogen hormones are classified as important hormones for human body relating to many system operations. They are responsible for

supporting regulate reproduction, bone health, function of heart and blood circulates, intellectual behavior, successful pregnancy and gastrointestinal systems. However, the hormones can affect to human health when above safety level of 0.3 mg/day (equivalent to 5 μg/kg bw/day, bw = body weight) is entered into human body according determined the NOAEL (No-observed adverse-effect level) (Jeong, Kang, Lim, Kang, & Sung, 2010; Plotan, Elliott, Frizzell, & Connolly, 2014). The value of this safe level is still disputable subject, various acceptable values have been reported as shown in Table 1. There was determination of the steroid estrogens, E1 and E2 in drinking water treatment works (DWTWs) in China. The study found that E1 and E2 were detected in 53 out of 62 DWTWs and 31 out of 62 DWTWs, respectively (Fan, Hu, An, & Yang, 2013). The maximum detected concentrations of E1 and E2 were 0.1 and 1.7 ng/L, respectively (Fan et al., 2013), It was the first investigation of estrogens in drinking water that may affect to human health.

Table 1 Safe level daily intake for human via food (μg/day)

	E1	E2	EE2	REFERENCE
Adult/60 kg	NDA	3	NDA	Lu et al., 2012
Child/10 kg	NDA	0.5	NDA	Lu et al., 2012
Human/kg bw/day	NDA	5	NDA	Plotan et al., 2014
Men	1	NDA	NDA	Shargil et al., 2015
Women	50	NDA	NDA	Shargil et al., 2015
Adult	NDA	0.0041	0.0028	Wenzel et al., 2003
Infant	NDA	0.0016	0.0011	Wenzel et al., 2003

NDA = no data available

It is acceptable that if steroidal estrogens pollute in the environment and contain in the human food, they can have fatal adverse effects to human health. If the estrogens are accumulated at higher concentration than the safe thresholds, they can increase the risk of breast cancer in women (Moore et al., 2016), prostate cancer in men (Nelles et al., 2011), and increase occurring of cardiovascular diseases in human

(Wocławek-Potocka et al., 2013). Estrogens have been listed as carcinogens in the US National Toxicology Program (Liang & Shang, 2013). However, the predominant intracellular estrogen is E2 which frequently was found in the environment. It prefers to bind with estrogen receptors (ER α and β) in tissues leading to promotion of cell proliferation (or decrease apoptosis) that can induce cancers. Indeed, breast cancer patients are often administered a drug that can prevent this binding. The exact cause is the relation of estrogens in the environment and breast cancer. Therefore, screening of estrogen contamination (together with all EDCs) in water environment is important part in the production of drinking water and to provide clean water to consumers (Gee, Rockett, & Rumsby, 2015). Fernández et al. examined the risk of estrogens found in the environment on breast cancer (Fernández et al., 2004). They measured 16 organochloride pesticides and total xenoestrogen levels in adipose tissue of 198 women which expression of breast cancer diagnosis, and compared to 260 control women without breast cancer. A risk for breast cancer was increased in the leaner women, especially a sub-group of leaner post-menopausal women. More recently, a study showed that environmental estrogens are correlated with progression of breast cancer (Treviño, Wang, & Walker, 2015).

2.1.3 Determination of estrogens

As illustrated in Figure 1, estrogens are a group of polycyclic ring structure chemicals containing a framework of carbon molecules. The most important physicochemical properties of some estrogens are listed in Table 2 (Briciu, Kot-Wasik, & Namiesnik, 2009). Up to now, the applicable methods used to investigate and distinguish natural and synthetic estrogens in water samples are gas chromatography (GC) and HPLC couple with mass spectrometry (MS), following the general principles in Figure 4. MS is the most suitable technique for the detection and quantification of trace estrogens mixture in complex samples simultaneously. These common techniques, therefore, are recognized as the most sensitive, uncomplicated, and reliable instruments in environmental science.

Table 2 Physicochemical properties of steroidal estrogens

Steroidal Hormone	Molecular weight (g/mol)	Melting point (°C)	H_2O solubility (g/L)	Half-life (h)	Hydrophobicity
E1	270.36	254–256	3	19	4.6
E2	272.38	1173–179	3.6	36	3.5
E3	288.39	282	NA	NA	2.3
EE2	296.40	183	0.1	36 ± 13	3.7

NA = Not available

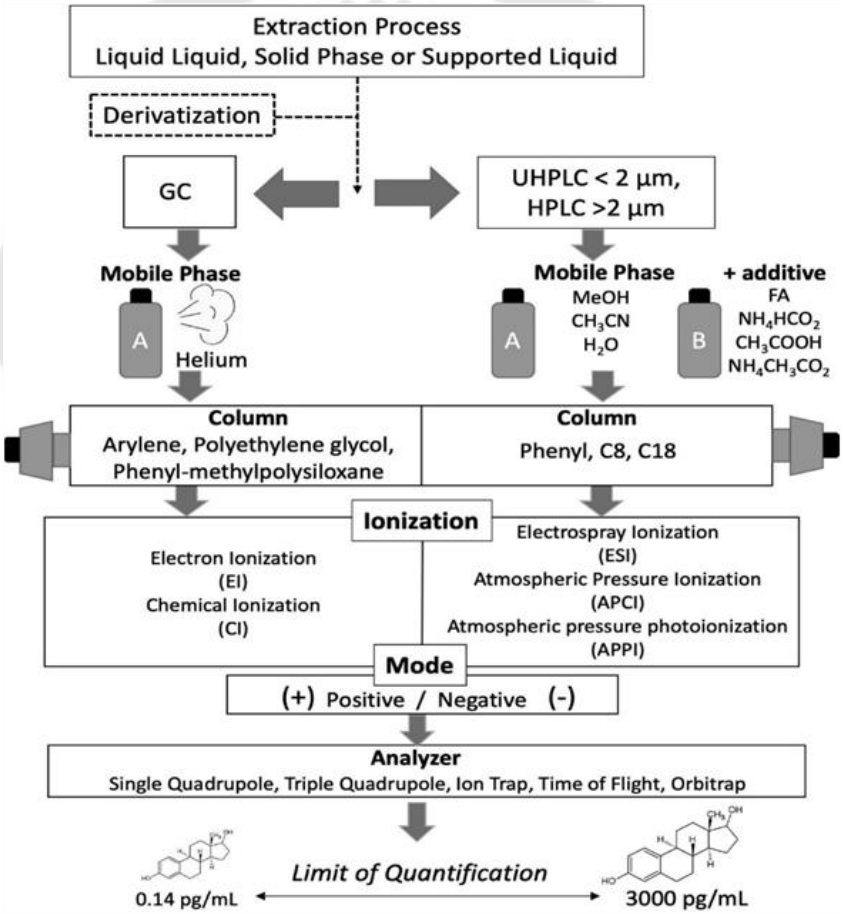

Figure 4 Flowchart for analysis principle of estrogens by GC-MS and LC-MS

Source: Denver, Khan, Homer, MacLean, & Andrew. (2019). Current strategies for quantification of estrogens in clinical research p. 105373.

2.1.3.1 Gas chromatography (GC) method

In GC, an inert gas (usually helium) is employed as the mobile phase with many different injection modes available (Petrovic, Eljarrat, de Alda, & Barceló, 2002). The stationary phase is a viscous liquid on an inert support overlaying on the capillary tubing walls of column which has various types useful for estrogen separation. Columns coating with phenyl groups have be typically applicable as stationary phases for determination of estrogens. During determination process, analytes are firstly vaporized and dissolved into the stationary phase after injected into the tube. Afterward, they are volatilized and derivatized for efficient phase transfer of analytes. On the capillary column, the derivatized steroids are resolved depending on the relative affinities and the temperature gradient of the GC oven, typically using 45–300 °C (Petrovic et al., 2002). The derivatization of the OH group of the steroid structure has the effect on the stability, sensitivity, and precision of GC detection because it can improve volatility and increase the efficiency of thermal decomposition. In couple with the MS analysis, which performs with electron impact (EI) and chemical ionization (CI), negative ion mode was employed for the trace analysis of estrogens at low concentrations in the GC–MS approaches (Prokai-Tatrai, Bonds, & Prokai, 2010). Although GC–MS provides high sensitivity than LC-MS, it takes a long period for detection run (30 min – 60 min), prepare sample with many complicated steps, require high thermal apply, and complex splitting of precursor ions within the MS source. Moreover, disadvantage of using GC is that we should be avoid direct injection of an aqueous matrix because of the decreasing of system and column performance. Thus, reliable and efficient sample preparation steps are required (LaFleur & Schug, 2011). The study demonstrated that using GC–MS/MS cooperated solid phase extraction (SPE) with derivatization agent MSTFA mixture (N-methyl-N-trimethylsilyl-trifluoroacetamide, ammonium iodide, and ethanethiol) was useful for determination of natural and synthetic estrogens in water at trace levels (0.25–5 ng/L), and provided recoveries in water of 105 ± 20%, and R2 $\geq$ 0.94 (Noppe et al., 2007).

2.1.3.2 High performance liquid chromatography (HPLC) method

It has been suggested that the need for direct analysis of complex water samples can be augmented by liquid phase separation methods, involving capillary electrophoresis (CE) and HPLC, which is an analytical technique typically used in many laboratories carrying on trace analysis (LaFleur & Schug, 2011). For the estrogen analysis with HPLC, separation systems compose of octadecyl chemically bonded with stationary phases and aqueous ACN associated to mobile phases (Alda & Barceló, 2001), eventually combined with buffers (Katayama et al., 2007). In operation with the mass spectrometry (MS) detection, a phenyl-based phase couple with a mobile phase based on methanol containing has been suggested for the analysis of E1, βE2, E3 and EE in environmental samples, which correlated to the separation efficiency and the sensitivity of MS detection (Hu, Zhang, & Chang, 2005). On this reason, a similar estrogens can be separated on a bonded stationary phase in a small diameter column (Morishima, Hirata, Jinno, & Fujimoto, 2005).

The advantages of using HPLC couple with MS (knowing as HPLC–MS), involving high sensitivity, specificity, and fast analysis, have been frequently applied in recent research. Efficiency of many types of detectors such as refractive index (RI), ultraviolet (UV), diode array detection (DAD), fluorescence (FL), chemiluminescence (CL), and electrochemical (EC) has been studied for determination of steroid estrogens (LaFleur & Schug, 2011). However, using of MS detector required high cost of analysis run. Alternatives to HPLC-MS, high-performance liquid chromatography operating with UV detector (HPLC-UV) which required lower cost of analysis has been suggested for estrogen analysis. This technique has detection limits applicable to the concentration level of estrogens frequently found in water samples from wastewater treatment plants and natural resources (Liz, Amaral, Stets, Nagata, & Peralta-Zamora, 2017). The limit of detection (LOD) of HPLC-UV technique for estrogen analysis was previously reported in the range of 0.3 to 1.1 μg/L, corresponding to a trace level (low-μg/L) of estrogens in natural waters. Especially, the LOD of HPLC-UV corresponds to the βE2 concentration in

wastewater treatment plant, which has been reported in the level of 1.9 to 2.2 µg/L (Penalver, Pocurull, Borrull, & Marcé, 2002).

2.2 Heavy metal

The term “heavy metals” refers to any metallic elements that their density is higher (at least four to five times) than water density at the same conditions (Duruibe et al., 2007; Ferguson, 1990; Garbarino et al., 1995). Heavy metals express positive charge and are classified into group I to III in the periodic table, including cadmium (Cd), antimony (Sb), arsenic (As), bismuth (Bi), cerium (Ce), chromium (Cr), copper (Cu), gallium (Ga), gold (Au), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), cobalt (Co), nickel (Ni), platinum (Pt), silver (Ag), tellurium (Te), thallium (Tl), zinc (Zn), tin (Sn), uranium (U) and vanadium (V) (Glanze, 1996). Heavy metals are toxic or poisonous even at very low concentration of discharging to the environment (Duffus, 2002; Lenntech, 2004). Up to date, heavy metal contamination in environment is a growing problem and has been concerned based on ecological and global public health. The heavy metals can be released from domestic, agricultural, industrial, and technological activities to aquatic environments. Other source of heavy metals such as geogenic, pharmaceutical, domestic effluents, and atmospheric sources (He, Yang, & Stoffella, 2005). However, the major sources of heavy metals are the mining lands (Battarbee et al., 1988; Hutton & Symon, 1986; Nriagu, 1989). Metals from those sources can release to the surface waters by municipal transportation, industrial wastewater and runoff from agricultural and mining operations. Therefore, it is essential to detect the heavy metals contamination in water bodies, in order to evaluate the risk assessment of environmental hazards and protect entering into food chain. The studies showed that water bodies around mining sites represented high content of heavy metals which exceed standard level for drinking water (Garbarino et al., 1995; Peplow, 1999). The limits of some heavy metals in the environments regarding to the United State Environmental Protection Agency (USEPA) are shown in Table 3 (Duruibe et al., 2007).

Table 3 Maximum concentration for heavy metal in air, soil, and water

Heavy metal	Max. conc. in air (mg/m^3)	Max. conc. in sludge (soil) (mg/Kg or ppm)	Max. conc. in drinking water (mg/l)	Max.conc. in H_2O supporting aquatic life (mg/l or ppm)
Cd	0.1-0.2	85	0.005	0.008δ
Pb	-	420	0.01π (0.0)	0.0058δ
Zn2	1, 5*	7500	5.00	0.0766δ
Hg	-	<1	0.002	0.05
Ca	5	Tolerable	50	Tolerable >50
Ag	0.01	- -	0.0	0.1
As	- -	- -	0.01	- -

(Value in bracket is the desirable limit; πWHO; [1]adapted from U.S. – OSHA; [2]EPA, July 1992; δUSEPA, 1987; Georgia Code, 1993; Florida Code, 1993; Washington Code, 1992; Texas Code, 1991; North Carolina, 1991; *1 for chloride fume, 5 for oxide fume; - - no guideline available)

2.2.1 Heavy metal toxicity

Cell metabolism, cell detoxification as well as cell damage repair systems in organisms, heavy metals and metal compounds have been found to damage organelles in the cells and their components including plasma membrane, lysosome, mitochondrial, endoplasmic reticulum (ER), nuclei in nucleus, and some enzymes (Squibb & Fowler, 1981). Metal ions can directly form ionic and coordination bonds with DNA, nuclear proteins or other cell components. This results in DNA damage and conformational changes and may induce the cell cycle modulation, apoptosis or carcinogenesis (Beyersmann & Hartwig, 2008; Chang, Magos, & Suzuki, 1996; Wang & Shi, 2001). For examples, metals such as arsenic, chromium, cadmium, mercury, and lead which are most toxicity and carcinogenicity, have been known to induce many types of cell damage, even at low contents of exposure. Even though some mechanisms of toxicity and carcinogenicity induced by heavy metals are not clearly defined, each metal have

distinct properties that provide its specific toxicological effect on human health. Contamination of each heavy metal is described as followings.

Cadmium

Cadmium was found to be mainly released during industrial operations such as mining, smelting, pigment manufacture, battery production, stabilizer synthesis, and alloy making. Cadmium can enter the human body through several pathways, including working in metal industries, smoking cigarettes, consuming contaminated food, and working in a workplace contaminated with cadmium (ASTDR, 2008; Davison et al., 1988; Satarug et al., 2003). In food, Cd is present as trace amounts in leafy vegetables, potatoes, seeds and grains, kidney and liver, as well as shrimp, shellfish, and crab (Mascagni, Consonni, Bregante, Chiappino, & Toffoletto, 2003).

Cadmium can severe irritate pulmonary and gastrointestinal, and fatal when inhaled air or ingested food. After acute ingestion within 15 to 30 min, manifestations such as abdominal pain, nausea, feeling of burning, vomiting, muscle cramps, salivation, vertigo, consciousness loss, shock, and convulsions will usually arise (Singhal, Merali, & Hrdina, 1976). Acute poisoning of Cd can also cause erosion in gastrointestinal system, injury in pulmonary, hepatic or renal and coma, depending on the way of ingestion (Singhal et al., 1976; Waalkes & Rehm, 1992). It has been speculated that Cd may be the cause of cell damage through the ROS generation, which results from the single-strand DNA break and synthesis disruption of nucleic acids and proteins (Blom, Harder, & Matin, 1992; Ferianc & Farewell, 1998). In vitro studies showed that free radical-dependent DNA damage and cytotoxic effects can be induced with a trace amount of Cd (0.1 to 10 mM) (Rossman, Roy, & Lin, 1992; Smith, Dwyer, & Smith, 1989). In vivo studies in mice model also illustrated that Cd modulates male reproduction at a concentration of 1 mg to 1 kg of body weight (Thevenod & Jones, 1992). The International Agency for Research on Cancer and the U.S. National Toxicology Program have reported an evidence showing that Cd and Cd compounds are a human carcinogen. This originates from repeated detection of an association

between occupational Cd exposure and lung cancer (ASTDR, 2008). Thus, the lung represents the most target tissue of carcinogenesis in human from Cd exposure. However, there are other target sites of carcinogenesis from Cd in animals such as adrenals, the hemopoietic system, testes, prostate, liver, kidney, hematopoietic system and stomach (Stohs & Bagchi, 1995; Waalkes & Rehm, 1992).

Lead

Lead, a metal with color of bluish-gray, presents in trace quantity in the earth's shell. However, human activities such as fossil fuels flaming, mining, and industrial fabrication releasing of high levels of Pb. Lead is used in many different activities relating to industry, agriculture, and domestic applications, including lead-acid batteries production (83%), ammunition production (3.5%), oxides for paint, glassware, color pigments and chemicals (2.6%), and plate lead (1.7%) in 2004 (Salazar & McNutt, 2012). Human can obtain Pb such as lead-contaminated dust particles or aerosols by inhalation, and lead contaminated in food, water, and paints by ingestion. However, Pb exposure has decreased significantly originating from the use of non-lead gasoline, and the reduce used of lead in the productions of residential paints, cans for food and drink container, and water pipe system (Pirkle et al., 1994; Pirkle et al., 1998).

Lead is the poisonous substance that express adverse effects to many organs in the body involving the kidneys, liver, as well as many systems, for example, central nervous, hematopoietic, endocrine, and reproductive systems (Pirkle et al., 1994). Instantaneous exposure to Pb causes brain and kidney damages, and gastrointestinal diseases, while exposure to lead in long-time may cause negative impact on the central nervous system, blood and blood pressure, kidneys, and vitamin D metabolism (Andrews, Savitz, & Hertz-Picciotto, 1994; Apostoli, Kiss, Porru, Bonde, & Vanhoorne, 1998; Corpas et al., 1995; Flora, Saxena, Gautam, Kaur, & Gill, 2007; Hermes-Lima, Pereira, & Bechara, 1991; Jiun & Hsien, 1994). One of the major mechanisms of Pb in human toxic is that Pb can interact with proteins instead of calcium because it can inhibit or mimic the mechanisms of calcium in human body (Pirkle et al.,

1994). Within the skeleton, Pb can combine to the mineral instead of calcium and form the interaction to biological molecules resulting on the interfering their biological activities. Moreover, lead can also bind to enzymes at the position of sulfhydryl and amide groups leading to interfering the configuration of enzymes and decreasing enzyme activities. Lead may also inhibiting enzyme activity by competition with cations for active sites (Bechara et al., 1993). There are experimental studies that indicate the carcinogenicity of Pb in rats, mice (Lin, Lee, Chen, & Lin-Shiau, 1994; Yang, Wang, Chang, & Liu, 1999) and human (Dipaolo, Nelson, & Casto, 1978). It was found that Pb can induce mutations in genetic coding and exchange in sister chromatid (Roy & Rossman, 1992; Wise, Orenstein, & Patierno, 1993). Moreover, studies in vitro and in vivo have demonstrated that Pb compounds provoke genetic damage through a variety of mechanisms involving inhibition of DNA synthesis and repair, induced DNA breaks, oxidative damage, and interaction with DNA-binding proteins and tumor suppressor proteins (Dopp, Hartmann, Florea, Rettenmeier, & Hirner, 2004).

Zinc

Zinc is a metal element found in all living organisms because it is a necessary element for all living being not exclude human organism. Replication and translation of genetic material require Zn containing proteins and enzymes (Galdes & Vallee, 1983). Human body requires 4 – 10 mg/day of Zinc depending on age and other conditions, such as pregnant women need to obtain Zn up to 16 mg/day. Zinc ingesting into the human body mainly comes from food. Taken zinc in normal dose is not toxic to human being, but excess amount of taken zinc is considered to be toxic as cause impairment of growth and reproduction (Nolan, 1983). The poison of zinc, additionally, has been found to express as same signs as poisoning of Pb (McCluggage, 1991). The expression of zinc toxicosis can be observed in the form of clinical signals such as vomiting, diarrhea, bloody urine, icterus (yellow mucus membrane), liver failure, kidney failure and anemia (Fosmire, 1990).

Copper

Copper is a common element found in nature. Widely use of Cu by human causes the contamination into watercourse such as any industrial activities, i.e. mining and smelting of copper, corrosion of metal-containing substances, production of metal steels, electrical industry, agriculture and settling of sludge in water pipe (Hussain et al., 2017). Copper is necessary for all organisms in trace level because it participates in many metabolic pathways. Copper is also an important nutrient, although only a small amount of copper is essential for good health (Araya et al., 2006). In the body, copper is found approximately 75-100 mg which is the third element of the most abundant trace element (iron, zinc, and copper) (Willis et al., 2005). In human body, Cu can be found in every tissue. Copper is primarily present in the liver, and small amounts found in the brain, heart, kidney, and muscles (Osredkar & Sustar, 2011). Copper is a powerful inhibitor of enzymes. One of many functions of Cu is an enzyme cofactor of many proteins such as ceruloplasmin, cytochrome oxidase, dopamine β-hydroxylase, superoxide dismutase and tyrosinase (Ashish, Neeti, & Himanshu, 2013).

Adult human can endure to copper up to 12 mg daily, while children cannot tolerate that amount. Children are easily be affected from toxicosis of copper. Kidney failure has been reported in young children who were exposed to high copper concentrations (Hussain et al., 2017). Indeed, Cu in metallic state is not poisonous but some of its salts are poisonous. For example, elevated levels of Cu(II) can adverse effects on the kidney and liver resulting in the upset of children and adults (Ashish et al., 2013). Copper toxicity increases in which its level in the kidney raises. Copper begins to accumulate in the liver and interferes with the liver's ability to detoxify the increased levels of copper in the body. Then, it results in negative effect to the nervous and reproductive systems, disrupt the function of the adrenal glands and connective tissue, and decrease the learning ability of the newborn, etc. (Ashish et al., 2013). A maximum content of copper of 2 mg/L in drinking water has been recommended by Swedish that does not cause acute toxicosis in adults (Hussain et al., 2017) BIS 10500 (2012),

however, has suggested that 50 µg/L of copper in drinking water was acceptable; or not exceed 1500 µg/L in case of no alternative source of water that have low quantity of copper (Hussain et al., 2017).

Iron

Iron is the most abundant element found in the earth's shell and expresses the most abundant compared to other heavy metals. Main form of Fe that frequently presents in the environment is Fe(II) or Fe(III) Iron can be generally found in surface waters in the form of salt containing Fe(III) when the pH is more than 7. Iron is a necessary element in human diet because it is an integral composition of cytochromes, porphyrins and metalloenzymes (Hussain et al., 2017).Iron toxicity from ingestion is a common poisoning which is particularly dangerous to infants and children. Toxicity of iron on life-threatening mainly occurs from pediatric taken of potent adult preparations, such as prenatal vitamins. Acute iron ingestion leading to hazard in adults usually arises from suicide attempts (Sane, Malukani, Kulkarni, & Varun, 2018). Ingestion of elemental iron less than 20 mg/kg has no effect on health but ingestion of 20-60 mg/kg results in moderate symptoms. If 60 mg/kg of iron or more was ingested, it can cause acute poison and lead to severe morbidity and fatality (Madiwale & Liebelt, 2006). Additionally, ingestion of Fe is a direct cause of corrosion in the gastrointestinal mucosa, leading to nausea, vomiting, stomach pain, and diarrhea. Significant fluid and blood loss can lead to a decrease in the volume of blood in human body. Hemorrhagic necrosis of the gastrointestinal tract at mucous membrane can decrease local blood flow leading to hematemesis, perforation, and peritonitis. In living cell, Fe poisons metabolism in many tissues such as heart, liver, and central nervous system. Free Fe atoms coming into cells will accumulate in the mitochondria and can cause the cell death through the disruption of oxidative phosphorylation, catalyzes lipid peroxidation, and forming free radicals in the cells (Baranwal & Singhi, 2003).

Nickel

Although many animal species, microorganisms, and plants need to require nickel, obtaining of overdose of nickel up too little or too much can toxic to them (Cempel & Nikel, 2006). The average abundance of Ni in the earth's crust, soils, streams, and groundwater is 1.2, mg/L, 2.5, mg/L, 1 µg/L, and <0.1 mg/L. Typically, main sources of nickel are pyrrhotite and garnierite. However, in some places related to human activities, opportunity to find high level of nickel can arise such as around mining, emission of smelters, burning fuel from coal and oil, sewage, phosphate fertilizers and pesticides (Gimeno-García, Andreu, & Boluda, 1996). Nickel compounds are important and used in modern industry, metal coating, production of batteries in the type of nickel–cadmium, and electronic device production. Nickel mixed to other metals to be nickel alloys are used in the production of tools, machinery, cast coins, jewelry, armaments, appliances, and medical prostheses. These industrial productions are main sources of nickel contamination to the environment, including the recycling of products that contain nickel as well as disposal of nickel-containing wastes. Nickel can leach from rocks and sediments resulting in increasing Ni concentrations in water in both dissolved forms and suspended insoluble particles. In deep-sea water, it usually found that nickel concentration is in the range of 0.1 to 0.5 ppb, while nickel content in surface water is typically reported in the level of 15 to 20 ppb (Friberg, Nordberg, & Vouk, 1979).

The most common nickel toxicity is skin sensitization. Nickel is the most common cause of allergic contact dermatitis (Cavani, 2005; Clarkson, Friberg, Nordberg, & Sager, 2012; Kitaura, Nakao, Yoshida, & Yamada, 2003), and cause of immediate and delayed hypersensitivity (Das & Buchner, 2007). The International Agency for Research on Cancer (IARC) and the U.S. Department of Health and Human Services studied the effect of nickel on employees and laboratory animals and found that all nickel compounds, except for metallic nickel, can induce the cancer in human, therefore, they have been classified as human carcinogens (Health & Services, 1994; Smoke & Smoking, 2004). This was supported by evidence of cancer detection in

nickel-associated workers such as nickel refinery, nickel mining and smelting, nickel alloy manufacturing, stainless steel manufacture, and nickel-cadmium battery production, all of which are connected to nickel in various species (Das, Das, & Dhundasi, 2008). *In vivo* study demonstrated that various nickel species can induce the carcinogenic activity in different level depending mainly on the solubility of the nickel compounds. The compounds with low solubility or not dissolve in water such as NiS, NiO and Ni_3S_2 express high degree of carcinogens because they strongly remain in the tissues while nickel compounds with easily dissolve in water, i.e. $Ni(Oac)_2$, $NiCl_2$, $NiSO_4$, are consider as low carcinogenic potential (Denkhaus & Salnikow, 2002).

2.2.2 Determination of heavy metals

As described previously, toxicosis of heavy metals can occur in the human beings and the aquatic animals even obtain into a body at low concentrations. It is therefore important to accurately determine the concentration of the metals in water samples. Widely used techniques reasonable for determination of heavy metals in a water sample have been reported such as Graphite Furnace Atomic Absorption Spectrometry (GFAAS), Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES), Atomic Emission/Fluorescence Spectrometry (AES/AFS), Inductively Coupled Plasma-Mass Spectrometry (ICP-MS), and Flame Atomic Absorption Spectrometry (FAAS) (Burham, Abdel-Azeem, & El-Shahat, 2009; Helaluddin, Khalid, Alaama, & Abbas, 2016). In this research, FAAS was utilized to quantitatively determine the amount of heavy metals in a water sample because it is inexpensive and simple usage.

In the trace analysis at part per million (ppm) level, FAAS is a good precision technique used to determine heavy metal concentration in a sample. FAAS offers air-acetylene and/or nitrous oxide flame atomizer. Figure 5 illustrates the FAAS diagram. The principle of AAS technique can be described as follows. Electrons in atom are excited from ground state to higher energy states by irradiation of light. After that, electrons will emit the adsorbed energy in the form of light during de-excitation and go back to the ground state. The emitting light of electrons from different atoms has the

different frequencies which are the characteristic of the particular atom. The energy gap between ground and exciting state or transition line of electron is very small leading to high sensitivity of the technique. The process of technique starts from making sample to be atomized by using the atomizer of the instrument. The irradiation of light was measured before applying to the atomized sample and then the radiation flux was measured again after exposing sample to radiation. The absorbance was determined from the ratio of the fluxes before and after sample exposing and used to convert into concentration of analyte from the relationship expressed as the Lambert-Beer law. FAAS technique can be used to measure the concentration of analyte in short period, normally in one sample consuming 10–15 s, and provides very good precision (repeatability) (Harvey, 2009). Moreover, the effect of moderate interferences such as background interference is easily normalized by the application of light scattering. This phenomena is one of the advantages of using flame as thermal energy and the use of deuterium based lamp (Eka, Retno, & Rohman, 2012). FAAS can be applied for the determination of heavy metals in various samples which contain numerous matrices.

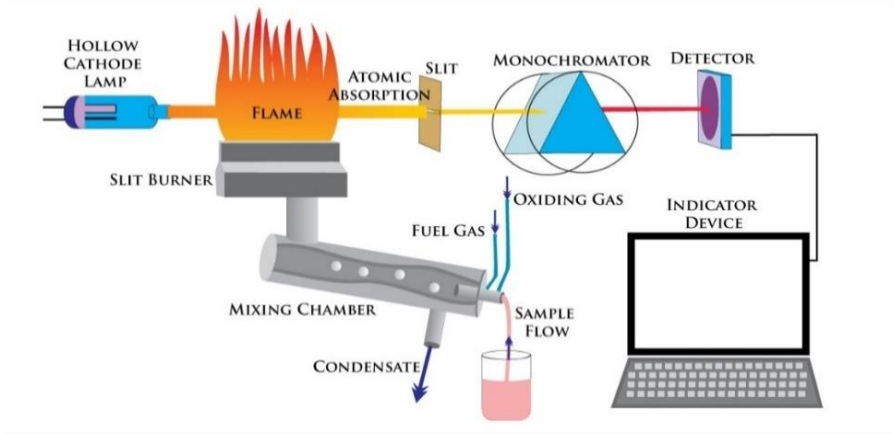

Figure 5 Schematic diagram of an atomic absorption spectrometer

Source: Deepak. (2014). On-line Certificate Program on Atomic Absorption Spectroscopy – Join Now. Retrieved April 20, 2020, from https://lab-training.com/2014/11/28/launch-certificate-program-atomic-absorption-spectroscopy.

2.2.3 Estrogenicity of heavy metals

Heavy metals are not only very poisonous and persistent in nature, but they are also reported to have an endocrine-disrupting potential, causing disruptions in the endocrine system (Zhu, Kusaka, Sato, & Zhang, 2000). Endocrine disrupting chemicals (EDCs) that modulate the action of the female hormone E2 are defined as estrogenic EDCs. Estrogenic EDCs are either hormonal estrogens or chemicals which produce biological responses in exposed organisms similar to those produced by the E2, called estrogenicity (Swart & Pool, 2013). The estrogenicity of heavy metals has been widely investigated and found that the main activities of heavy metal displaying their estrogenic effect are interaction with steroid receptors and interruption the binding of cognate ligands. In 1992, Predki and Sarkar reported the substitution between several metals (such as Cu, Co, Ni, Cd) and zinc in the zinc fingers domain of the DNA binding protein of the human estrogen receptor (hER) (Predki & Sarkar, 1992). Then, *in vitro* and *in vivo* studies have indicated the estrogenicity of Cd that it can travesty the function of estradiol in estrogen-responsive breast cancer cell lines, and can also activates ER-alpha by interaction to the receptor at binding site domain, exerting effects characteristic of the endogenous hormone (Stoica, Katzenellenbogen, & Martin, 2000). This showed that heavy metals try to act as a ligand and bind to the receptors of the target cells, for example, interaction of cadmium, vanadium, chromium, lead, nickel, and copper with the ERa in Mcf-7 breast cancer cells similar to the function of estrogen (Martin et al., 2003). Choe et al. investigated the estrogenicity of numerous heavy metals and their species from proliferation of MCF-7 breast cancer cells (estrogen receptor dependent transcriptional expression assay) and reported the estrogenic activity in terms of relative efficiency respected to E2 as shown in Table 4 (Choe et al., 2003).

Table 4 The estrogenicity (relative efficiency) of various heavy metals

Compounds	Relative efficiency (%)	Concentration
17β-estradiol	100	1 nM
Antimony chloride	60.9	1 μM
Arsenic oxide	5.6	1 μM
Barium chloride	46.8	1 μM
Bis (tri-n-butyltin)	93.5	100 nM
Cadmium chloride	73.8	1 μM
Chromium chloride	46.4	1 μM
Cobalt chloride	3.3	1 μM
Cupric acetate	4.4	1 μM
Cupric chloride	Not reactive	1 μM
Dimethyl mercury	4.2	1 μM
Lead acetate	25.0	1 μM
Lead nitrate	10.5	1 μM
Lithium chloride	7.5	1 μM
Lithium hydroxide	34.6	1 μM
Magnesium chloride	2.0	1 μM
Manganese chloride	9.1	1 μM
MMT	3.8	1 μM
Mercuric chloride	4.4	1 μM
Potassium chromate	5.6	1 μM
Potassium tellurite	Not reactive	1 μM
Sodium molybdate	9.3	1 μM
Sodium selenate	26.0	1 μM
Sodium selenite	7.1	1 μM
Stannous chloride	15.0	1 μM
Titanium chloride	3.1	1 μM
Tungstic acid	Not reactive	1 μM
Zinc chloride	6.6	1 μM

2.3 Estrogenicity and mutagenicity testing

Steroid estrogens and heavy metals constantly pollute in surface waters which increase the risk of toxicity to aquatic animals and human. To investigate the adverse effects of these pollutants, biological tests were developed for demonstration the potential hazard of the contaminants.

2.3.1 YES assay

Yeast estrogen screen (YES) assay is a method widely used in many biological laboratory for investigation of estrogenic activity in environmental samples, *i.e.* river waters, effluents, wastewater and landfill leachates by measuring the activity of estrogen-receptor in yeast *Saccharomyces cerevisiae*-RMY326 (Collins, McLachlan, & Arnold, 1997; Parrella, Lavorgna, Criscuolo, & Isidori, 2013; Spengler, Körner, & Metzger, 2001; Sumpter, 2005). In YES assay, a recombinant yeast strain was developed with a human estrogen receptor gene (hER). Therefore, the yeast strain with hER can establish β-galactosidase from the binding of estrogen response element regulated-expression plasmid (lac-Z) and hER. This β-galactosidase expression is used to determine the receptors' activity (Arnold, Robinson, Notides, Guillette Jr, & McLachlan, 1996; Routledge & Sumpter, 1996). The principle of YES assay starts from transporting estrogen into the cell. Then, estrogen receptors are generated from the response of cell to the entering estrogen. Estrogen will bind to receptors, in which two couples of them can form as a dimer. The estrogen response element can bind to the dimer and induce the transcription of lac-Z mRNA to produce β-galactosidase enzyme. Finally, the enzyme catalyzes a substrate in the culture media to produce more reaction product, which is CPRG (chlorophenol red β-D-galactopyranoside). The expression of CPRG is measured by using a spectrometer, which determines the intensity of the colorimetric response of CPRG at specific light absorbance wavelength peaks (Legler et al., 2002). The estrogenic activity (EA) can be determined from the activity of the enzyme β-galactosidase through a correction for cell density to growth ratio measured

turbidity of media at 600–630 nm and a red product absorbance at 540–550 nm (Fent, Escher, & Caminada, 2006):

$$EA = A_{575(sample)} - (A_{620(sample)} - A_{620(blank)}), \quad (2.1)$$

where, $A_{575(sample)}$ and $A_{620(sample)}$ represent the absorbance of the sample measured at 575 and 620 nm, respectively, and $A_{620(blank)}$ denotes the turbidity of yeast in the assay medium.

2.3.2 Ames test

Several tests are available for the mutation/genotoxicity detection of water samples including water from natural sources and wastewater treatment plants. However, the use of biological analysis with bacteria has proven to be very effective for monitoring due to its high sensitivity, inexpensive cost, reliability, and fast analysis. Among the microbial assays, Ames test is most commonly used to examine the mutagenicity of river waters and sediments (Çakmak & Demir, 2018; Černá et al., 1996; Vargas, Motta, & Henriques, 1993). The test utilizes bacteria *Salmonella typhimurium* to evaluate the ability of DNA mutation in the tested bacteria of a sample. In this test, mutation/genotoxicity can be determined from the number of histidine-independent revertant in selected *Salmonella* strains after exposure to tested chemicals or mutagens as shown in Figure 6. Typically, histidine cannot be synthesized in *Salmonella typhimurium*. However, *Salmonella typhimurium* is responsive to mutations because there are no normal repair mechanisms. The bacteria, therefore, needs to survive on plates lacking histidine by synthesize histidine from the mutation mechanism (i.e., a back mutation or reversion). This reversion can occur by bacteria itself or induce by a mutagen (Rodríguez, Piccini, Sosa, & Zunino, 2012). The mutagenicity of chemicals is determined from a number of colonies expressing a mutation induced from a mutagen comparing to a spontaneous mutation colonies (Rodríguez et al., 2012). In control plate, there are very few numbers of colonies displaying spontaneous mutation on histidine

encoding gene. In this assay, the different strains of *Salmonella typhimurium* were used to detect various type of mutagens. For example, base-pair substitution mutagens were detected in TA100 and TA1535 strains, frameshift mutagens were detected in TA97 and TA98, and oxidative mutagens and intact excision repair mechanisms were monitored in TA102 strain (Vargas et al., 2001). However, the strains TA98 and TA100 are reasonable to be used in the Ames test because they are very sensitive. In other words, strains TA98 and TA100 can respond to many types of mutagenic compounds. From these reasons, strains TA98 and TA100 have been suggested as the basic strains utilized in the bioassay based on DIN38415-4 (1999) and ISO 16240 standards (2002) (ISO13829, 2002; ISO13829, 2000).

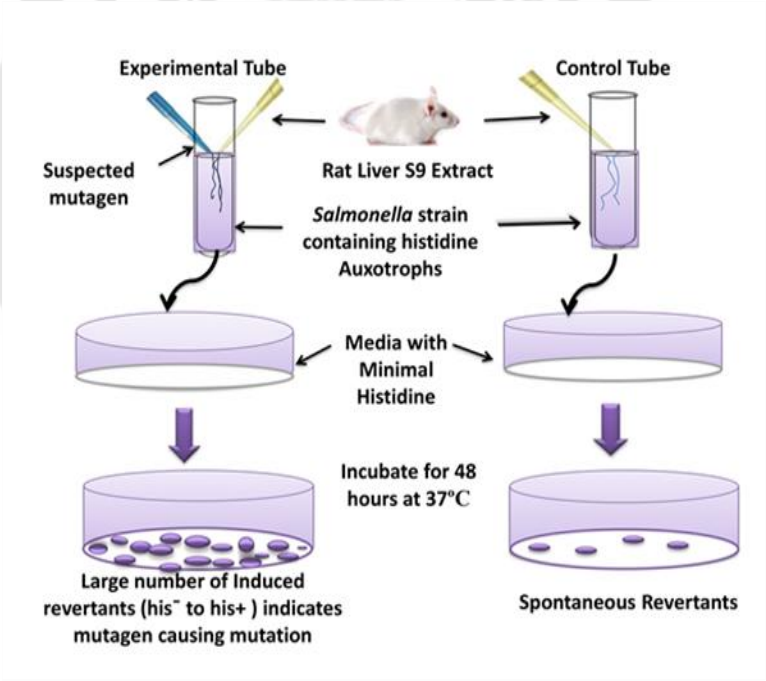

Figure 6 Genetic approach for assessing the mutagenicity in Salmonella strains

Source: Vijay, Gupta, Mathur, Suravajhala, & Bhatnagar. (2018). Microbial Mutagenicity Assay: Ames Test p. e2763.

CHAPTER 3
EXPERIMENTAL

3.1 Description of study area

The water samples were collected from the Saen Saep canal in Bangkok, Thailand. Five samples were collected from each site, which including Panfa Leelard Pier, Prasanmit Pier, Phatu Nam Pier, Watklang Pier, and Wat sriboonreung Pier as show in Figure 7. Khlong Saen Saep is a canal located in the central region of Thailand, connecting the Bang Pakong River in Chachoengsao Provinces to Chao Phraya River. Part of the canal is used for public transport by an express boat services in Bangkok. The canal is 72 km long through 21 districts and connects to more than 100 smaller canals. The Saen Saep canal have the buildings and residences beside along the canal, which including hospitals, restaurants, markets, hotels, condominiums, department stores, and homes. It has, therefore, received pollutant and discharged waste water from various sources.

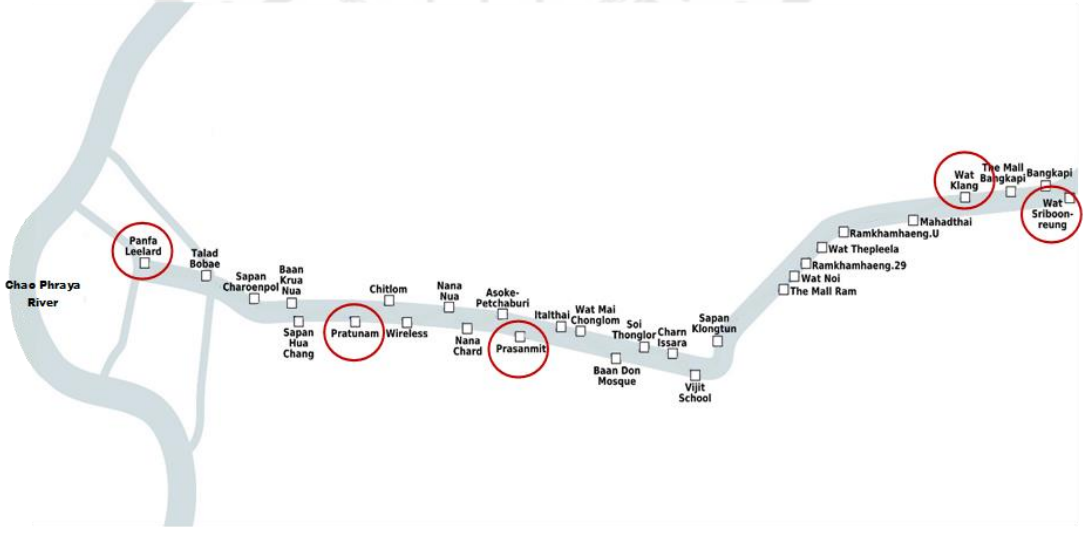

Figure 7 The location of sampling point along the Saen Saep canal

3.2 Materials and instruments

Standard of E2 (>97.0% purity) was purchased from TCI (Tokyo, Japan). Acetonitrile, ethyl acetate, hexane, methanol and iron standard for atomic absorption (J. T. Baker, Phillipsburg, NJ, USA) were purchased from Carlo Erba (Val de Reuil, France). Ammonia solution, 37% hydrochloric acid, 65% perchloric acid, 40% hydrogen peroxide solution, 67% nitric acid and chloroform were purchased from QRëc (New Zealand). Diethyl ether was purchased from PanReac AppliChem (Spain). HPLC grade acetonitrile was obtained from Fisher chemical (Seoul, Korea). Nickel standard for atomic absorption was purchased from Sigma-aldrich (St. Louis, MO, USA). Copper and zinc atomic absorption were purchased from Certipur® (Merck Millipore, Germany). Lead and cadmium standard for atomic absorption were purchased from Loba hemie (India). Ultrapure water with resistivity of 18.2 MΩ·cm at 25 °C was prepared from a Synergy® UV system (Millipore, Molsheim, France). 0.45 μm pore size hydrophobic polytetrafluoroethylene (PTFE) syringe filters were purchased from CNW technologies (Shanghai, China). Oasis® HLB 6cc (200 mg) SPE cartridges (Waters, Milford, USA) were purchased from Waters corporation. 12-port Prep SPE vacuum manifold (Fisher Scientific, Fair Lawn, USA). Whatman filter paper no. 42 was purchased from VWR International (Mississauga, ON, Canada).

Chromatographic measurement was performed with Agilent 1100 series HPLC equipped with revers phase 4.6x150 mm Zorbax extend-C18 column (Agilent, CA, USA) with 5 μm particles, using injection volume of 20 μL and UV-vis detector with a wavelength of 280 nm. The mobile phase was acetonitrile (ACN)–water (45:55, v/v) prepared at a flow rate of 1.0 mL/min. The concentrations of heavy metals were analyzed with Perkin Elmer AAnalyst 300 flame atomic absorption spectrometer (FAAS).

3.3 17β-estradiol (E2) determination methods

3.3.1. Preparation of standard curve

A stock standard solution (200 mg/L) of E2 was prepared by dissolving 10 mg E2 in 50 mL of 45% ACN. Standard solutions were stored at –20 °C. Calibration standards of E2 at concentrations of 1.5, 3, 5, 10, 20, 50, 100 and 200 mg/L was prepared by suitable dilution of stock standard solution in 10 mL volumetric flask. After standard solutions of E2 were prepared, filtered with 0.45 μm PTFE syringe filters and kept in amber glass bottles.

3.3.2. Analysis Instrumentation

Standard solutions of E2 were analyzed by using an Agilent 1100 series HPLC system equipped with a UV-vis detector set at 280 nm was used. Separation was accomplished using a ZORBAX Extend-C18 column (150 × 4.6 mm, 5-μm particle size). The mobile phase was a mixture of ACN-water (45:55, v/v) at a flow rate of 1.0 mL/min (Moon & Myung, 2016) and the volume of 20 μL was injected (Table 5).

Table 5 HPLC conditions

Parameters conditions	Conditions
Column	C18, 150 × 4.6 mm, 5 μm
Mobile phase	A: acetonitrile, b: water
Isocratic	A: b = 45:55
Flow rate	0.8 mL/min
Injection volume	20 μL
Wavelength	280 nm

3.3.3 Limit of detection (LOD) and quantification (LOQ)

LOD is the lowest concentration level in a sample that can be identified but not quantified from background noise. The lowest concentration of analyte that can be determined with acceptable precision and accuracy is referred to as the LOQ (Yilmaz &

Kadioglu, 2012). For HPLC measurement, LOD and LOQ of E2 were determined under the chromatographic conditions in Table 5 by injecting progressively low concentrations of the standard solution. The lowest concentrations giving the signal to noise ratio (S/N) at 3:1 and 10:1 were defined as LOD and LOQ, respectively (Yilmaz & Kadioglu, 2017).

3.3.4 Liquid-liquid extraction (LLE) procedure

The LLE procedure was carried out with three different methods for comparison as followings.

Method A: 10 mL of standard E2 solution, 2 mL of ACN (dispersive solvent), 500 μL of chloroform (extraction solvent) were added into 50 mL conical tube. The solution was mixed by vortex for 20 s until fine size spray droplets appeared. Then, continuously centrifuged for 10 min at 4000 rpm. The sediments of extraction solvent scattering in the tube would move to the bottom. The sedimented fraction at the bottom of sample tube was transported into amber glass bottles with a syringe (Moon & Myung, 2016).

Method B: 5 mL of extraction solvent, a mixture of diethyl ether and hexane (75:25), was added into 10 mL of standard E2 solution in a separatory funnel using an autopipette. The mixture of diethyl ether/hexane solvent and sample was gently shaken for 2 min. After the layer of water and organic solvent completely separated, the organic phase was transferred into amber glass bottles.

Method C: A procedure is similar to method B but using ethyl acetate as extraction solvent (Yilmaz & Kadioglu, 2012).

Afterward, all organic phase was evaporated with rotary evaporator at 40 °C. Then, the solutions were blow to dryness using a stream of nitrogen gas (99.99%). The dry residues were dissolved in 1 mL of 100% acetonitrile and filtrated with 0.45 μm pore size PTFE syringe filter prior to analysis by HPLC-UV. The extraction efficiency was

determined by recovery which can be calculated by Equation (3.1) (Magnusson, 2014). An appropriated extraction method giving highest recovery was selected for the determination of E2.

$$\%recovery = ((C1-C2)/C3) \times 100, \quad (3.1)$$

where C1 = corrected concentration of spiked sample in mg/L, C2 = corrected concentration of non-spiked sample in mg/L, and C3 = concentration of spike added into sample in mg/L.

3.3.5 Solid phase extraction (SPE) procedure

The sample extraction by SPE technique was performed using 200 mg HLB Oasis cartridges fitted to a 12-position solid phase extraction vacuum manifold. The cartridges were conditioned before loading 5 mL of samples, then the cartridges were washed and eluted using organic solvent listed in Table 6. The eluates were collected in amber glass vials, evaporated using a rotary evaporator at 40 °C and dried with N2 stream. The dry residues were dissolved in 1 mL of 100% ACN and filtrated with 0.45 μm PTFE syringe filter prior to analysis by HPLC-UV.

Table 6 Three different methods for SPE pre-treatment

Extraction step	Method X	Method Y	Method Z
Conditioning of cartridges	5 mL methanol 5 mL DI water	3 mL diethyl ether 3 mL methanol 3 mL water	5 mL Ethyl acetate 5 mL DI water
Washing	10% methanol	3 mL methanol: water (40:60), 3 mL water 3 mL methanol: 2% NH_4OH in water	20% ethyl acetate
Elution	3×3 mL methanol	3×3 mL MeOH: diethyl ether (10:90)	3×3 mL ethylacetate
Reference	(Bistan et al., 2012)	Adapted from guideline: Oasis sample preparation application notebook	(Bistan et al., 2012)

3.3.6 Liquid-liquid-solid phase extraction (LLE-SPE procedure)

Concentration of E2 in collected water sample from natural water environment is frequently lower than LOD of HPLC-UV technique. Therefore, pre-concentration step was applied before the extraction step as followings. Firstly, 1 L of 1, 3, and 5 mg/L E2 solutions were pre-concentrated with LLE according to the procedure explained in Section 3.3.4. The dry residues were dissolved in 5 mL of ACN with sonification. After that, the solution was extracted with SPE as illustrated in Section 3.3.5. The condition of this pre-concentration step was optimized by varying the concentration of ACN as 35%, 45%, 50%, and 60%.

3.4 Heavy metal determination methods

3.4.1 Preparation of standard solution for calibration curve

Standard substances of cadmium (Cd), copper (Cu), iron (Fe), nickel (Ni), lead (Pb), and zinc (Zn) were prepared by serial dilution at the following concentrations:

Cd, Cu, and Zn: 0.031, 0.062, 0.125, 0.250, 0.500, and 1.000 mg/L

Fe: 0.078, 0.156, 0.312, 0.625, 1.250, and 2.500 mg/L

Ni and Pb: 0.156, 0.312, 0.625, 1.250, 2.500, and 5.000 mg/L

An example of a serial dilution series was prepared from a stock solution to generate a standard curve is shown in Figure 8.

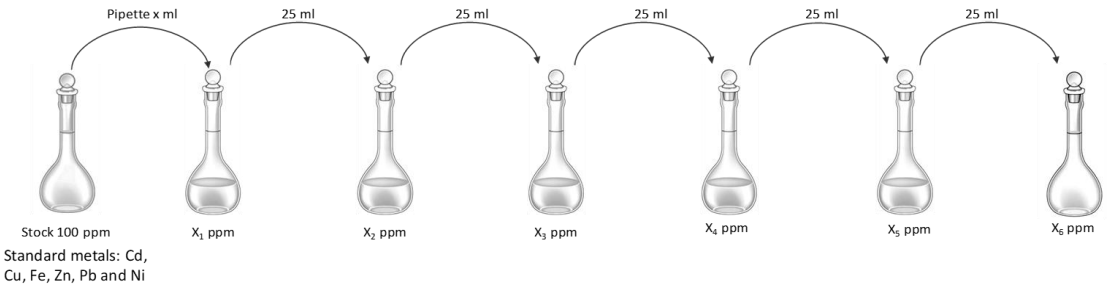

Figure 8 Preparation of standard solutions by serial dilution method

3.4.2 Method for LOD and LOQ

For the determination of LOD and LOQ of each heavy metal with FAAS technique, standard solutions of Cd, Cu, Fe, Ni, Pb, and Zn were prepared as described in section 3.4.1. After that the absorbance of standard solutions of heavy metals were determined with the following conditions in Table 7 before generated the calibration curve by plot between absorbance values (y-axis) and concentrations (x-axis). The sample blank solutions were prepared (1% nitric acid) and determined the absorbance of 10 sample blanks. Then, the standard deviations of 10 signal responses of blank reagent were calculated. The LOD and the LOQ were determined by calibration curve method using the following equations (Guideline, 2005; Shrivastava & Gupta, 2011):

$$LOD = 3Sa/b \quad (3.2)$$

$$LOQ = 10Sa/b, \quad (3.3)$$

where Sa is the standard deviation of the response of the sample blanks and b is the slope of the calibration curve.

Table 7 FAAS conditions

Metals	Wavelength (nm)	Current (mA)	Slit width (nm)	Flame
Cu	324.8	15	0.7	Acetylene / air
Cd	228.8	4	0.2	
Fe	248.3	30	0.2	
Zn	213.9	15	0.2	
Ni	232.0	25	0.2	
Pb	217.0	10	0.2	

3.4.3 Comparison of digestion methods for heavy metal determination

Standard solutions of heavy metals were prepared at the concentration as follows:

Fe: 0.625, 1.250 and 2.500 mg/L Cu: 0.250, 0.625 and 1.000 mg/L

Cd: 0.500, 1.000 and 2.000 mg/L Pb: 0.625, 1.250 and 2.500 mg/L

Ni: 0.625, 1.250 and 2.500 mg/L Zn: 0.125, 0.250 and 0.625 mg/L.

The prepared standard solutions of Fe, Cd, Cu, Pb, Ni and Zn were poured into 125 mL Erlenmeyer flask, and digested by using three different digestion methods in triplicate as followings.

1) Nitric (65% HNO_3) – hydrochloric (37% HCl) acid digestion (Radulescu et al., 2014)

Adding 2 mL of 65% HNO_3 and 1 mL of 37% HCl to the standard solutions (in Erlenmeyer flask).

2) Aqua regia (HCl: HNO_3 of 3:1) - perchloric (Conc. $HClO_4$) (Ogoyi, Mwita, Nguu, & Shiundu, 2011)

Adding 2 mL of Aqua regia and 250 µL of 70% $HClO_4$ to the standard solutions.

3) Nitric acid – 1 hydrogen peroxide (1% H_2O_2) (Gouda, 2016)

Adding 2 mL of 65% HNO_3 and 1 mL of 1% H_2O_2 to the standard solutions.

The mixture was boiled on a hot plate until a light color clear solution indicating the digestion is complete. The digestion occurred until the volume was reduced to less than 1 mL or as low as possible. After digestion the standard solutions were cooled to room temperature, filtered with Whatman No. 42 filter paper and adjusted to 25 mL with 1% of Nitric acid. Absorbance of Cd, Cu, Fe, Pb and Zn were determined using FAAS with the experimental conditions in Table 7. Finally, the recovery

percents of Cd, Cu, Fe, Pb and Zn were calculated. Percent recovery is calculated by the following equation 3.1 (EURACHEM, 2014). Then the percent recoveries from various digestion methods were compared in order to obtain an appropriated digestion method used in the determination of heavy metals.

3.5 Determination of the concentration of E2 in the Saen Saep canal

3.5.1 Water sampling

For each sampling site, 1 L of grab samples were collected from surface water (<0.5 m depth). Water samples were accumulated in clean 1 L amber glass bottles and kept on ice in the dark for protection from sunlight before transported to the laboratory in order to avoid degradation of E2 in water sample. The samples were extracted within 24 hours (Bergamasco et al., 2011).

3.5.2 LLE-SPE procedure

After the water samples were collected from the Saen Saep canal as described previously, the samples were extracted using LLE techniques with method B followed by SPE technique with method Z. 1 L of water sample was filtered through 0.45 µm pore size glass microfiber filter and pre-concentrated with LLE techniques which has the following steps: A mixture of diethyl ether and hexane (75:25) was rapidly added into 1 L of water sample in a separatory funnel. The mixture of diethyl ether/hexane solvent and sample was gently shaken for 5 min. After the layer of water and organic solvent completely separated, the organic phase was transferred into 2 L amber glass bottles and evaporated close to dryness using a rotary evaporator with a water bath temperature of 40 °C. Then, the samples were blow to dryness using a stream of 99.99% purity nitrogen (N_2) gas. The dry residues were dissolved in 5 mL of 45% acetonitrile (ACN) with sonification. After that, the solution was extracted with SPE. Solid phase extraction was performed using 200 mg HLB Oasis. Briefly, conditioning of the cartridges was performed with 5 mL of ethyl acetate followed by 5 mL of DI water. After loading the dry residues solution, the cartridges were washed with 5 mL of 20% ethyl

acetate. Elution was performed using 3×3 mL of ethyl acetate. The eluates were collected in amber glass vials, evaporated using a rotary evaporator at 40 °C and dried with N_2 stream. The dried residues were dissolved in 500 μL of DMSO and filtrated with 0.45 μm PTFE syringe filter prior to analysis by HPLC-UV.

3.6 Determination of the concentration of heavy metals in the Saen Saep canal.

3.6.1 Water sampling

Water grab samples from the Saen Saep canal were collected within 0.5 m from water surface. Representative 1 L of sample were collected in polyethylene bottles, previously washed with 10 % HNO_3 solution. To avoid oxidation of sample at headspace, the bottles were filled up with water and then sealed with parafilm. Water samples were stored approximately at 0-4°C in ice box container. After samples were returned to laboratory, they were acidified to pH 2.0 adding 2 mL of 67 % nitric acid to prevent metal ion precipitation, and kept in a refrigerator until needed for analysis (Elhamili, Abokhshim, Elaroud, & Elbaruni, 2016).

3.6.2 Digestion of Water Samples and analysis (External standard Method)

The digestion procedure for water samples was performed by transferring 25 mL of water samples into a 250 mL Erlenmeyer flask. Then 2 mL of aqua regia (HCl 37% : HNO_3 65% = 3:1) and 250 μl perchloric acid were added into the flask. On a hotplate, the mixture was boiled and evaporated to the smallest volume feasible. During digestion, do not allow the sample to dry out. After cooling to room temperature, the flask was washed with 1% HNO_3 and filtered using Whatman No. 42 filter paper. Then the filtrates were transferred into 25 mL volumetric and diluted with 1 % HNO_3. The solutions were analyzed for heavy metals by FAAS with the experimental conditions in Table 7. After that the concentration of Cd, Cu, Fe, Pb and Zn were calculated from linear equation of calibration curve.

3.6.3 Digestion of Water Samples and analysis (Standard addition method)

The method of standard addition is utilized when the sample matrix also contributes to the analytical signal, resulting in an inability to compare the analytical signal between sample and standard using the conventional calibration curve methodology. Procedurally, here are the steps for preparing the samples for analysis in standard addition: the water samples were pipetted 5 mL of water samples each pier into 25 mL of volumetric flask 6 bottoms (S_0–S_5). Heavy metal standards for each of the heavy metals Cd, Cu, Fe, Pb, and Zn were prepared to be added to the water samples. Stock solutions of each heavy metal were prepared at a concentration of 100 mg/L in a 10 mL volume flask, adjusted with 1% HNO_3, and added to the sample. Tables 8-9 summarize the amounts of heavy metals added from the stock solution for each heavy metal in order to achieve various heavy metal concentrations. Then the mixtures were diluted with 1 % HNO_3 and were analyzed for heavy metals by FAAS with the experimental conditions in Table 7. After that the signals of Cd, Cu, Fe, Pb and Zn were plot between absorbance values (y-axis) and concentrations (x-axis) in order to determine x-intercept, which the x-intercept gives the concentration of the unknown.

Table 8 Preparation of Cd and Cu for standard addition to samples of water samples

Water sample	S0	S1	S2	S3	S4	S5
Sample volume (mL)	5	5	5	5	5	5
Heavy metal concentration (mg/L)	0	0.1	0.2	0.4	0.6	0.8
Concentration of Heavy metal stock solution (mg/L)	100	100	100	100	100	100
µL added from stock	0	25	50	100	150	200

Table 9 Preparation of Fe, Ni Pb and Zn for standard addition to samples taof water samples

Water sample	S0	S1	S2	S3	S4	S5
Sample volume (mL)	5	5	5	5	5	5
Heavy metal concentration (mg/L)	0	0.2	0.4	0.6	0.8	1.0
Concentration of Heavy metal stock solution (mg/L)	100	100	100	100	100	100
µL added from stock	0	50	100	150	200	250

3.7 Bioassays

3.7.1 Yeast estrogen screen assay (YES assay) procedure

YES assays were determined to compare the estrogenic activities of samples tested with E2, the positive control, in order to calculate the E2 equivalent (EEQ) values of the samples. In this test, recombinant Saccharomyces cerevisiae yeast was used. The yeast was transfected with the human estrogen receptor (hER) gene, which was supplemented by an expression plasmid carrying promoter sequences and the lac-Z reporter gene (encoding the enzyme β-galactosidase). In this system, the hER is expressed when it binds to estrogen response elements (ERE), causing the reporter gene Lac-Z to be expressed and β-galactosidase to be released into the medium. As a result, the original yellow color of chlorophenol red-β-D-galactopyranoside (CPRG) is converted into red (chlorophenol red), which can be quantified colorimetrically at 570 nm (Xiao et al., 2017; Yi et al., 2015). In this study, using cells were provided from the XenoScreen YES/YAS kit, Xenometrix (YES Strain, Art. No. N05-230-E). The testing procedure is as follows:

1. Yeast Culture Preparation

5 mL of growth medium was added into 50 mL conical tube, followed by the yeast cell on the filter disk from the test kit. The yeast cells were incubated on an orbital shaker set at 31°C with shaking at 100 rpm for 2-4 days or until clearly turbid. The yeast culture was inspected under a microscope. If there are clumps of yeast cells, use a serological pipette to disseminate the yeast cells. After that, 200 µL of yeast culture was transferred to a well on a 96-well microplate and the OD690 was measured, with the mean value being at least 0.2. Preparation stock of yeast cells as shown in Figure 9.

2. Sample and Controls Preparation

Preparation of the agonist controls by 100 µL DMSO was added to the vial "17β-estradiol" ("E2", red label). Then, the solution was mixed thoroughly for ~30 seconds, which gives a 1x10-6 M stock solution of E2. For sample preparation, the test sample was dissolved in DMSO using the highest soluble concentration (4.9×10-6 M).

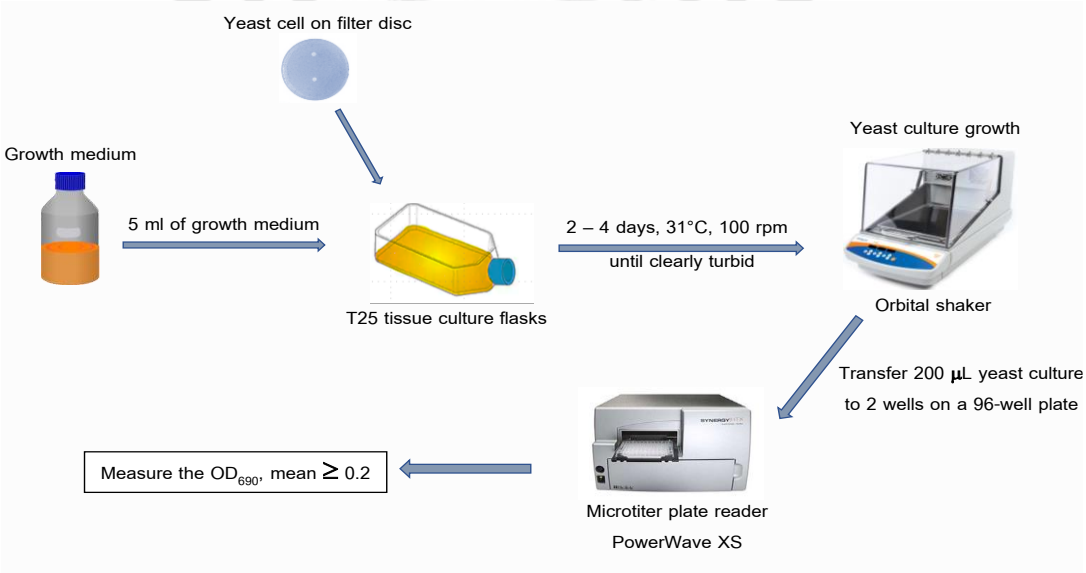

Figure 9 Preparation stock of yeast cells for estrogenicity testing

3. Assay Medium Preparation

The YES plate assay medium (prepared for growing yeast cells) is prepared in a 50 mL conical tube. 0.2 mL of CPRG substrate solution and 8 mL of growth medium were added to the tube and mixed well. 80 μL of assay medium was transferred to each well of the plates (column 1-8) and cover with the lids.

4. Preparation of Dilution Plate

Dilution plate layout is as shown in Figure 10. Preparation of first dilution plate, 50 μL of DMSO was added to all pink wells as shown in Figure 10a. Afterward, 73 μL of the reconstituted E2 (1×10^{-6} M) was added to well H of column 1, which contained the 100x concentrated stock dilutions utilized as estrogenic positive controls. The wells of 2H, 3H, and 4H were added 73 μL of Standard E2 solution (1×10^{-6}M), water extract, and raw water, respectively. Then, using a pipette, transfer and mix 23 μL from row H to G of column 1 to prepare the dilution series, and continue up to and including row B, discarding the final 23 μL. The well A of column 1 wasn't added anything due to the solvent control. The tips were changed for each step of dilution and mixed with at least 5 strokes of the pipette. Repeat the preceding steps to prepare the dilutions of standard E2, water extract, and raw water, respectively, in columns 2 to 4, but all the way to row A. (8 dilutions). After that, 180 μL of growth medium was filled into all wells in columns 1 to 4 (yellow wells) to prepare a second dilution plate, as illustrated in Figure 10b. 20 μL of the first dilution plate's filled wells were transferred to the second dilution plate and mixed thoroughly, using the second dilution plate for the preparation of the assay plate.

(a)

	E2	SP1	SP2	SP3								
	1	2	3	4	5	6	7	8	9	10	11	12
A	50	50	50	50								
B	50	50	50	50								
C	50	50	50	50								
D	50	50	50	50								
E	50	50	50	50								
F	50	50	50	50								
G	50	50	50	50								
H	73	73	73	73								

(b)

	E2	SP1	SP2	SP3								
	1	2	3	4	5	6	7	8	9	10	11	12
A	20	20	20	20								
B	20	20	20	20								
C	20	20	20	20								
D	20	20	20	20								
E	20	20	20	20								
F	20	20	20	20								
G	20	20	20	20								
H	20	20	20	20								

Figure 10 Schematic presentation of 96-well plates for (a) first dilution plate and (b) second dilution plate layout

5. Preparation of Assay Plates

The wells of the assay plates were filled with 80 μL of assay medium (columns 1-8). After that, 20 μL of the prepared dilution plate was transferred to the assay plates by using a pipette set at a volume of 20 μL as follows: 20 μL of the dilution plate of column 1 (positive control) was transferred into columns 1 and 2 of the assay plate. Standard E2 was prepared by transferring 20 μL of the dilution plate from column 2 to columns 3 and 4 of the assay plate. Later, for estrogenic activity testing of samples, 20 μL of the dilution plate columns 3 (water extract) was moved to columns 5 and 6 of the assay plate, and 20 μL of the dilution plate columns 4 (raw water) was transferred to columns 7 and 8 of the assay plate. Prior to performing, the yeast cells were pipetted up and down to dissolve clumps, added into a 20 mL growth medium and mixed well. Afterward, 100 μL of yeast cells were added to all wells of the correlating assay plates before closing the plate with a breathable plate sealer. The plate was transferred to a plastic container with moist wet wipes and closed the lid to prevent evaporation from the wells. Then, the plate was incubated at 30 °C on a rotating platform at approx. 150 rpm for 48 hr. Check the plates after 48 hours and removed the plate sealers without spilling. The plate was read at 690 nm for growth and at 570 nm for expression of β - galactosidase. The procedures for the YES assay are shown in Figure 11.

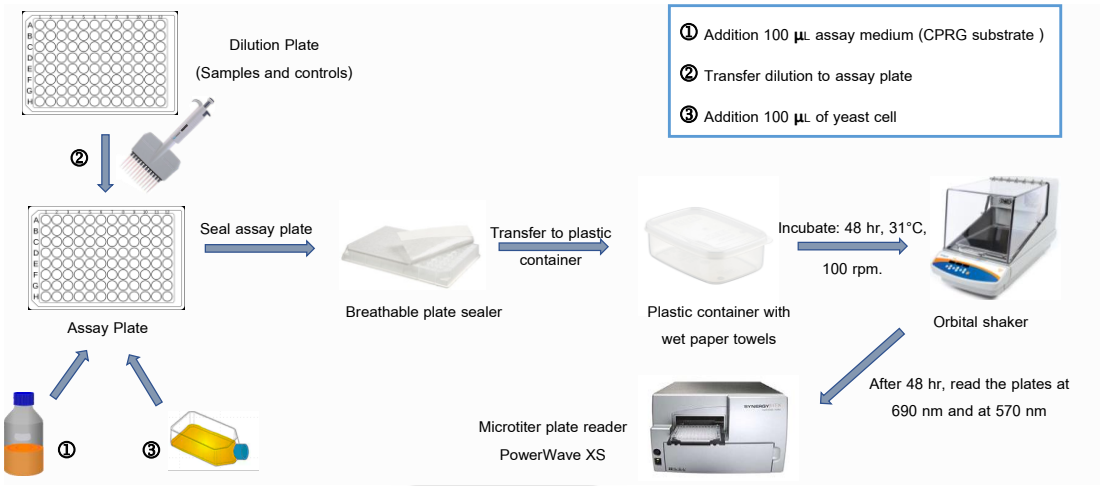

Figure 11 Schematic presentation of procedures for estrogenic activity testing

3.7.2 Ames test

The Ames test was used to investigate the mutagenicity of E2 and heavy metal. In this test, histidine-dependent strains of *Salmonella typhimurium* were utilized, which could revert to histidine independence and grow as viable colonies after being exposed to a suitable mutagen (Yi et al., 2015). Mutagenicity was assayed with *Salmonella typhimurium* strains, TA98 and TA100, without exogenous metabolic activation (–S9). Frameshift mutations were detected by strain TA98, whereas base-pair substitutions were detected by strain TA100. Before performing the experiment, fresh bacteria were prepared as follows:

Preparation of cells

The freezing cells of *Salmonella typhimurium* strains TA98 and TA100 (–80 °C) were removed from the freezer and rapidly scraped a small inoculum from the surface with a sterile loop. The inoculum was transferred to 20 mL NB in a 50 mL conical tube and incubated overnight at 36 °C with shaking at 300 rpm prior to performing the experiment. After incubation, the conical tube was centrifuged at 4,000 rpm for 5 min. Discarded supernatant of NB while the remainder of precipitate cell was resuspended

by adding 5 mL NB and mixed by vortex mixer. Then, adjusted the OD_{600} until approximately equals to 0.1 by dilution with NB. Afterward, diluted only stain TA100 for 1000 times with NB.

Mutagenicity test procedure

After the *Salmonella typhimurium* were prepared, all GM plates and corresponding sterile glass test tubes for each test condition were labeled prior to the experiment. Each experiment contained a series of triplicate plates for negative solvent control (DMSO), positive control (for testing without metabolic activation), three concentrations of the test substance (E2: low, medium, and high concentrations), and dilutions of the test sample as shown in Figure 12.

For all conditions, 2 mL of molten top agar (supplemented with 0.05 mM histidine and 0.05 mM biotin) was added in sterile glass test tubes. Then, added 0.1 mL of DMSO for negative control, 0.1 mL of positive content (4-Nitro-o-phenylenediamine and sodium azide) for positive control, 0.1 mL of E2 for the test standard substance, 0.1 mL of extracted water sample from the Saen Saep canal for test sample. Afterward, 0.1 mL of overnight culture of the *Salmonella typhimurium* strain was added. The contents and bacteria in sterile glass test tubes were mixed preferably by mild vertexing. Poured the mixture of each test tube onto the surface of the corresponding GM plate and gently swirled to evenly distribute on the molten top agar. Afterwards, the top agar was hardened, inverted plates and placed in 37 °C incubator for 48 h. After incubation, revertant colonies were counted in order to express the test results in terms of number of revertant colonies per plate.

Negative and positive controls were included in each test. The negative controls only had bacteria and DMSO without test samples. The positive control for testing without metabolic activation were 4-Nitro-o-phenylenediamine and sodium azide

for TA98 and TA100 respectively (Parrella et al., 2013). The mutation ratio (MR) was calculated as the number of histidine-positive (His+) revertants induced by the sample divided by the number of spontaneous His+ revertants in the negative control. Generally, a twofold increase in the MR in a test sample was considered as a positive mutagenic response (Yi et al., 2015).

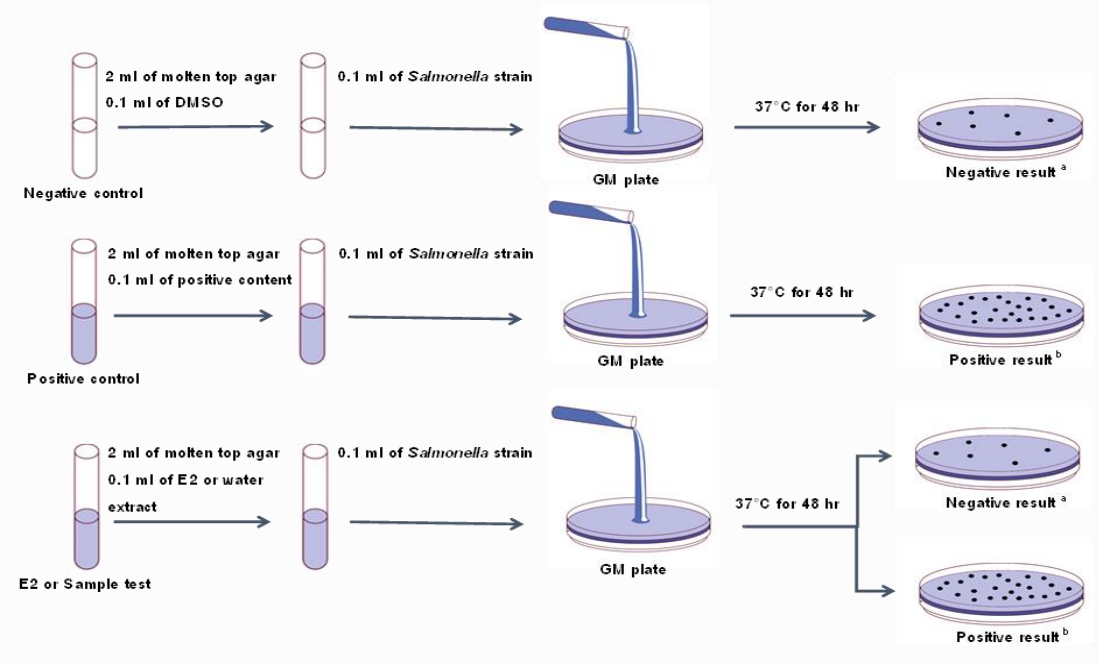

Figure 12 Mutagenicity was assayed with *Salmonella typhimurium strains*, TA98 and TA100, without metabolic activation

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Determination of the concentration of E2 and heavy metals contaminating in the Saen Saep canal

4.1.1 Optimization of E2 determination methods with HPLC-UV technique

4.1.1.1 Method Validation

The chromatogram of standard E2 solutions measured by HPLC-UV method are showed in Figure 13. Peaks of E2 appeared clearly at approximately 4.9 min without overlapping peaks. This takes a little more retention time than previous work that peak of E2 occurred at 3.54 min (Aydoğmuş, Yılmaz, Yörüsün, & Akpınar, 2015). Calibration curve associated with the chromatogram was showed in Figure 14. The linearity range of E2 was found at 1–200 mg/L (n = 3), correlation coefficients (R2) were 0.9999, indicating good linearity. Other calibration curve parameters (regression equation, slope, and intercept) were illustrated in Figure 14. The LOD and LOQ of E2 detected with HPLC-UV were 0.125 and 0.50 mg/L, respectively, which were higher than that of other techniques such as GC-MS (LOD = 0.03, LOQ = 0.09 ng/L (Zhou et al., 2009)), LC-MS/MS (LOD = 0.5, LOQ = 2.0 ng/L (Guo et al., 2013)). From the limitation of this methods, therefore, the development of pre-treatment process is necessary for this research.

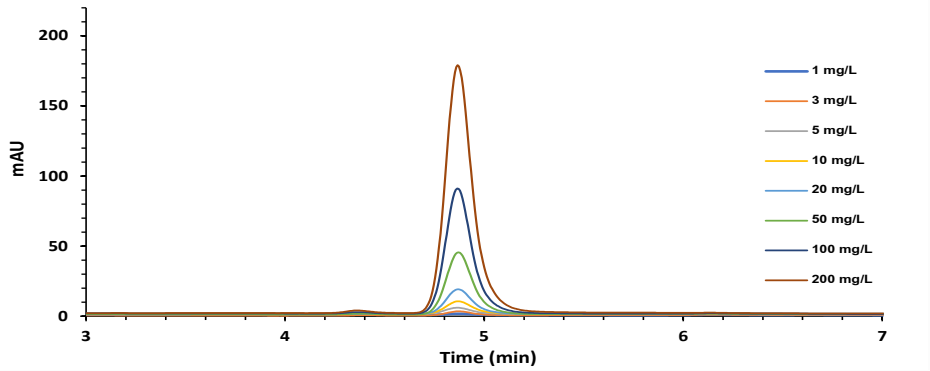

Figure 13 Chromatogram of standard E2 solutions measured by HPLC-UV

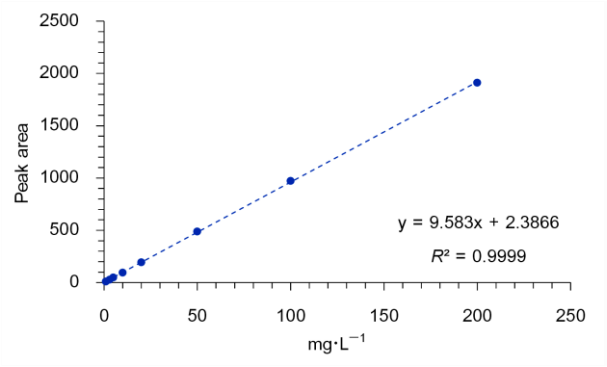

Figure 14 The **calibration curve** of standard E2 solutions were found at 1–200 mg/L

4.1.1.2 Extraction efficiencies of LLE and SPE

Recoveries of LLE and SPE were evaluated with the same E2 concentration (20 mg/L) and the results were shown in Table 10. LLE with method B provided the highest recovery of 85.63% while methods A and C gave recoveries of 74.89% and 73.15% , respectively. This result suggested that using diethyl ether and hexane in the ratio of 75:25 as extraction solvent was applicable for the LLE method. For the SPE, it was found that use of method Z represented higher recovery (92.55%) than methods X (72.99%) and method Y (68.94%), and LLE. This result indicated that SPE can approach good extraction efficiency when using the condition of method Z listed in Table 10 and reasonable for pre-treatment process in the E2 determination with HPLC-UV.

Table 10 Recovery of E2 (mg/L) standard extracted by LLE and SPE

Methods		Concentration found (Mean ± SD)	% RSD	% Recovery
LLE	A	14.98 ± 1.22	8.13	74.89
	B	17.13 ± 0.34	1.98	85.63
	C	14.63 ± 0.96	6.57	73.15
SPE	X	14.02 ± 0.25	14.59	72.99
	Y	13.22 ± 0.66	13.79	68.94
	Z	18.09 ± 0.25	18.51	92.55

The SPE with method Z was used to extract E2 in standard solutions for corroboration the efficiency. The results showed in Table 11 indicates that recoveries were acceptable which higher than 85% and RSD lower than 3, representing the high performance on E2 extraction comparing to other conditions of SPE providing recoveries of 41-72% (Liz et al., 2017), 83% (Melo & Brito, 2014) , and 85.2% (Aufartová, Torres-Padrón, Sosa-Ferrera, Solich, & Santana-Rodríguez, 2012). This confirmed the high extraction efficiency of SPE on the E2 extraction when using the method Z. However, the disadvantage of SPE extraction is the inability to extract E2 at ng/L concentration level, which is the most typical E2 concentration found in water resources in the environment (Ruchiraset & Chinwetkitvanich, 2014). Therefore, a preconcentration of water sample with LLE was required before extraction by SPE method.

Table 11 Extraction efficiency of SPE with method Z

E2 added (mg/L)	Concentration found (Mean ± SD)	% RSD	% Recovery
0.25	0.22 ± 0.006	2.60	86.39
0.50	0.44 ± 0.004	0.82	87.58
5.0	4.55 ± 0.053	1.18	90.57
20	18.06 ± 0.343	1.90	90.30

4.1.1.3. Extraction efficiencies of LLE-SPE procedure

The condition of pre-concentration step was the concentration of acetonitrile (ACN). ACN was used to dissolve the dry residues obtained from LLE pre-concentration. However, large amount of ACN prevents the absorption of E2 on the sorbent of SPE, E2 will be partly eluted in the step of sample loading in SPE cartridge. Figure 15 shows the effect of ACN concentration on recovery and found that 45% ACN provided highest recovery and lowest RSD comparing to other concentrations.

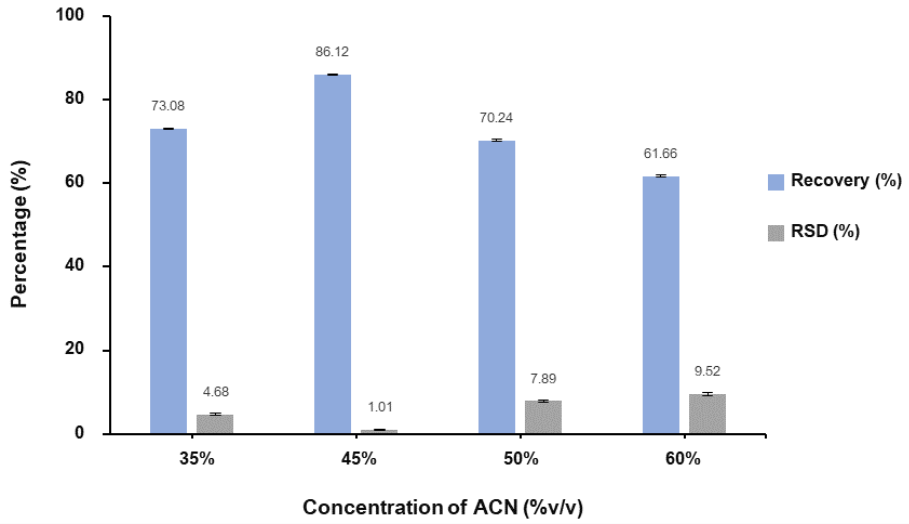

Figure 15 Effect of concentration of ACN on recovery of E2 and RSD

From the previous results, it was found that the optimum conditions for the extraction process are i) use of LLE with method B for pre-concentration of E2 solution, ii) use 45% ACN for dissolve the dry residues, and iii) use the SPE with method Z for extraction of E2 before HPLC-UV measurement. This optimum LLE-SPE pre-treatment was used to extract E2 from the standard E2 solution. The results shown in Table 12 indicated that after pre-treatment, the quantity of E2 can be determined even though at very low concentration (0.5 mg/L) with RSD of 1.94% and recovery of 84.40% which is in the acceptable ranges of the U.S. Environmental Protection Agency (55–108%) (USEPA, 2007) the European Commission (70–120%) (European Commission, 2015), and more than previous studies. Moreover, recovery was found to increase when the concentration of E2 increase. Additionally, in step i), LLE can be used for the large volume of water sample (>1,000 mL) while SPE can be loaded with water sample of 100–500 mL (Andrási, Helenkár, Vasanits-Zsigrai, Záray, & Molnár-Perl, 2011; Gatidou, Thomaidis, Stasinakis, & Lekkas, 2007; Mol, Sunarto, & Steijger, 2000), and extraction with LLE was faster than SPE because in SPE, sample was slowly infused into a cartridge with a flow rate of 10-60 mL/ min (Kim, Do, Yeh, & Cunningham, 2014; Ruchiraset & Chinwetkitvanich, 2014).

Table 12 Extraction efficiency of LLE-SPE optimum condition

E2 added (mg/L)	Concentration found (Mean ± SD)	% RSD	% Recovery
0.5	0.84 ± 0.702	1.94	84.40
3.0	2.56 ± 0.646	1.61	85.32
5.0	4.30 ± 0.909	1.34	86.03

A pre-treatment procedure was optimized involving 3 steps: i) use of LLE with method B (diethyl ether: hexane of 75: 25 extraction solvent) for pre-concentration, ii) use 45% ACN for dissolve the dry residues, and iii) use the SPE with method Z (conditioned with 5 mL ethyl acetate and 5 mL DI water, washed with 20% ethyl acetate, and eluted with 3x3 mL ethyl acetate) for extraction of E2 before HPLC-UV measurement. For this optimum procedure, standard E2 solutions were used for recovery and RSD evaluation, with results of 84.4–86.3% and RSD of 1.34–1.94% for E2. The pre-treatment methodology developed in this research gave the pre-concentration factor more than 100 times, allowing to determine the E2 in ng/L. This originates from the benefit of using the LLE that can extract E2 from a large volume of sample. Moreover, in this method, sample clean-up was performed in two steps before HPLC-UV measurement (from LLE and SPE), resulting matrix was more eliminated from water sample which given the clear background for HPLC analysis then target E2 peak was easily and accurately detected by HPLC-UV4.1.2 Optimization of determination of heavy metal concentration with FAAS technique.

4.1.2 Optimization of E2 determination methods with HPLC-UV technique

4.1.2.1. Method validation

FAAS technique was used to determination the concentration of Cd, Cu, Fe, Ni, Pb, and Zn standard solutions prepared in ppm or mg/L. After FAAS

measurements, calibration curves of the standard solutions were established. Then, LOD and LOQ values were determined.

1. Calibration curves of metal standard solutions

The absorbance of standard solutions of heavy metals were determined with FAAS method. The obtained calibration curves were shown in Figure 16. Linear equations and values of correlation coefficients (R2) were illustrated in Table 13. The relationship between the absorbance and the concentrations of metal standard solutions demonstrated the linearity with the values of R2 of 0.9995, 0.9998, 0.9993, 0.9996, 0.9997, and 0.9999 for the standard solutions of Cd, Cu, Fe, Ni, Pb, and Zn, respectively.

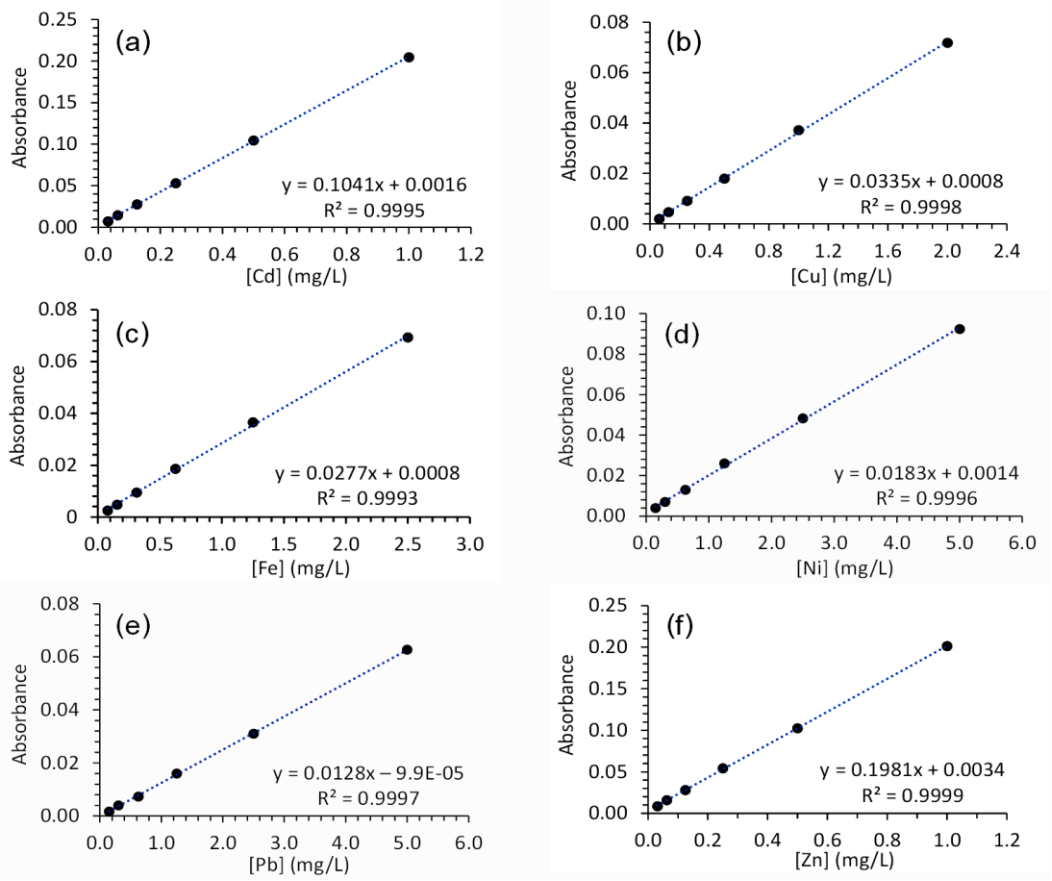

Figure 16 Calibration curves of standard solutions of (c) Cd, (d) Cu, (e) Fe, (f) Ni, (g) Pb, and (h) Zn

Table 13 Linear equations and correlation coefficients (R^2) of calibration curves

Metal	Concentration of standard series solutions (mg/L)	Linear equation y = mx + c	R^2
Cd	0.031, 0.062, 0.12, 0.25, 0.50, 1.0	y = 0.0649x + 0.0006	0.9997
Cu	0.031, 0.062, 0.12, 0.25, 0.50, 1.0	y = 0.0330x – 0.0002	0.9998
Fe	0.078, 0.16, 0.31, 0.62, 1.2, 2.5	y = 0.0263x + 0.0008	0.9993
Ni	0.16, 0.31, 0.62, 1.2, 2.5, 5.0	y = 0.0168x + 0.0006	0.9994
Pb	0.16, 0.31, 0.62, 1.2, 2.5, 5.0	y = 0.0127x – 0.0001	0.9999
Zn	0.031, 0.062, 0.12, 0.25, 0.50, 1.0	y = 0.1507x + 0.0020	0.9996

2. Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ of the FAAS technique for determination of heavy metal concentrations were investigated by measuring the absorbance of blank solutions (n = 10). Then, mean of absorbance values was calculated and used to compute the LOD and LOQ by using Eqs. 3.2 and 3.3, respectively. The LOD and LOQ of FAAS method for determination of the metal quantity were obtained and listed in Table 14. The results showed lowest values of LOD and LOQ for concentration analysis of Zn of 0.011 ± 0.0007 and 0.036 ± 0.0007, respectively. On the other hand, determination of Pb concentration provided the highest values of LOD and LOQ of 0.171 ± 0.0007 and 0.569 ± 0.0007, respectively. If the sample concentrations are below LOQ, the laboratory does not necessary to mention the LOQ in the test report. In this case the results should be expressed as below LOQ or not detectable (ND).

4.1.2.2 Comparison of digestion methods for heavy metal determination

In order to obtain the appropriate method for digestion a water sample in the heavy metal determination with FAAS, three different digestion methods were employed to digest standard solutions of metals in three concentrations as described in Section 3.4.3. The recoveries measured after digestion were used to determine the digestion efficiency of the methods. The results of the recoveries showed

Table 14 LOD and LOQ of FAAS method for determination of heavy metal concentrations

Metals	LOD (mg/L)	LOQ (mg/L)
Cd	0.013 ± 0.0004	0.043 ± 0.0004
Cu	0.015 ± 0.0002	0.051 ± 0.0002
Fe	0.046 ± 0.0004	0.153 ± 0.0004
Ni	0.095 ± 0.0006	0.319 ± 0.0006
Pb	0.171 ± 0.0007	0.569 ± 0.0007
Zn	0.011 ± 0.0007	0.036 ± 0.0007

that Method 2 demonstrated high recovery of Cu, Fe, Ni, and Pb than other methods, while high Cd and Zn recoveries were received from Method 3 and Method 1, respectively. However, recoveries of Cd and Zn from Method 3 and Method 1 were found to be higher than that from Method 2 only 2-5% and appeared in some concentrations as shown in Table 15. The recovery obtained from Method 2 were satisfactory with a mean percentage recovery of 95.94% (Methods 1 and 3 of 86.28% and 87. 5% , respectively). This mean percentage recovery was above the 95% threshold and consequently the results produced by the FAAS were reproducible. From Table 15, the recoveries of almost heavy metals determined from the digestion Method 2 expressed above 90 % with relative standard deviations below 10% (except for Cu at 0.25 and 0.62, and Zn at 0.12 mg/L) showing the acceptable efficiency of the method.

Table 15 percentage recoveries of heavy metals from three digestion methods

Metals	Concentration (mg/L)	Recovery (%)		
		Method 1	Method 2	Method 3
Cd	0.5	95 ± 0.011	97 ±0.0065	97 ±0.023
	1	96 ± 0.033	97 ± 0.022	98 ± 0.021
	2	92 ± 0.030	96 ± 0.070	94 ± 0.043

Table 15 (Continuous)

Metals	Concentration (mg/L)	Recovery (%)		
		Method 1	Method 2	Method 3
Cu	0.25	62 ± 0.016	86 ± 0.016	59 ± 0.028
	0.62	74 ± 0.030	88 ± 0.12	71 ± 0.028
	1.0	63 ± 0.084	90 ± 0.025	68 ± 0.028
Fe	0.62	89 ± 0.030	98 ± 0.024	90 ± 0.060
	1.20	83 ± 0.048	103 ± 0.048	89 ± 0.10
	2.50	75 ± 0.14	92 ± 0.29	79 ± 0.13
Ni	0.62	82 ± 0.067	96 ± 0.045	101 ± 0.021
	1.2	96 ± 0.12	98 ± 0.045	94± 0.12
	2.5	94 ± 0.20	100 ± 0.027	97 ± 0.098
Pb	0.62	95 ± 0.059	111 ± 0.088	106 ± 0.039
	1.2	86 ± 0.13	106 ± 0.067	95 ± 0.029
	2.5	99 ± 0.14	102 ± 0.059	82 ± 0.12
Zn	0.12	92 ± 0.0075	87 ± 0.0073	85 ± 0.0050
	0.25	90 ± 0.0035	90 ± 0.0054	82 ± 0.017
	0.62	90 ± 0.0035	90 ± 0.0042	88 ± 0.0063

4.1.3 Determination of the concentration of E2 in the Saen Saep canal

Water samples were collected from Wat Sriboonreung Pier, Wat Klang Pier, Prasan Mit Pier, Pratu Nam Pier, and Panfa Leelard Pier from December 2020 to March 2021. The pre-treatment process was performed as described in Section 3.4.2 but using dimethyl sulfoxide (DMSO) instead of the mixture of acetonitrile and water because the samples need to be further used in a bioassay. After that the concentrations of E2 in the water samples were determined with HPLC technique by detection the peak at retention time of 4.8 min in HPLC chromatogram which was according to the signal from standard solution of E2 as illustrated in Figure 17. The results shown in Table 16 indicate that the

water samples from Wat Sriboonreung Pier had the highest E2 content in all sampling periods and was found to be highest on December 2020 of 11.89 μg/L, followed by water samples from Wat Klang, Panfa Leelard, and Prasan Mit Piers, respectively. The concentration of E2 in water sample from Pratu Nam sampling site was not detectable. From January to March 2021, the concentration of E2 in water samples was in the sequence of Wat Sriboonreung > Prasan Mit > Wat Klang > Panfa Leelard > Pratu Nam. Table 16 shows that the concentrations of E2 in water samples were variable in each sampling site and period due to urban activities, waste water discharge, sea level, and weather conditions. For example, the E2 levels in water sample taken from Wat Klang, Prasanmit, Pratu Nam, and Panfa Leelard Piers on March 2021 were higher than other months, due to the beginning of Summer in Thailand. The concentration levels of E2 observed in this work were also similar to the studies conducted within China, where the concentrations of E2 determined in the dry season were higher than other periods, due to the reduced of water volume in river leading E2 levels to increase (Liu et al., 2009).

From Table 16, concentrations of E2 in water samples collected from the Saen Saep canal can be detected by using HPLC technique and found to be in the range of 0.63 – 11.89 μg/L. The E2 levels in the samples taken from Wat Sriboonreung were in the range of 3.59 ± 1.05 to 11.89 ± 1.56, Prasan Mit 0.63 ± 1.30 to 4.20 ± 2.32, Wat Klang 1.98 ± 2.77 to 2.88 ± 2.45, Panfa Leelard 1.59 ± 1.21 to 2.71 ± 5.17, and Pratu Nam 1.25 ± 1.94 to 1.78 ± 1.82 μg/L. The E2 concentration found in water samples from the Saen Saep canal was obviously higher than that detected in water sample from other sampling site near urbanization such as Sankheap Canal (Chonburi Province) and Payun Canal (Rayong Province) which found the E2 levels of 62.98 ± 5.03 and 35.53 ± 2. 99 ng/L, respectively (Ocharoen, Boonphakdee, Boonphakdee, Shinn, & Moonmangmee, 2018). However, the concentration of E2 in water samples from the Saen Saep canal determined in this study was consistent with the study of Ruchiraset and Chinwetkitvanich (2014) who reported markedly high levels of E2 of 1.32 – 2.01 μg/L in water from the Chaophraya River which flows from the center of Bangkok to the Gulf of Thailand (Ruchiraset & Chinwetkitvanich, 2014). Seriously, the levels of E2 in the

Saen Saep canal and also the Chaophraya River exceed the predicted no effect concentration (PNEC) of E2 as a level beyond 2 ng/L can adversely affect to aquatic organisms (Caldwell, Mastrocco, Anderson, Länge, & Sumpter, 2012).

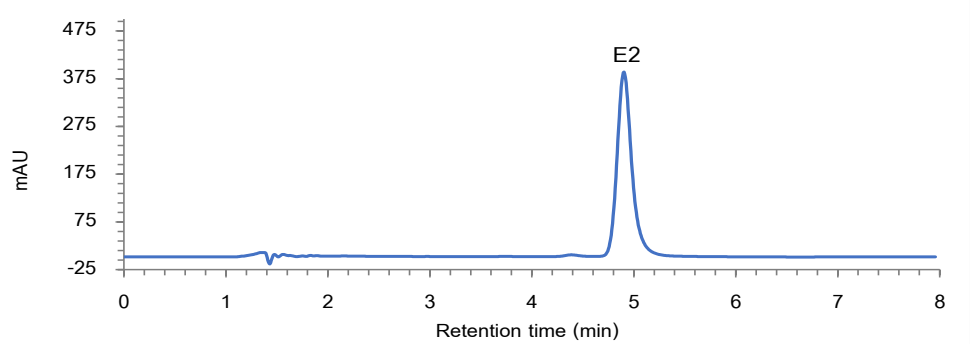

Figure 17 Peak Chromatogram of 5 mg/L standard solution of E2 was extracted

Table 16 Concentration of E2 in water samples from the Saen Saep canal detected from December 2020 to March 2021

Sample	Concentration (µg/L)			
	12/20	01/21	02/21	03/21
Wat Sriboonreung	11.89 ± 1.56	7.41 ± 0.78	3.59 ± 1.05	4.75 ± 2.00
Wat Klang	2.52 ± 3.82	2.40 ± 2.11	1.98 ± 2.77	2.88 ± 2.45
Prasanmit	0.63 ± 1.30	3.16 ± 1.48	2.83 ± 1.25	4.20 ± 2.32
Pratu Nam	ND	1.25 ± 1.94	1.43 ± 1.75	1.78 ± 1.82
Panfa Leelard	1.59 ± 1.21	1.68 ± 2.45	1.67 ± 1.69	2.71 ± 5.17

ND =Not detectable

Investigation of E2 degradation

The decomposition rate of E2 was studied in order to investigate the suitability of the sample storage method. The series of concentrations of E2 standard solutions were prepared in the sequence of 1.0, 3.0, 5.0, 10.0, 20.0, and 50.0 mg/L as

shown in the Figure 18, and measured the absorbance with an HPLC. Then, the prepared E2 standard solutions were kept in an amber glass vial at -20 °C. The absorbance was measured every day for 7 days to analyze the decomposition of E2.

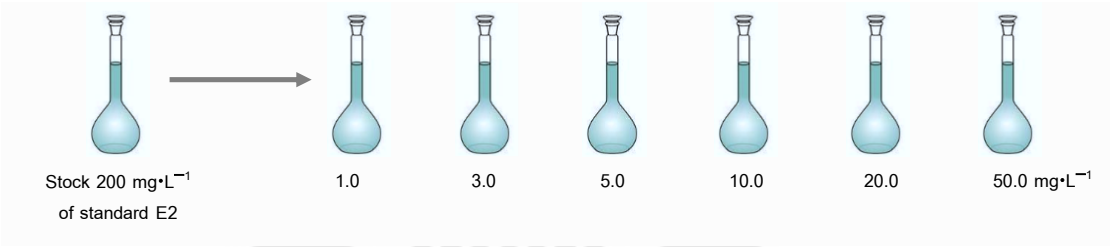

Figure 18 Preparation of E2 standard solutions in the concentration of 1.0, 3.0, 5.0, 10.0, 20.0, and 50.0 mg/L

From the absorbances of E2 measured with HPLC on different days, the peak area in chromatogram of the E2 standard solution was determined. The results showed that the peak areas were approximately the same for 7 days as shown in Table 17. The percentage of relative standard deviation (%RSD) was found to be in the range of 0.34 – 2.56. The calibration curves plotted between the concentration of E2 standard solutions and the peak areas demonstrated a linear relationship with R^2 greater than 0.9995. Additionally, the linear equations showed the approximately same values of the slope and y-intercept for day 1 to day 7 as shown in Figure 19. This result suggests that the sample storing method is suitable for preserve the water sample for 7 days without E2 degradation, which typically about 30% of initial concentration was degraded after 24 h and 70% after 48 h (Zheng, Wang, Bi, & Liu, 2011). Therefore, this storing method was used to store samples from water sources for analysis the E2 content and the effect of E2 in a bioassay.

Table 17 Peak area of E2 std solution measured at Day 1 – Day

Concentration (mg/L)	Peak area							Mean ±SD	%RSD
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7		
1.0	11.01	11.43	11.53	11.49	11.01	11.56	10.89	11.27 ± 0.29	2.56
3.0	31.71	33.56	33.63	34.04	33.04	32.46	33.79	33.18 ± 0.83	2.51
5.0	51.17	52.60	53.55	53.64	52.51	51.47	51.89	52.40 ± 0.96	1.83
10	102.84	110.63	112.15	114.35	112.84	113.45	111.50	111.11 ± 3.85	3.46
20	206.80	205.82	206.62	207.81	206.80	205.78	207.32	216.71 ± 0.74	0.34
50	557.93	564.46	566.04	566.13	567.93	557.23	557.45	562.45 ± 4.71	0.84

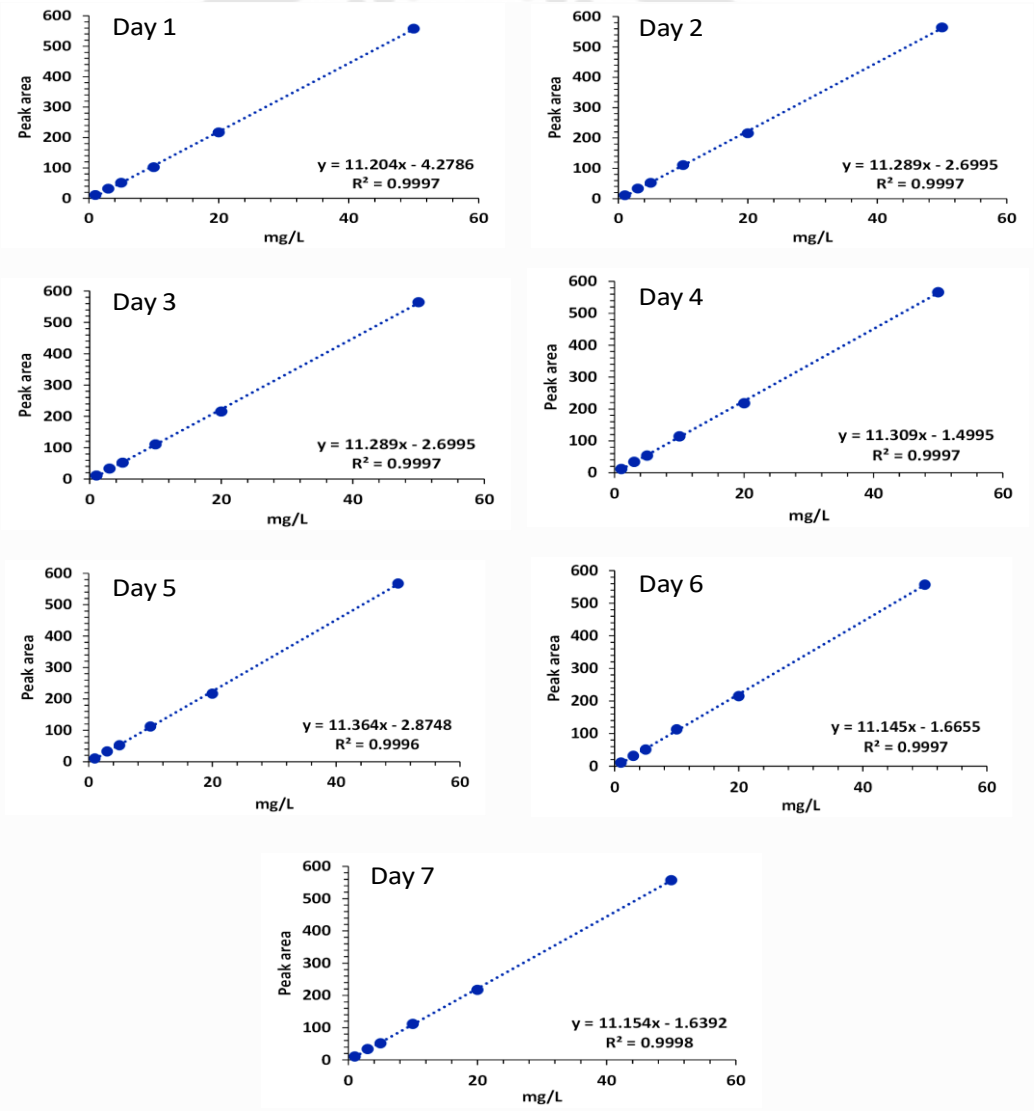

Figure 19 Peak area vs. concentration of E2 standard solution measured at 1 – 7 days

4.1.4 Determination of the concentration of heavy metals in the Saen Saep canal.

From the comparison of digestion efficiency, Method 2 provided more efficiency than Methods 1 and 3. Thus, determination of the quantity of heavy metals in water samples collected from the Saen Saep canal was performed regarding to the digestion Method 2. After digestion step, FAAS was used to measure the concentration of Cd, Cu, Fe, Ni, Pb, and Zn by using two methods, external standard and standard addition, for comparison.

4.1.4.1 Determination of the heavy metal concentration by external standard method

The external standard method, absorbance of water samples measured with FAAS were converted to the concentration of heavy metals by using the linear equations as shown in Table 18. The concentrations of Cd, Cu, Fe, Ni, Pb, and Zn in water sample collected from 5 areas, Wat Sriboonreung, Wat Klang, Prasan Mit, Pratu Nam, and Panfa Leelard Piers, were listed in Table 18. The results showed the contamination of Cd, Cu, Fe, and Zn in all sampling areas, while the quantities of Ni and Pb could not be able to detect. The concentration level of Fe was found to be higher than that of other metals in the range of 0.696-1.9 mg/L, and highest in water sample from Prasan Mit Pier. The results illustrated the contamination of heavy metals in the sequence of Fe > Zn > Cu > Cd. Moreover, a sampling area that contained the highest level of heavy metal was the sample from Prasan Mit Pier. The concentrations of all heavy metals were highest than other areas, which equal to 0.022 ± 0.0089, 0.063 ± 0.0058, 1.9 ± 0.0010, and 0.12 ± 0.0056 mg/L for Cd, Cu, Fe, and Zn, respectively.

Table 18 Concentrations of heavy metals in water samples from the Saen Saep canal measured by external standard method

Metal	Concentrations (mg/L)				
	Wat Sriboonreung	Wat Klang	Prasan Mit	Pratu Nam	Panfa leelard
Cd	0.013 ± 0.0059	0.016 ± 0.0051	0.022 ± 0.0089	0.016 ± 0.0051	0.015 ± 0.015
Cu	0.030 ± 0.012	0.016 ± 0.010	0.063 ± 0.0058	0.060 ± 0.012	0.053 ± 0.0058
Fe	1.08 ± 0.0219	0.696 ± 0.0264	1.9 ± 0.0010	0.891 ± 0.0146	1.29 ± 0.0917
Ni	ND	ND	ND	ND	ND
Pb	ND	ND	ND	ND	ND
Zn	0.074 ± 0.0046	0.079 ± 0.0046	0.12 ± 0.0056	0.094 ± 0.0064	0.10 ± 0.0059

ND = Not detectable

4.1.4.2 Determination of the heavy metal concentration by standard addition method

In order to investigate the effect of matrix in water sample on the determination of heavy metal concentration with FAAS technique, the standard addition method was utilized and compared to the external standard method. In the standard addition method, a standard solution of heavy metal was added into a water sample as described in Section 3. 5. 3. The absorbances of water sample were measured and plotted as a function of concentration of standard solution. Figures 20–24 show the standard curves associated with the standard addition method of each heavy metal measured from water samples collected from Wat Sriboonreung, Wat Klang, Prasan Mit, Pratu Nam, and Panfa Leelard Piers, respectively. The standard curves indicated a linear relationship between the absorbance and concentration of added standard solutions. The values of y-intercept of a straight line shown in the graphs were the absorbance of water sample without standard addition. If the y-intercept equals to zero or negative value, it means that the technique cannot detect such heavy metal. For

example, Ni and Pb in the water samples from Wat Sriboonreung Pier cannot be determined as displayed in Fig. 20 (d) and (e).

From the linear equation of the standard curve obtained from the addition of metal standard solutions in Figure 20–24, the x-intercepts were the concentrations of heavy metals in the water sample and shown in Table 19. The results displayed that concentrations of Ni and Pb were undetectable. This is because the signal response of the water samples without standard addition (y-intercept) is very small (near zero) or negative. The heavy metals found in the water samples were Cd, Cu, Fe, and Zn in the sequence of Fe > Zn > Cu > Cd, which corresponds to the metal content measured with the external standard method (Table 18). Differently, Cd and Cu were found to be the most contaminated in water samples from the Wat Klang, while Fe and Zn were found to be most contaminated in water samples from Prasan Mit Pier.

From the determination of the heavy metal contamination by FAAS technique, both external standard and standard addition methods suggested that four heavy metals comprising Cd, Cu, Fe, and Zn can be determined in water samples collected from the Saen Saep canal. The most contaminated was Fe followed by Zn and Cu, respectively. Cadmium was found to have the least contamination. For the other two metals, Ni and Pb, they cannot be measured by using either the external standard or the standard addition methods, as there may be no contamination of that metal in the water source or the contamination is less than the LOQ of the FAAS instrument, making it not reasonable to measure the amount of Ni and Pb contamination in the water samples. The concentrations of Cd, Cu, Fe, and Zn determined from external standard and standard addition methods are shown in Figure 25 for comparison.

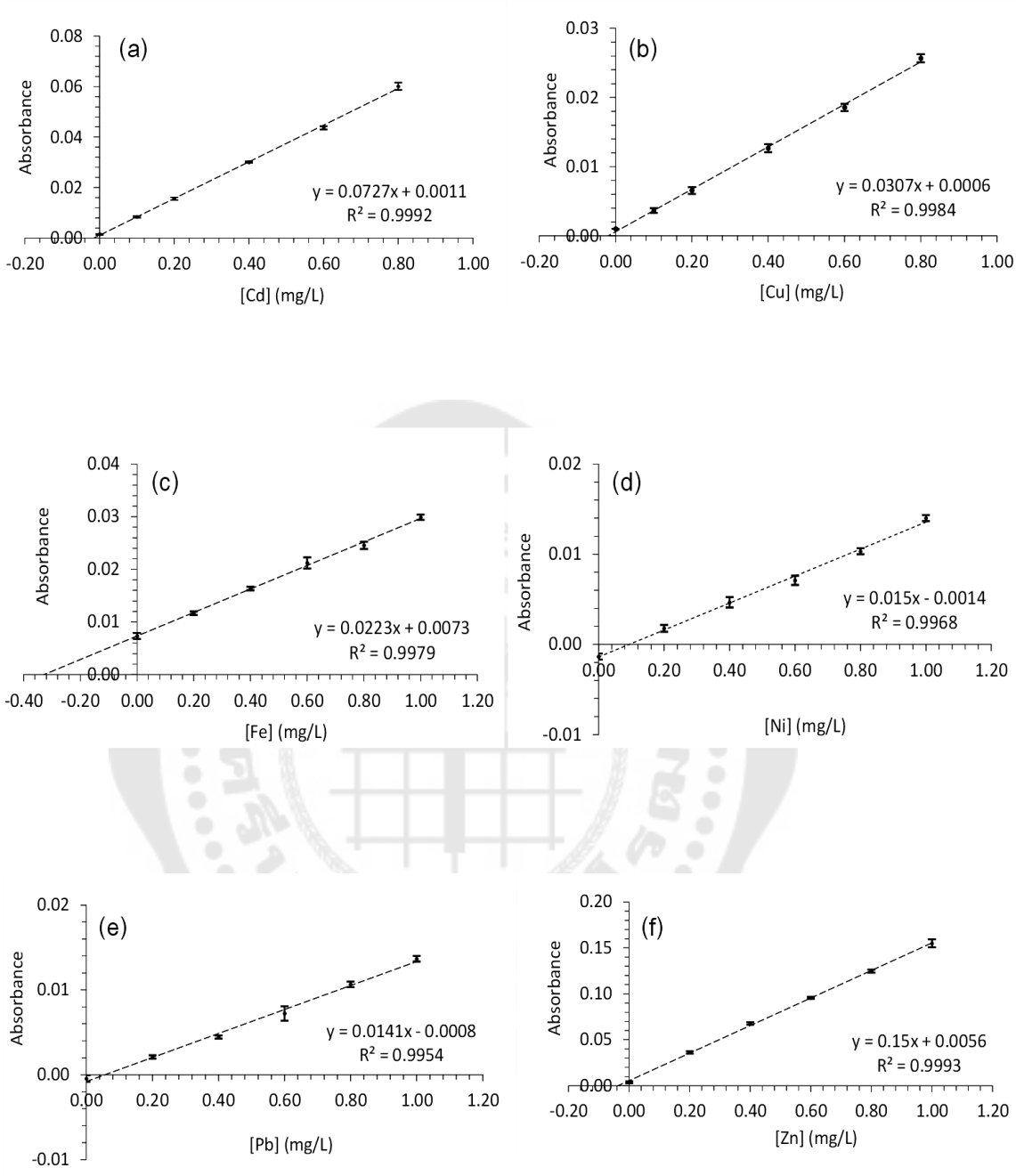

Figure 20 Determination of (a) Cd, (b) Cu, (c) Fe, (d) Ni, (e) Pb, and (f) Zn in water samples from Wat Sriboonreung Pier by the method of standard addition

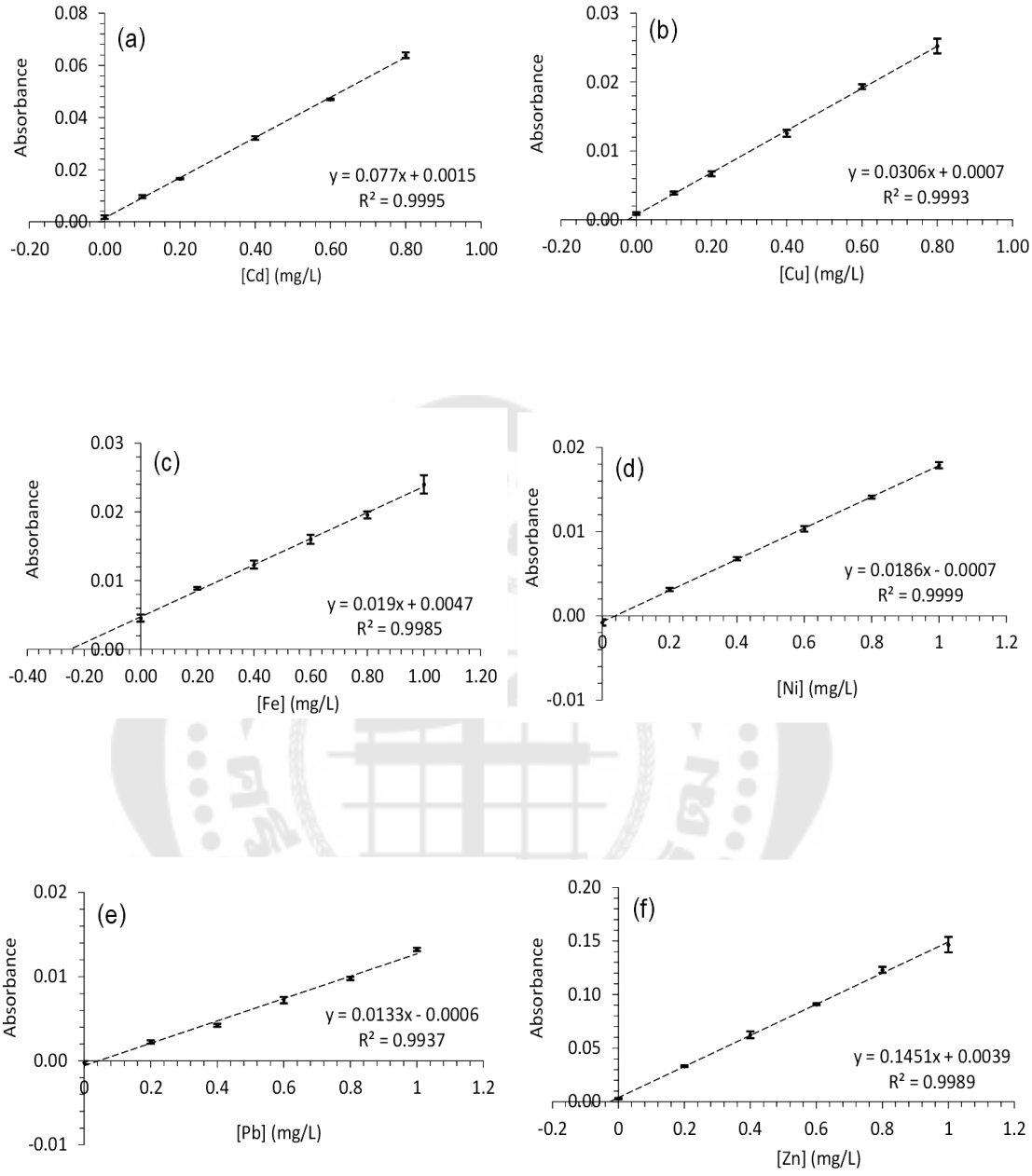

Figure 21 Determination of (a) Cd, (b) Cu, (c) Fe, (d) Ni, (e) Pb, and (f) Zn in water samples from Wat Klang Pier by the method of standard addition

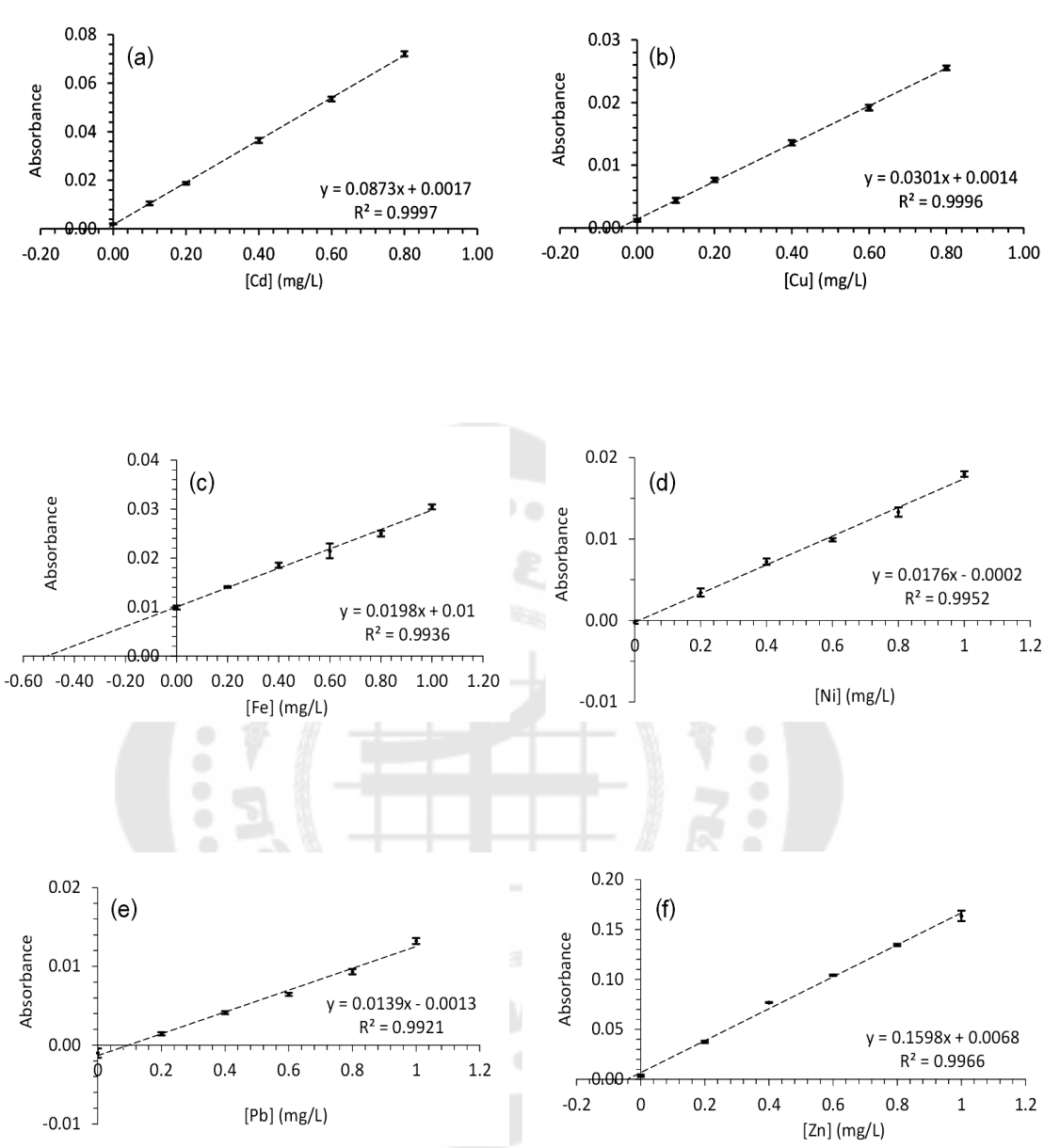

Figure 22 Determination of (a) Cd, (b) Cu, (c) Fe, (d) Ni, (e) Pb, and (f) Zn in water samples from Prasan Mit Pier by the method of standard addition

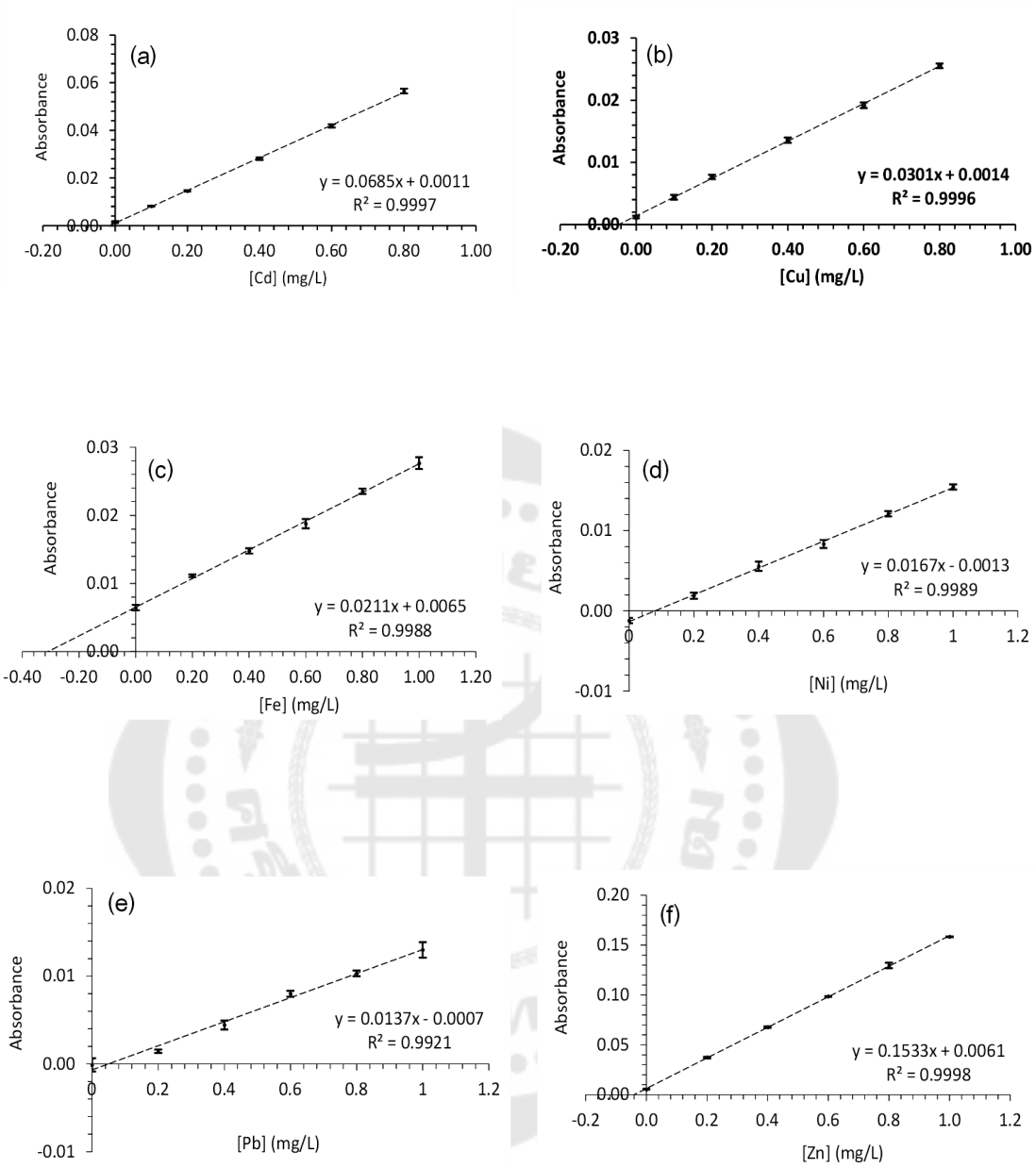

Figure 23 Determination of (a) Cd, (b) Cu, (c) Fe, (d) Ni, (e) Pb, and (f) Zn in water samples from Pratu Nam Pier by the method of standard addition

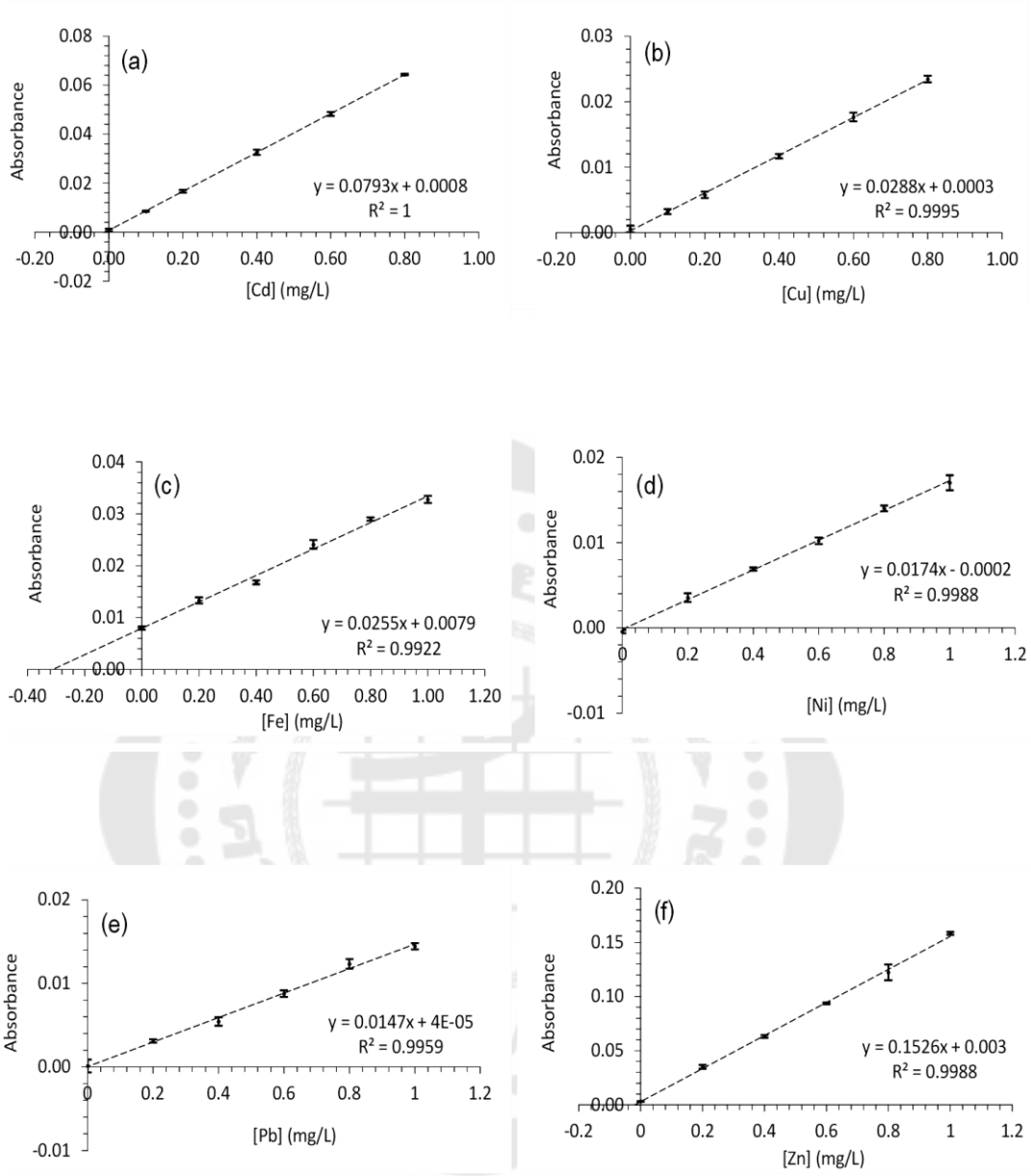

Figure 24 Determination of (a) Cd, (b) Cu, (c) Fe, (d) Ni, (e) Pb, and (f) Zn in water samples from Panfa Leelard Pier by the method of standard addition

Table 19 Concentrations of heavy metals in water samples from the Saen Saep canal measured by standard addition method

Metals	Concentrations (mg/L)				
	Wat Sriboonreung	Wat Klang	Prasan Mit	Pratu Nam	Panfa leelard
Cd	0.015	0.020	0.018	0.016	0.010
Cu	0.020	0.023	0.046	0.019	0.011
Fe	0.327	0.271	0.505	0.308	0.310
Ni	ND	ND	ND	ND	ND
Pb	ND	ND	ND	ND	ND
Zn	0.037	0.027	0.043	0.040	0.020

* ND = Not detectable

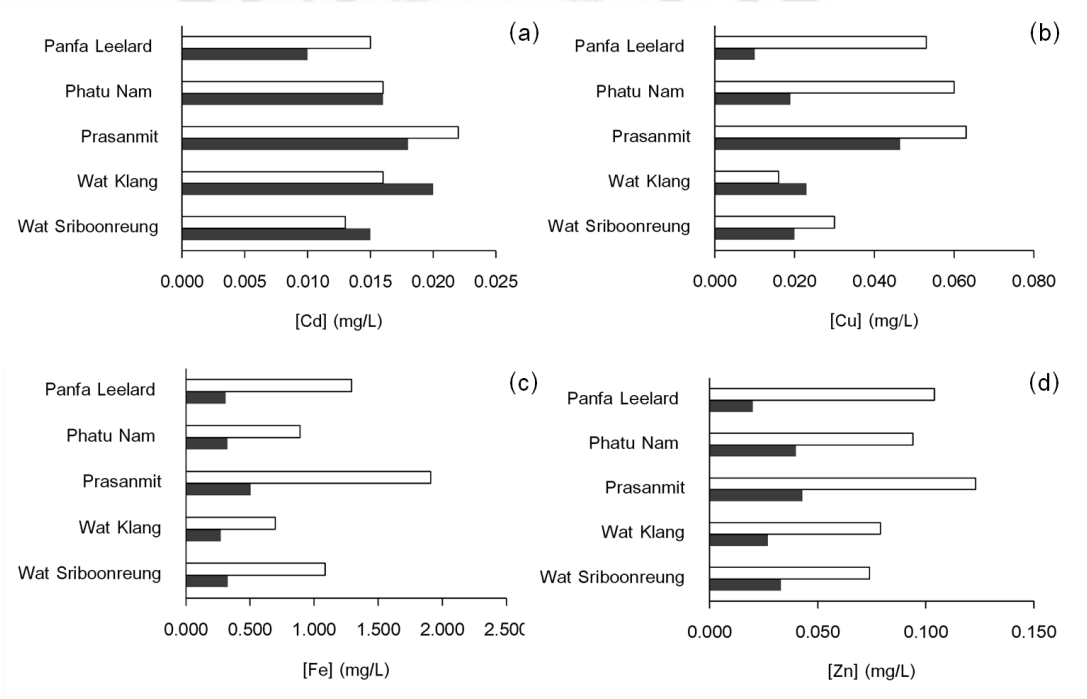

Figure 25 Concentrations of (a) Cd, (b) Cu, (c) Fe, and (d) Zn determined from external standard (□) and standard addition (■) methods

Cadmium (Cd) is not necessary heavy metals for living things which has the most toxic effect compared to other heavy metals and expresses as non-degradable

and persistent (Bazrafshan, Mostafapour, Esmaelnejad, Ebrahimzadeh, & Mahvi, 2016). Cadmium can adversely affect human health on both acute and chronic disorders. The well-known "itai-itai" disease has been comprehended that originates from exposure to cadmium. Other harmful effects of Cd also include damaging on renal damage, emphysema, hypertension, and testicular atrophy. The limit concentration of cadmium in drinking water was suggested by WHO in the value of 0.003 mg/L (Organization, 2008). As shown in Table 18 and 20, the concentration of Cd in surface water sample collected from Prasan Mit Pier displayed the highest value of 0. 022 mg/L compared to other sampling sites. This maximum value was higher than 0. 003 mg/L (maximum concentration from WHO's recommendation). Additionally, the concentrations of Cd observed in all sampling area were found to be higher than Cd limitation in drinking water (0.003 mg/L).

Copper is a metal typically containing in the natural resources. However, the manifest effect of copper in human body is acting as powerful enzyme inhibitor. One of many functions of Cu is an enzyme cofactor for ceruloplasmin, cytochrome oxidase, dopamine β-hydroxylase, superoxide dismutase and tyrosinase (Ashish et al., 2013). The amount of copper that adult human can receive may reach to 12 mg a day, while children are very lower than that. When the copper content in water resources is higher than the threshold value, it can become hazard to an aquatic organism (Hall, Scott, & Killen, 1997). From Table 18 and 20, the concentration of Cu in the Sean Seap canal ranges from 0.001 to 0.063 mg/L, which is not exceed the suggestion limit introduced by WHO (2 mg/L) (Organization, 2008). Water sample from Prasan Mit Pier showed higher concentration of copper than copper concentration in other sampling area. The minimum concentration of Cu was found at Panfa leelard Pier of 0.011 mg/L

Among metals, iron is the most abundant element found in the nature and is absolutely is the most plentiful heavy metal. Iron toxicity from ingestion is a

common poisoning and easily endanger to children. The toxicity to human life is frequently related to an intentional activity such as suicide attempts. Exposure to Prenatal vitamins was reported as one of the causes of iron ingestion in adults (Sane et al., 2018). Ingested iron has acute effects directly to corrosion in the gastrointestinal mucosa, leading to nausea, vomiting, abdominal pain, and diarrhea. In this work, the concentration of Fe in water samples from the Saen Saep canal was highest at Prasan Mit Pier in the concentration of 1.9 mg/L that exceeded the limit concentration of Fe in drinking water from WHO's recommendation (0.3 mg/L) (Organization, 2008). The minimum concentration of Fe was 0.271 mg/L at Wat Klang Pier.

Zinc is a metal element found in all living organisms because it is a necessary element for all living being not exclude human organism. Replication and translation of genetic material require Zn containing proteins and enzymes (Galdes & Vallee, 1983). Human body requires 4 – 10 mg/day of Zinc depending on age and other conditions, such as pregnant women need to obtain Zn up to 16 mg/day. Zinc ingesting into the human body mainly comes from food. Taken zinc in normal dose is not toxic to human being, but excess amount of taken zinc is considered to be toxic as cause impairment of growth and reproduction (Nolan, 1983). The poison of zinc, additionally, has been found to express as same signs as poisoning of Pb (McCluggage, 1991). The expression of zinc toxicosis can be observed in the form of clinical signals such as vomiting, diarrhea, bloody urine, icterus (yellow mucus membrane), liver failure, kidney failure and anemia (Fosmire, 1990). The most common sampling site is Prasan Mit Pier, in which the concentration of 0.12 mg/L was detected as maximum content of zinc. While, the minimum level of 0.02 mg/L was detected in water sample from Panfa leelard sampling area. From the study, it was indicated that the concentration of Zn in the Saen Saep canal was not effect on human health because it was within the limit value of Zn in drinkable water of 3 mg/L (Organization, 2008).

4.2 Mutagenicity and estrogenic activity of E2 and heavy metals of water samples in Saen Saep canal

4.2.1 Mutagenicity testing

Ames test

The mutagenicity of E2 in the water sample from the Saen Saep canal was examined with the Ames test without S9 mix (–S9). The water sample collected from Wat Sriboonreung Pier was used as representative sample of water in the Saen Saep canal because it contained the highest level of E2 concentration. The mutagenic activity tests of E2 extracted from the water sample (E2 extract) was performed against E2 standard solution. In the test assay, the standard solutions of E2 and the E2 extract were diluted with DMSO to give a concentration ranges of 0.1-5 and 0.75-1.25 μg/plate, respectively. Table 20 shows the mean number of revertants/plate, and the The mutation ratio (MR) after treatment with E2 standard solution and E2 extract observed in *Salmonella typhimurium* strains TA98 and TA100 in the absence of S9. In TA98, 4-Nitro-o-phenylenediamine was utilized as a positive control in order to confirm the responsibility of the genotype to mutagens (Parrella et al., 2013). The MR value of 4-Nitro-o-phenylenediamine equals to 9.51 which is more than twofold increase and considered as a positive mutagenic response (China, 2015). The MR values of standard E2 at concentrations of 5, 2.5, and 0.1 μg/plate were also listed in Table 20. as 0.93, 0.84, 0.87, respectively, indicating that there are no mutagenic effects of the E2 standard solutions on TA98. Similarly, the values of MR of the E2 extracts at concentrations of 1.25 and 0.75 μg/plate respectively were 0.99 and 1.06 which lower than two. This suggesting that the mutagenic activity of the E2 extract was similar to that of the negative controls (DMSO). For the test performed with TA100, sodium azide was employed as a positive control and showed the MR value of 2.59. This confirmed that TA100 can respond to a mutagen. Similar to TA98, the MR values of E2 standard solutions and E2 extracts were less than two, representing the non-mutagenic activity. The results from the Ames test demonstrated that E2 in the concentration level found in

the Saen Saep canal did not induce the frame-shift mutations in TA98 and base-pair substitutions in TA100.

The mutagenicity of heavy metals was determined in the TA98 strain with the metal concentration that exhibited 50% and 90% inhibition in bacteria growth as follows: 20-120, 160-200, 160-200, and 120-160 mg/L for Cd, Cu, Fe, and Zn, respectively. The results showed that the mentioned concentrations did not present mutagenic activity in TA98 strain for both with and without S9 enzyme (Wong, 1988). Additionally, Orešcanin et al. investigated the mutagenicity of heavy metals found in sediment extracts in the concentration of 110-610, 80-110, and 72-110 ng/g for Fe, Cu, and Zn. The test results displayed the MR values lower than 2, indicating that the heavy metals in that concentration ranges did not induce mutagenic effect in bacteria strains TA98 and TA100 (Orešcanin, Franekic-Colic, Durgo, & Valkovic, 2002).

Table 20 Ames test results for water sample from Wat Sriboonreung Pier of Saen Saep canal

Sample	Ames test without S9 mix					
	TA98			TA100		
	Dose μg/plate	Revertant/ plate	MR	Dose μg/plate	Revertant/ plate	MR
DMSO	–	23.67 ± 6.51	–	–	213.33 ± 23.29	–
4-Nitro-o-phenylenediamine	2.5	225 ± 13.89	9.51	–	–	–
Sodium azide	–	–	–	1.5	552.33±97.01	2.59
Standard E2	5	22 ± 1.73	0.93	5	195 ± 5.29	0.91
Standard E2	2.5	20 ± 2.0	0.84	2.5	207.33 ± 5.03	0.97
Standard E2	0.1	20.67 ± 4.73	0.87	0.1	203.33 ± 29.54	0.95
E2 extract	1.25	23.33 ± 3.79	0.99	1.25	217.67 ± 14.84	1.02
E2 extract	0.75	25 ± 3.61	1.06	0.75	179.33 ± 25.01	0.84

4.2.2 Estrogenic activity

YES assay

Estrogenic activities of the E2 extracted from water sample collected from the Wat Sriboonreung Pier was determined regarding to YES bioassay using XenoScreen YES/YAS assay kit. The estrogenic activity was determined from the β-galactosidase activity, which can be investigated by measuring the absorbance of the water extracted. After transfer the β-galactosidase into the medium, it will change the yellow substrate CPRG (Figure 26a) into purple product (Figure 26b). This activity can be determined with the absorbance measuring at 570 nm and read the plates at 690 nm for growth of yeast cells. The value of OD_{570} is directly associated with the amount of secreted β-galactosidase and the activity of the tested samples reacting to the active site of enzyme receptor. The estrogenic potency of the E2 extracted from water sample was expressed as estradiol equivalent (EEQ). This correlated to the concentration of E2 which expresses the same response as the sample in the assay. In this study, E2 in the concentration ranges of 4.22–13,347.6 ng/L (in extract) and 67 ng/L (in raw water) were used for estrogenic activity tests against positive control, standard E2 in the concentration of 2.724—2,724 ng/L. The results revealed that the EEQ of E2 extract equals to 4.12 ng/L.

The value of EEQ in the E2 extract suggested that the estrogenic activity might has an effect on aquatic organisms in terms of disrupting the ordinary operation of hormonal and endangering the quality of water resource. This is because the EEQ of E2 extract was higher than 1 ng/L, which previously proposed as a tentative long term predicted no-effect concentration (PNEC) for freshwater life (China, 2015). The aquatic organisms live in the water resources that present EEQ value larger than 1 ng/L might have adverse effect on the reproductive system (Jiang et al., 2012). However, the E2 extract was the pre-concentrated water sample to increase the E2 concentration of 2000 times. Then, the E2 concentration in the extract was higher than that in raw water. The estrogenic activity of raw water, therefore, did not express as shown in Figure 26. The

color response of the yeast screen in column 7 and 8 was similar to that in the wells 1A and 2A, which were negative controls. In this YES bioassay, the EEQ of raw water cannot be determined.

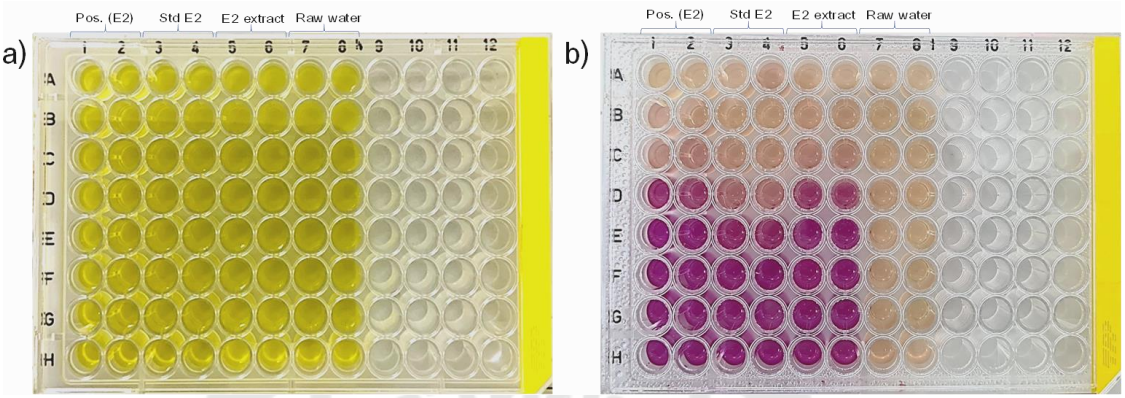

Figure 26 Plate showing (a) the background color of the medium and (b) the response of the yeast screen to estrogenic substants comprising positive control (standard E2, row 1-4), E2 extract (row 5-6), and raw water (row 7-8)

4.3 Risk assessments

From the concentrations of heavy metals found in the water samples, risk assessment can be performed based on the maximum concentration levels of heavy metals in drinking water proposed by WHO (Organization, 2008). The results demonstrate that water samples from all sampling sites have the risk from cadmium and iron because their concentrations were predominant to the recommendation limit (0.003 mg/L of Cd and 0.3 mg/L of Fe). This indicates that long-term ingestion of water from the Sean Seap canal can adversely affect on renal damage, emphysema, hypertension, and testicular atrophy from cadmium. Additionally, there are harmful effects from iron to corrosion in the gastrointestinal mucosa, leading to nausea, vomiting, abdominal pain, and diarrhea, if a large quantity of the water has been ingested into the body, especially to children. Determination of the concentrations of copper and zinc in the water samples found that copper and zinc levels were lower than the limitation recommended in

drinking water (2 mg/L of Cu and 3 mg/L of Zn). Therefore, the toxicitis from copper and zinc were not found in water from the Sean Seap canal. However, there were previously reported that Cu(II) and Zn(II) can increase the mutagenic activities of mutagens 4-nitroquinoline 1-oxide (4NQO) and 2-aminoanthracene (2-AA), respectively (Fujii, Yano, & Takeshita, 2016). Direct drinking water from the Sean Seap canal is still not recommended. However, water from the Sean Seap canal may be utilized for irrigation purpose because almost all heavy metals (except for cadmium) presented their concentrations within the standard limits of irrigation water suggested by FAO of 0.01, 0.20, 5.0, and 2.0 mg/L for Cd, Cu, Fe, and Zn, respectively (Ayers & Westcot, 1985).

For the mutagenicity of heavy metals, their risk assessment can be carried out using previous studies because the concentrations of heavy metals found in the Sean Seap canal were lower than that used for mutagenic test. Concentrations of heavy metals in standard solutions of 20-120, 160-200, 160-200, and 120-160 mg/L respectively for Cd, Cu, Fe, and Zn, and 110-610, 80-110, and 72-110 ng/g for Fe, Cu, and Zn in sediment extracts were used in the Ames test (Orešcanin et al., 2002). The results showed that the heavy metals in the mentioned concentration ranges did not induce mutagenic effect in bacteria strains TA98 and TA100 for both with and without S9 enzyme. Therefore, it can be implied that heavy metals in the concentration levels found in the Sean Seap canal have no mutagenic effect to aquatic organisms.

The risk assessment based on E2 in the water samples was performed from the Ames and YES assays. The results from the Ames test demonstrated that E2 in the concentration level found in the Saen Saep canal did not induce the frame-shift mutations in TA98 and base-pair substitutions in TA100. The estrogenic activities of the E2, investigated from YES assay, demonstrated the EEQ of water extract equals to 4.12 ng/L which might has an effect on aquatic organisms because it was higher than PNEC value. However, the estrogenic activity of raw water did not express (EEQ of raw water cannot be determined). This indicates that at typical concentration of E2, water in the Saen Saep canal can be identified as non-estrogenic activity.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In this study, chemical analyses and biological tests are utilized for the analytical detection of heavy metals and 17β-estradiol (E2) and risk assessments of the water samples collected from the Saen Saep canal in five sapling sites comprising Panfa Leelard, Prasanmit, Phatu Nam, Watklang and Wat sriboonreung Piers.

The amount of E2 and heavy metals (Cd, Cu, Fe, Ni, Pb, and Zn) were determined by using a HPLC-UV and a FAAS, respectively. Before the measurements, a pre-treatment process was optimized. For the E2 determination, a preconcentration of water samples with LLE (method B: extraction solvent was a mixture of diethyl ether and hexane (75:25)) was performed followed with the method Z of SPE extraction (conditioning of cartridges: 5 mL Ethyl acetate and 5 mL DI water; washing: 20% ethyl acetate; and elution: 3×3 mL ethyl acetate). This pre-treatment step showed the recovery of E2 higher than 84%. Determination of E2 content with HPLC-UV indicated that concentrations of E2 in water samples collected from the Saen Saep canal were in the range of 0.63 – 11.89 μg/L. The E2 levels in the samples taken from Wat Sriboonreung were in the range of 3.59 ± 1.05 to 11.89 ± 1.56, Prasan Mit 0.63 ± 1.30 to 4.20 ± 2.32, Wat Klang 1.98 ± 2.77 to 2.88 ± 2.45, Panfa Leelard 1.59 ± 1.21 to 2.71 ± 5.17, and Pratu Nam 1.25 ± 1.94 to 1.78 ± 1.82 μg/L. The water samples from Wat Sriboonreung Pier had the highest E2 content in all sampling periods and was found to be highest in December 2020 of 11.89 μg/L, followed by water samples from Wat Klang, Panfa Leelard, and Prasan Mit Piers, respectively. From January to March 2021, the concentration of E2 in water samples was in the sequence of Wat Sriboonreung > Prasan Mit > Wat Klang > Panfa Leelard > Pratu Nam.

Similarly, digestion step was required for the heavy metal determination with FAAS. The recovery obtained from Method 2 (use of Aqua regia and perchloric) were satisfactory with a mean recovery of 95.94% and the all detected heavy metals showed

a recovery above 90% with relative standard deviations below 10%. Thus, determination of the quantity of heavy metals in water samples collected from the Saen Saep canal was performed regarding to the digestion Method 2. After digestion step, FAAS was used to measure the concentration of Cd, Cu, Fe, Ni, Pb, and Zn by using two methods, external standard and standard addition. In the first method, there were contamination of Cd, Cu, Fe, and Zn in all sampling areas, while the quantities of Ni and Pb could not be able to detect. The concentration level of Fe was found to be higher than that of other metals in the range of 0.696-1.9 mg/L, and highest in water sample from Prasan Mit Pier. The results illustrated the contamination of heavy metals in the sequence of Fe > Zn > Cu > Cd. Moreover, a sampling area that contained the highest level of heavy metal was the sample from Prasan Mit Pier, which contained 0.022 ± 0.0089, 0.063 ± 0.0058, 1.9 ± 0.0010, and 0.12 ± 0.0056 mg/L of Cd, Cu, Fe, and Zn, respectively. For the standard addition technique, concentrations of Ni and Pb were undetectable while the concentrations of heavy metals were found in the sequence of Fe > Zn > Cu > Cd, which corresponds to the metal content measured with the external standard method. Differently, Cd and Cu were found to be the most contaminated in water samples from the Wat Klang, while Fe and Zn were found to be most contaminated in water samples from Prasan Mit Pier.

Finally, the mutagenic and estrogenic activities of E2 was examined with the Ames test and YES bioassay, respectively. In the Ames test, the water sample collected from Wat Sriboonreung Pier was used as representative sample of water in the Saen Saep canal because it contained the highest level of E2 concentration. The mutagenic activity of the E2 extract was similar to that of the negative controls observed in TA98 and TA100 which provided the MR values less than two, representing the non-mutagenic activity. The results from the Ames test demonstrated that E2 in the concentration level found in the Saen Saep canal did not induce the frame-shift mutations and base-pair substitutions in *Salmonella typhimurium* TA98 and TA100, respectively. Estrogenic activities of the E2 extracted from water sample collected from the Wat Sriboonreung Pier was determined regarding to YES bioassay. The estrogenic

potency of the E2 extracted from water sample was expressed as EEQ. The E2 extracts in the concentration range of 4.22—13,347.6 ng/L and the raw water in the concentration of 67 ng/L were used for estrogenic activity tests against positive control. The results revealed that the EEQ of E2 extract equals to 4.12 ng/L while EEQ of the raw water did not be determined. This result was reasonable because the E2 content in the extract was higher than that in raw water in the Saen Saep canal. The bioassays demonstrated that the E2 level found in the water collected from the Saen Saep canal did not express the mutagenic and estrogenic activities.

From the determination of heavy metals and E2 concentration and biological studies on mutagenic and estrogenic activities of heavy metals and E2, the assessment of risk of water in the Saen Saep canal was done. Based on bioassays, water in the Saen Saep canal was identified as non-estrogenic and non-mutagenic activities. The main hazards found in the water come from contaminations of cadmium and iron, in which the concentrations were higher than standard limit in drinking water. This research indicates that water in the Saen Saep canal is suitable for irrigation and transportation purposes and not recommended for direct consumption usage.

5.2 Recommendations

In the Ames test, the mutagenic activity of the E2 should be investigated by using *Salmonella* typhimurium strains TA98 and TA100 with S9 mix on order to determine the mutagenic activities of an active metabolite and/or final product from a metabolism process. Additionally, mutagenic activity of the E2 should be performed on both E2 extract and raw water.

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