AMERICAN UNIVERSITY OF BEIRUT

FATE OF 17α-ETHYNYLESTRADIOL IN THE PRESENCE OF VEGETABLE WASTES

by
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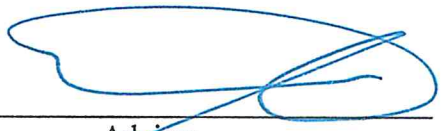

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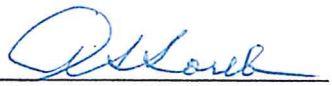

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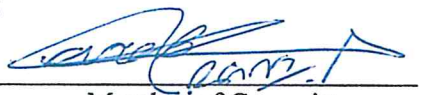

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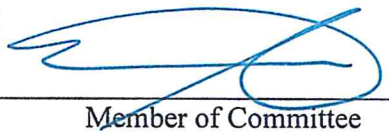

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AN ABSTRACT OF THE DESSERTATION OF

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Estrone (E1), Estradiol (E2), 17α-Ethynylestradiol (EE2), and Estriol (E3) are estrogens that can result in endocrine disrupting effects in human and wildlife populations, impacting reproduction and development. Estrogens display characteristics typical of organic compounds and are typically excreted in conjugated forms, with rapid conversion to the unconjugated molecules in the environment. Entry to the environment can occur via multiple pathways which minimize the possibility of source control as a remediation strategy and, as such responsibility for their removal falls on wastewater treatment plants. As expected the majority of research on the control of estrogens is reported in the municipal wastewater treatment literature. A recent study assessed the abiotic transformation of Estrogens in the presence of model vegetable matter (rabbit food). That study stipulated the catalytic or enzymatic polymerization of E1, E2, E3 and EE2 in the presence of rabbit food and dissolved oxygen. Greater than 80% reduction in the parent compounds was achieved for each target estrogen after 72 h of exposure. Interestingly testosterone, androstenedione and progesterone did not undergo any such transformation when exposed to the same conditions. It was speculated that the presence of a phenolic group in the estrogen skeleton was responsible for polymer formation. This study was undertaken to demonstrate a treatment process for the removal of estrogens from water matrices using vegetable matter.

Comparison of four extraction procedures Accelerated Solvent Extraction (ASE), conventional solvent based extraction, Soxhlet extraction, and ultra-sonication was carried out using environmentally relevant concentrations. ASE and ultra-sonication techniques proved the most efficient in extracting EE2 from the three substrates investigated (rabbit food (RF), Soil and Ottawa Sand. ASE yielded recoveries of EE2 of 81.2%, and 77.5% from RF and soil substrates, respectively. Sonication yielded comparable recoveries of 73.4, and 72.3 percent EE2 from RF and soil, respectively. These techniques proved to be accurate and less time-consuming producing reproducible results and eliminating the need for freeze-drying (lyophilizing). Soxhlet and conventional solvent-based extractions proved to be up to 20 times more time consuming than ASE and ultra-sonication extractions. Conventional solvent extraction results yielded recoveries of 53, and 78.2% EE2 from RF and soil substrates, respectively. Soxhlet extraction yielded 62.5, and 34.5% EE2 recoveries from RF and

soil substrates, respectively. Lyophilizing the substrates prior to extraction proved necessary in both conventional and Soxhlet extraction.

The efficiency of RF as a model vegetable material in the removal of EE2 was investigated using column experiments where flow rate and the mass of rabbit food utilized were varied. Experimental data showed that the attenuation capacity of RF for EE2 increased with increasing mass of the RF used and decreased with increasing flow rate. The attenuation capacity of RF ranged between 64 and 232 μg/g. Thomas and Yoon-Nelson models were used to analyze the column experimental data. The data collected showed the occurrence of abiotic transformation and provided a viable approach for implementing such a process in the treatment of EE2.

The fate of EE2 in the presence of various vitamins, minerals, and vegetables wastes was studied. Vitamins and minerals were tested individually to ascertain their potential to affect the observed transformation of EE2. The lowest observed transformation capacity, m_t, was for mint. On the other hand, carrot leaves, corn, and radish leaves had comparable m_t values to those of RF with radish leaves exceeding that of RF by approximately 86 ng/g. Transformation of EE2 was significantly enhanced upon exposure to radish leaves. The fraction of EE2 that was transformed was 68.3 and 58.9% for radish leaves and RF, respectively. Magnesium sulfate affected 18.8 % transformation of EE2. While the fraction of EE2 transformed in the presence of a mixture of fiber multivitamins and minerals reached 39.5. Premix resulted in a greater transformation of EE2 at 57 %.

CONTENTS

ILLUSTRATIONS

TABLES

ABBREVIATIONS

AC: Activated Carbon

AEM: Anion-exchange membrane

ASE: Accelerated solvent extraction

CEM: Cation-exchange membrane

DE: Diatomaceous earth

E1: Estrone

E2: Estradiol

E3: Estriol

EDC: Endocrine disrupting compounds

EE2: 17α-Ethynylestradiol

H: Henry's law Constant

HLB: Hydrophile-Lipophile balance

HRth: Hormone replacement therapy

ICP: Inductive coupled plasma

ISTD: Internal Standard

LC: Liquid chromatography

LDH: Layered double hydroxide

LOD: Limit of detection

LOQ: Limit of quantification

MIP: Molecularly imprinted polymer

MRL: Minimum reporting level

MRM: Multiple reaction monitoring

mRNA: Messenger RNA

MS: Mass spectroscopy

NF: Nano filter

NOM: Natural organic matter

PAHs: polycyclic aromatic hydrocarbons

POPs: Persistent organic pollutants

RF: Rabbit food

RO: Reverse Osmosis

RSD: Relative standard deviation

SE: Soxhlet extraction

SPE: Solid phase extraction

UF: Ultrafiltration

WW: Wastewater

WW: Wastewater

WWTP: Wastewater treatment plant

CHAPTER I

INTRODUCTION

Endocrine Disrupting Compounds (EDCs) comprise a diverse group of heterogeneous contaminants, with the common effect of altering the normal functioning of the endocrine system of living organisms (Al-Khateeb et al. 2014; Birkett and Lester 2003; Bolong et al. 2009; Muz et al. 2013). Due to their adverse impact on human and ecosystem health, EDCs are generating attention among scientific communities worldwide (Tan et al. 2007a). Estrogens are EDCs that display characteristics typical of organic compounds, including low solubility in water, high affinity to organic matter, and lipophilic traits that lead to ready diffusion through the cell membrane and into the cytoplasm of target cells. Although typically excreted in conjugated forms, quick conversion to unconjugated molecules occurs in the environment (Hanselman et al. 2003; Lai et al. 2000). Estrone (E1), Estradiol (E2), 17 α-Ethynylestradiol (EE2), and Estriol (E3) can result in endocrine disrupting effects in human and wildlife populations, affecting reproduction and development (Ogino et al. 2007; Sone et al. 2004; Sumpter and Jobling 1995). Research has shown high potency of Estrogens at concentrations as low as one ng per liter (Caldwell et al. 2010; Irwin et al. 2001; Jobling et al. 1995). The effects of human exposure to EDCs at varying concentrations remain unclear due to the deviation of these compounds from the typical linear dose-response relationships used in classic toxicology, where greater exposure to a chemical has increased health effects (Vogel 2004). On the other hand, impact of exposure to EDCs on wildlife is well documented and includes disturbance of the immunological system and fertility,

reproductive failure, feminization and masculinization, and altered sexual development (Hester and Harrison 1999). Fish species proved to be the most affected due to palpable impacts imparted by estrogens (Sumpter and Jobling 1995; Sumpter and Johnson 2005).

The spread of Estrogens and other EDCs in the Environment is well documented. According to Jobling et al. (1995) and Mes et al. (2005) human and animal excretion is cited to be the main source of steroidal hormones in aquatic environments. Johnson et al. (2000) studied the increase in concentration of estrogen secretions during pregnancy. The corresponding excretion levels of E1, E2, and E3 are 600, 259, and 6,000 pg per day, respectively, and among females who are taking birth control pills, the excretion of EE2 is around 35 pg per day. Additionally, the plant-processing industry also significantly contributes toward the presence of phytoestrogens in surface water bodies, this includes biodiesel production facilities (Lundgren and Novak 2009).

After excretion, these compounds enter the environment via multiple pathways including wastewater treatment plant (WWTP) effluents and run-off from manure applied to soils (Khanal et al. 2006). The majority of research on the environmental fate of estrogens is reported in the municipal wastewater treatment literature (Hamid and Eskicioglu 2012). Conventional WWTPs are considered effective in removing nutrients and solids from wastewater. Estrogens, on the other hand, follow a different pathway and are not completely removed (Auriol et al. 2007). Therefore, the residual concentrations of estrogens in treated sewage remain at levels that cause adverse effects on aquatic life and ecosystems (Auriol et al. 2006a; Auriol et al. 2006b).

The removal of estrogens in WWTP has been extensively studies. Ternes et al. (1999), reported on the presence of estrogens in WWTPs in Germany and Canada. E1, E2, and EE2 were found in several samples at concentrations ranging between 15 and 70

ng/L. Lishman et al. (2006), reported concentrations of E2 at 6–14 ng/L in the influent and less than 5 ng/L in effluent samples from facilities in Canada. Zorita et al. (2009), assessed WWTPs is southern Sweden and concluded that concentrations of EE2 below 10 ng/L (LOQ) were achievable after tertiary treatment. Additionally Braga et al. (2005) reported concentrations of estrogens in WWTP influents of 55, 22, and less than 5 ng/L of E1, E2, and EE2, respectively. In agreement with the aforementioned findings Janex-Habibi et al. (2009) conducted a study on thirteen wastewater treatment plants located in six countries and found that the influent water contained similar concentrations of Estrogens as in previous works. On the other hand, effluent water had a concentration of 2.8 ng/L for EE2 and a range of 1 to 73 ng/L for E1.

The first natural mechanism for removal/inactivation of estrogens is in the body, where estrogens are metabolized in the liver. E1, E2, and E3 are the natural estrogens that enter a series of reactions that eventually results in conjugation with polarizing substances, leading to loss of estrogenic activity (Matsushima et al. 2005; Zamek-Gliszczynski et al. 2006). The possibility of estrogens undergoing the same pathway in water matrices is possible, never the less de-conjugation and the return of estrogenic activity seems to be a more likely outcome (Lee et al. 2003). Light-induced estrogen degradation, which mainly includes photolysis in association with a photocatalyst such as TiO2 is reported to have a significant effect on reducing estrogens in water matrices. Both Ohko et al. (2002) and Nakashima et al. (2002) concluded that photo-degradation with a titanium dioxide (TiO2) catalyst displayed 99 and 98 percent degradation of estrogens after 30-min and 1 hour UV exposure, respectively.

Liu et al. (2009) explained that estrogens have a hydrophobic character and, as such concentrate on solid matrices such as sludge. Additionally soil sediments and bio-

solids were proven to have a similar impact on estrogens and other EDCs (Heidler and Halden 2008; Jones-Lepp and Stevens 2007). Consequently, the removal of estrogens from different matrices proved to be difficult to model. Sarmah et al. (2008) reported that the majority of studies focused on physio-sorption as the major mechanism for removal and underestimated potential abiotic transformations. Understanding the mechanisms involved in estrogen removal from water can aid in mitigating health and environmental risks resulting from exposure to EDCs. The need to develop a model for removal of estrogens from water matrices is importance. Most importantly, the understanding of abiotic transformation of estrogens as representative compounds of phenolic EDCs can be extrapolated to other micro-pollutants with similar chemical structure (BPA and alkylphenol surfactants, for example).

CHAPTER II

REVIEW OF LITERATURE

A. Introduction

EDCs comprise a diverse group of heterogeneous contaminants, with the common effect of altering the normal functioning of the endocrine system of living organisms (Al-Khateeb et al. 2014; Birkett and Lester 2003; Bolong et al. 2009; Muz et al. 2013). An endocrine disruptor is an exogenous substance or mixture that alters functions of the endocrine system that lead to adverse effects in an intact organism, or its progeny, or subpopulations. EDCs interfere with synthesis, secretion, transport, binding, action or elimination of natural hormones in the body, which are responsible for the maintenance of homeostasis, reproduction, development, and behavior (Chen et al. 2010; Vos et al. 2000; Zhang et al. 2014). According to Nash et al. (2004), EDCs represent a class of substances that interact with the endocrine system, potentially impairing cell proliferation and differentiation, which can consequently contribute to infertility. EDCs are a major environmental threat due to their adverse effects on human and ecosystem health. Consequently, they are generating attention among scientific communities worldwide (Tan et al. 2007a).

Sumpter and Johnson (2005) explained that the characteristics of EDCs enable this group to mimic endogenous hormones, and antagonize normal hormones, which alters the proper pattern of hormone synthesis or metabolism, or modifying hormone receptor levels. EDCs have the potential to interfere with normal reproduction and development, which is controlled by an array of hormonal signals. Studies showed that the exposure to low concentrations of EDCs could be linked to increased testicular cancer,

falling sperm counts, increased breast cancer, and early puberty (Blumberg et al. 2011; Hopert et al. 1998; Knez 2013; Sumpter and Johnson 2005; von Saal et al. 2010). Endocrine disruptors include both agonist (a chemical that can combine with a receptor to produce a physiologic reaction typical of a naturally occurring substance) and antagonist (a substance that interferes with the physiological action of other compounds (Kavlock et al. 1996; Lamb Iv et al. 2014). Pesticides, herbicides, PCBs, dioxins, alkylphenols, pharmaceuticals, and other synthetic compounds are considered EDCs. Although much attention has been focused on anthropogenic EDCs (xenoestrogens), natural hormones present a potentially significant and potent environmental load (Abargues et al. 2013). Estrogens are a major group of these EDCs, and are present in larger concentrations than other EDCs.

B. Estrogens as EDCs

The terms estrogen, estrogenic compound and estrogenic agent refer to the general group of steroid compounds that function as the primary female sex hormones while specific estrogens refer to the specific compounds. Steroidal compounds, including estrogens, represent a hormonal class generally synthesized from cholesterol. E1, E2, EE2, and E3 display molecular structures similar to cholesterol, with a five-carbon ring attached to three six-carbon rings (Figure 1).

These Estrogens display characteristics typical of organic compounds, including low solubility and high affinity to organic matter as summarized in Table 1. Lipophilic traits lead to ready diffusion through a cell membrane and into the cytoplasm of target cells. Although typically excreted in a conjugated form, quick conversion to unconjugated molecules occurs in the environment. The primary endogenous estrogen which binds to

the human estrogen receptors is Estradiol (E2), which can be oxidized in metabolic processes to form E1, and is further transformed into E3 (Hanselman et al. 2003; Lai et al. 2000). These three estrogens encompass the natural estrogens found in the environment (Hanselman et al. 2003).

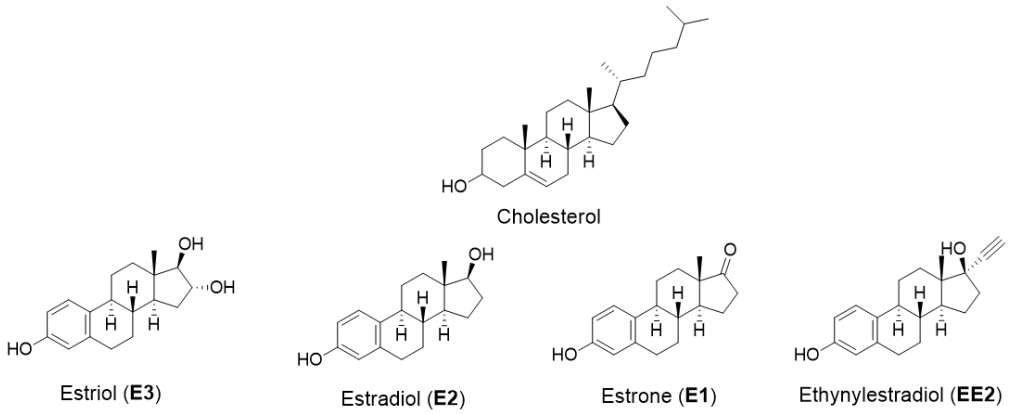


Figure 1. Structures of E1, E2, E3, EE2 and Cholesterol

Table 1. Physicochemical properties of Estrogens E1, E2, E3, and EE2 (Ying et al. 2002)

Estrogen	Molecular weight (g/mol)	Water solubility (S_w) (mg/L at 20 °C)	Vapour pressure (mm Hg)	Octanol–water partition coefficient (log K_{ow})
Estrone (E1)	270.4	13	2.3×10^{-10}	3.43
17β-Estradiol (E2)	272.4	13	2.3×10^{-10}	3.94
Estriol (E3)	288.4	13	6.7×10^{-15}	2.81
17α-Ethynylestradiol (EE2)	296.4	4.8	4.5×10^{-11}	4.15

C. Sources of Estrogens

Human and animal excretion was cited as the main source of steroidal hormones in the aquatic environment (Jobling et al. 2006; Mes et al. 2005). EDCs enter the

environment via multiple pathways including wastewater treatment plant effluents and run-off from animal feed-lots and farmland receiving animal manure, reaching the receiving waters. Excretion rates of the three natural hormones (E1, E2, and E3) are given in

Table **2**.

Table 2. Estimates of estrogen excretion by humans (per person) in µg/day (Johnson et al. 2000)

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

According to Johnson et al. (2000), pregnant women excrete up to 600, 259, and 6000 ug/day of E1, E2, and E3 respectively. The study reported the amount of EE2 secreted from females who are taking birth control pills is around 10.1 ug/day. Additionally the plant-processing industry, such as biodiesel production facilities, significantly contributes toward the presence of phytoestrogens in surface water bodies (Lundgren and Novak 2009). The majority of research in the field of environmental estrogens is in the municipal wastewater treatment literature. WWTPs are effective in removing nutrients and solids from wastewater, estrogens on the other hand, cannot be completely removed. After excretion, the natural and synthetic hormones and their metabolites eventually reach WWTP (**Figure 2**) (Hamid and Eskicioglu 2012).

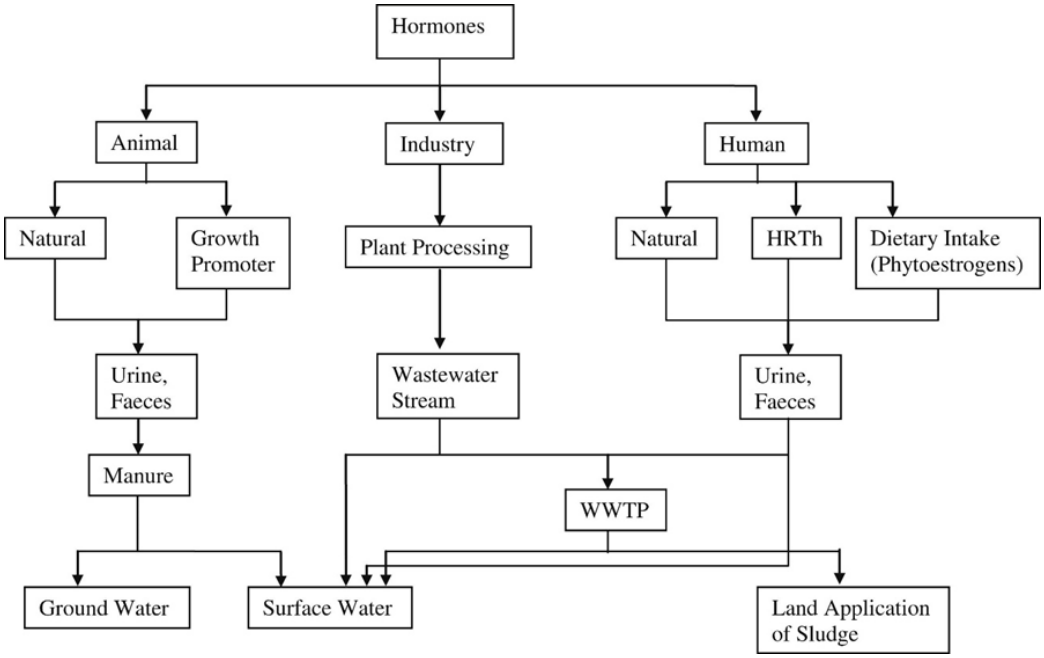


Figure 2. Sources and pathways of steroidal hormones in environment (HRTh: hormone replacement therapy, WWTP wastewater treatment plant) (Hamid and Eskicioglu 2012).

Increased population and livestock-farming practices are considered to be major sources of estrogens in the environment. Steroid drugs, used as growth promoters in livestock result in further increases in the levels of execration (Wise et al. 2011). It is important to note that different estrogens are common to different species of animals.

Table 3 summarized the daily excretion rate for estrogenic compounds for different livestock.

Table 3 Daily excretion rate of estrogenic compounds for different livestock

Species	Type	Total estrogen excreted in urine (µg/day)	Total estrogen excreted in feces (µg/day)	Total estrogen excreted (µg/day)
Cattle[a]	Calves	15	30	45
	Cycling cows	99	200	299
	Pregnant	320–104,320	256–7,300	56–111,620
Pig[b]	Cycling sow	82	21	103
	Pregnant	700–17,000	61	–
Sheep[a]	Cycling ewes	3	20	23
	Rams	3	22	25

a. (Lange et al. 2002), b. (Johnson et al. 2000)

Relatively high levels of steroid hormones occur in certain areas, as a result of the concentration of the population in large cities. Because of the widespread use of contraceptives or other drugs prepared with synthetic estrogens in hormone replacement therapy, the environmental presence of synthetic estrogens such as EE2 might have increased in recent decades (Zhang 2006).

D. Impact of Estrogens

Predicting the effects of human exposure to EDCs at varying concentrations is difficult, as EDCs often do not follow the typical linear dose-response relationships used in classic toxicology, where greater exposure to a chemical has a higher health risk (Vogel 2004). Hormones, including the estrogenic compounds E1, E2, EE2, and E3 can result in endocrine disrupting effects in human and wildlife populations, impacting reproduction and development (Ogino et al. 2007; Sone et al. 2004; Sumpter and Jobling 1995). Although endogenous, the potency of these compounds can lead to detrimental health

consequences at concentrations as low as one ng per liter (Caldwell et al. 2010; Irwin et al. 2001; Jobling et al. 1995; Routledge et al. 1998). Despite concerns over the presence of hormones in aquatic systems, a thorough understanding of both acute and chronic exposure to the hormones remains deficient.

EDCs are categorized with respect to origin of each compound (Burkhardt-Holm 2010; Esplugas et al. 2007; Hamid and Eskicioglu 2012; Hamid and Eskicioglu 2013):

1. Natural estrogenic/androgenic hormones: E2, E1, testosterone.
2. Synthetic hormones: EE2, diethylstilbestrol, 19-norethindrone.
3. Phyto- and mycoestrogens: daidzein, genistein, zearalenone.

1. Wildlife impact

The impact of exposure to EDCs on wildlife is well documented and includes disturbance of the immunological system and fertility, reproductive failure due to thinning of eggshells, feminization and masculinization, and altered sexual development, among many others (Hester and Harrison 1999). Much of the research conducted on higher-level organisms has focused on fish, partially due to the palpable impacts imparted by estrogens (Sumpter and Jobling 1995; Sumpter and Johnson 2005). Sumpter and Jobling (1995) authored a landmark paper describing vitellogenin synthesis in male fish maintained in municipal wastewater treatment effluent. This study also documented that a mixture of various estrogenic contaminants further enhanced the production of vitellogenin compared to each of the compounds when tested individually. More recent studies revealed the major health effects associated with exposure of different fish species to estrogenic compounds including altered sexual development, presence of intersex

species, changed mating behavior, with high incidence of intersexuality reported in wild roach (Hamid and Eskicioglu 2012; Hamid and Eskicioglu 2013; Pollock et al. 2010). Fathead minnows exposed to environmentally relevant concentration of EE2 in an experimental lake area exhibited feminization characteristics of the males through vitellogenin production that led to a near collapse of the species in the lake (Kidd et al. 2007). Early exposure to EE2 at a concentration of 9.86 ng/L resulted in diminished courting behavior of female zebrafish (Danio rerio) resulting in reduced female reproductive success (Coe et al. 2010; Kloas et al. 2009). Zebrafish embryos exposed to 2, 20, and 200 ng/L concentrations of progesterone, levonorgestrel, norethindrone, and the low-specificity PR antagonist RU-486 for up to 144 h exhibited altered expression levels for nPR mRNA, other steroid receptors, and steroidogenic enzyme genes, depending on the studied compound (Zucchi et al. 2012).

E1 and E2 led to a de-masculinization of male rainbow trout (Oncorhynchus mykiss) at a concentration of 25 ng/L, emphasing the potency of these compounds (Routledge et al. 1998). Estrogenic effects on other species were also prominent, amphibians also showed similar effects to fish. Laboratory studies have induced vitellogenesis in male red-eared turtles (Trachemys scripta) and African clawed frogs (Xenopus laevis) through 17β-estradiol injections (Palmer and Palmer 1995). A recent study on African clawed frogs experimentally exposed to gestagens and other steroid hormones as tadpoles or adults have altered reproduction, development, and behavior. It has been shown that addition of progesterone or a combination of progesterone and estrogen to water in which female Xenopus are maintained induces oocyte maturation or ovulation, respectively (Hoffmann and Kloas 2012; Ogawa et al. 2011).

2. Human health impact

Despite the compelling evidence on the adverse health effects of estrogenic hormones on humans, this issue is still debatable. Some studies have reported lower sperm count, declining male reproductive health and breast cancer as an aftermath of increased exposure to endogenous and exogenous estrogenic compounds (Delbes et al. 2006). Conclusive cause-effect relationships between environmental chemical exposure and adverse physiological impacts are difficult to establish due to the preponderance of unknown and conflicting variables. Evidence indicates detrimental trends in health and reproductive vigor exist in association with EDCs; however, the plethora of compounds that humans are exposed to makes it often infeasible to isolate the effects of a single compound. The following discussion on estrogenic impacts on humans will refer to estrogenic agents in general, unless otherwise specified. Suggestions for linkage between exposure to endocrine disruptor and human health implications include (Harrison et al. 1997):

1. Increased incidents of breast, testicular and prostate cancer
2. Reduced sperm counts and quality
3. Increased cases of cryptorchidism (undescended testes) and hypospadias (malformation of the penis)
4. Increased occurrence of polycystic ovaries in women.

Physiological alterations listed by Harrison et al. (1997) can be caused by exposure to estrogens. Examples of such alterations were presented in many studies; the incidence of breast cancer in industrialized nations has inarguably risen over the last several decades, with evidence suggesting that 17β-estradiol induces breast cancer (Colditz et al. 1995; Dickson et al. 1986; Yager and Davidson 2006). Reports of early

female maturity in humans are supported by the parallel occurrence of estrogenically induced premature udder development and lactation in heifers. In addition, premature human sexual development in Puerto Rico has raised concern that environmental estrogens now impact much of the population (Ball et al. 2000). Thereby, accumulation of evidence suggests that exposure to environmental components with estrogenic activity causes reproductive disorders in human populations (Delbes et al. 2006; Sikka and Wang 2008).

E. Fate in the Environment

A number of mechanisms influence the appropriation and dissipation of estrogenic compounds in the environment, which ultimately affects their fate and transport. Mechanisms such as photolysis, sorption and microbial degradation processes regulate persistence and mobility of estrogenic compounds. This section explores the mechanisms influencing the fate of E1, E2, E3, and EE2.

1. Conjugation de-Conjugation

In the human body, the liver metabolizes estrogens. E1, E2, and E3 are natural estrogens that enter a series of reactions that eventually result in conjugation. The metabolites are polarizing substances containing functional groups that tend to couple onto the structure of the estrogen (Matsushima et al. 2005; Zamek-Gliszczynski et al. 2006). Prior to conjugation the sources of E1, E2, and EE2 are Androstenedione and Testosterone. In the presence of the Aromatase enzyme androstenedione is converted to E1 as illustrated in Figure 3. Additionally, testosterone is converted to E2 via a similar

pathway (Lee et al. 2003). A family of monooxygenase enzymes, called the ‘cytochrome P450’, catalyzes a product of cyclo-alkane hydroxylation of E2, leading to the production of E3 (Matsushima et al. 2005). As illustrated in Figure 4, the iron atom, present on the active part of the cytochrome P450 coordinated to the nitrogen atoms of four linked pyrrole rings, characterizes the mechanism of such transformation (Lee et al. 2003).

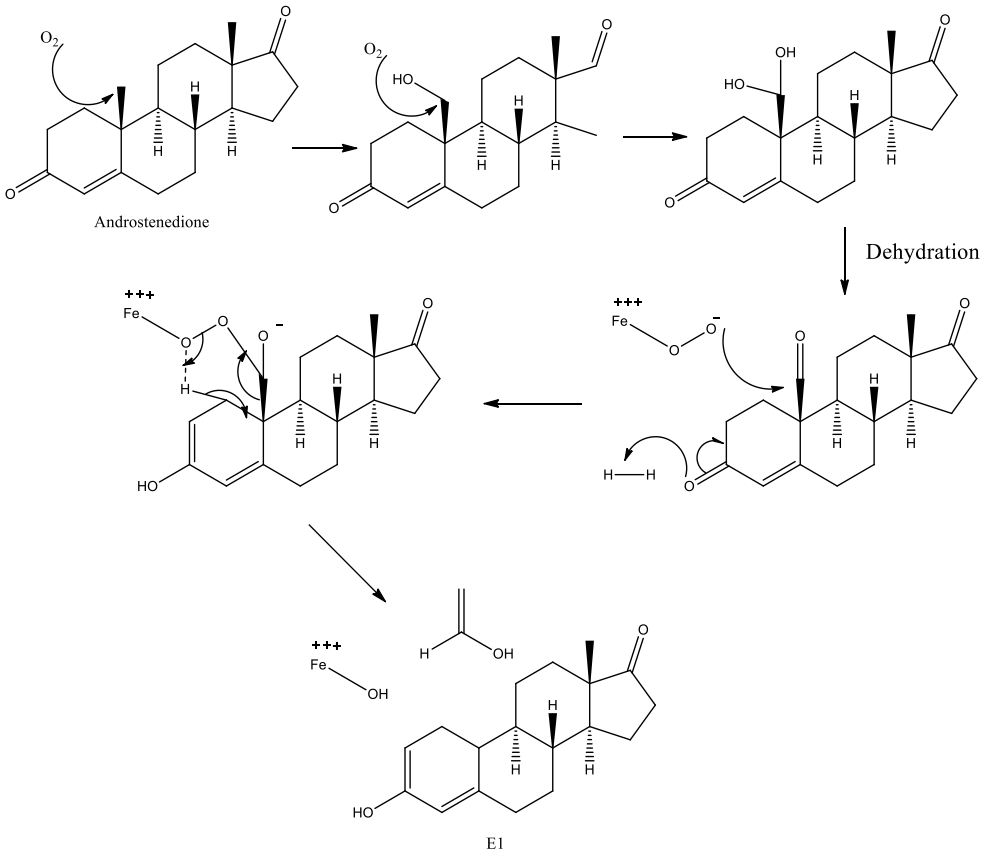


Figure 3. General reaction for the conversion of Androstenedione to estrone catalyzed by aromatase. Steroids are composed of four fused rings, aromatase converts a tail ring into an aromatic state (Khan and Khan 2013; Lee et al. 2003).

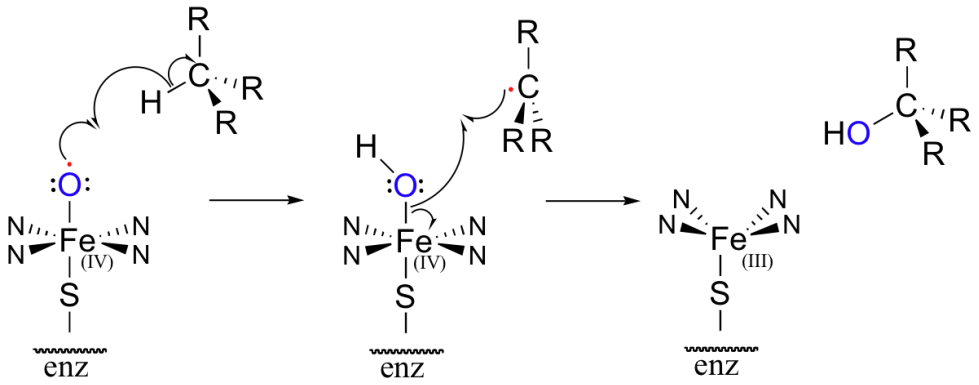


Figure 4. Alkane hydroxylation via cytochrome P450 catalyst (Lee et al. 2003)

Conjugation occurs in the presence of glucuronic acid and sulfuric acid which when bound to estrogens produce inactive polar conjugates that are later secreted via urination (Wlcek et al. 2014). A bacterium that synthesizes arylsulfatase is needed to deconjugate the sulfated estrogens, while one that synthesizes the glucuronidase enzyme is necessary for deconjugation of the glucuronated estrogens. A majority of the total estrogens produced by the body is excreted in conjugated form via the urinary route (Adlercreutz et al. 1987; D'Ascenzo et al. 2003). Prior to excretion, conjugated estrogens are formed by glucuronide (GLU) and/or sulfate (SUL) groups at the position(s) of C3 and/or C17. These polar conjugates are biologically inactive and more soluble in water when compared to their corresponding free or unconjugated counterpart. Studies on WWTP influent and effluents proved that free estrogen and sulfate conjugates are dominant over nonpolar conjugates, which leads to the conclusion that deconjugation is taking place (D'Ascenzo et al. 2003; Reddy et al. 2005). Earlier studies revealed that deconjugation is a natural process and bacteria such as Escherichia coli in the feces are able to deconjugate estrogens by synthesizing large quantities of the enzyme b-glucuronidase (Adlercreutz and Martin 1980).

The suggested mechanism for conjugation of E1 is shown in **Figure 5** and **Figure 6**. E2 E3 and EE2 have similar structure to E1 on the aromatic ring (phenolic functional group), and therefore such mechanisms are applicable to these estrogens. Estrogen sulfates and estrogen glucuronates exist for all three forms of estrogens, and include: E1-3S, E1-3G, E2-3S, E2-17G, E2-3G, E3-3S, E3-16G, and E3-3G; where the "G" and "S" designations refer to glucuronate and sulfate groups, and the preceding number refers to the carbon chain position (Johnson and Williams 2004a). Following similar pathways, the conjugates of EE2 are synthesized as illustrated in **Figure 7** (Kirilovas et al. 2007). Sun et al. (2005) proposed degradation pathway for E1 in the human body. These mechanisms include methylation, deoxidation, and hydroxylation as presented in Figure 8 (Sun et al. 2005).

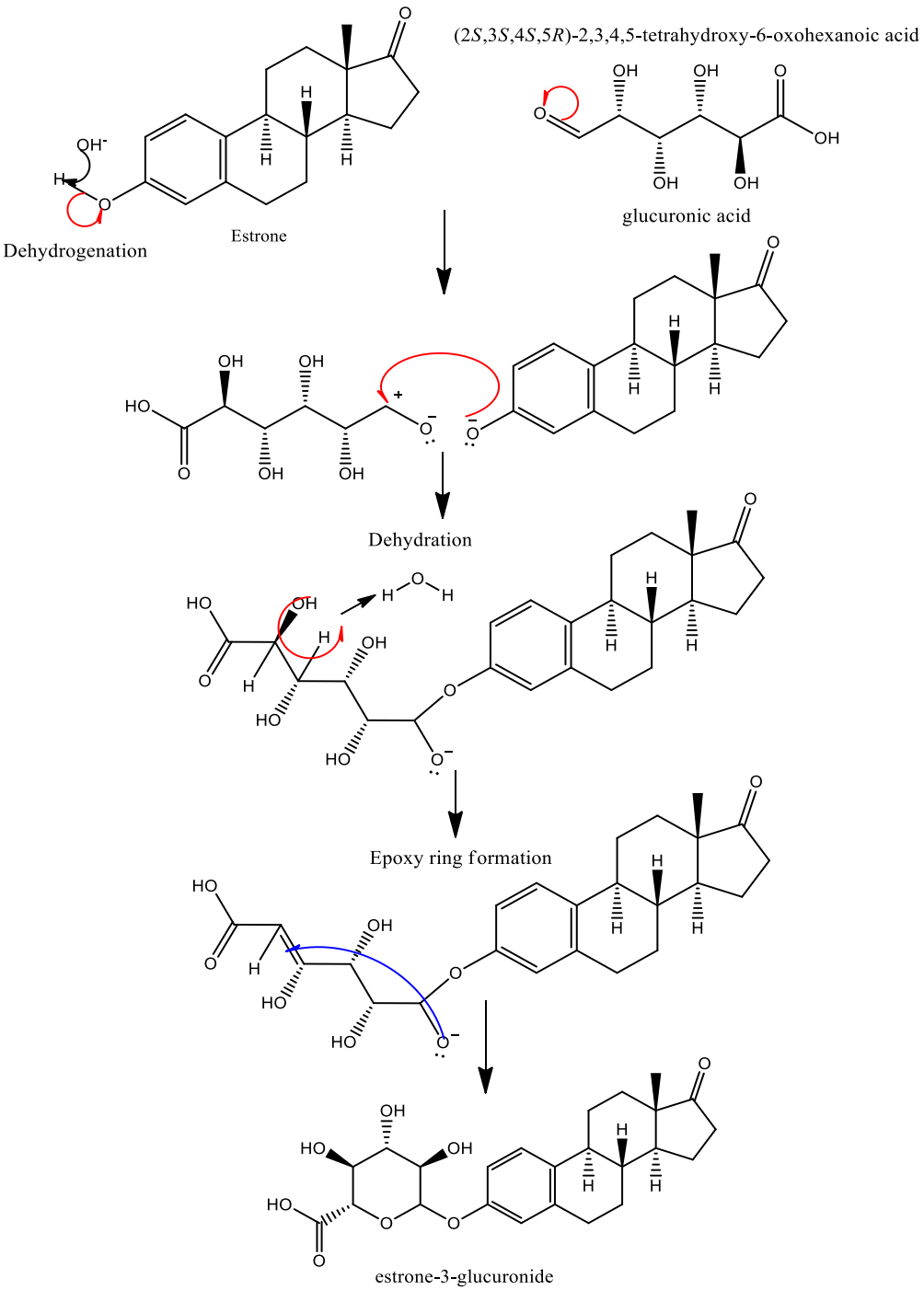


Figure 5. Mechanism of estrone and glucuronic acid (Johnson and Williams 2004a)

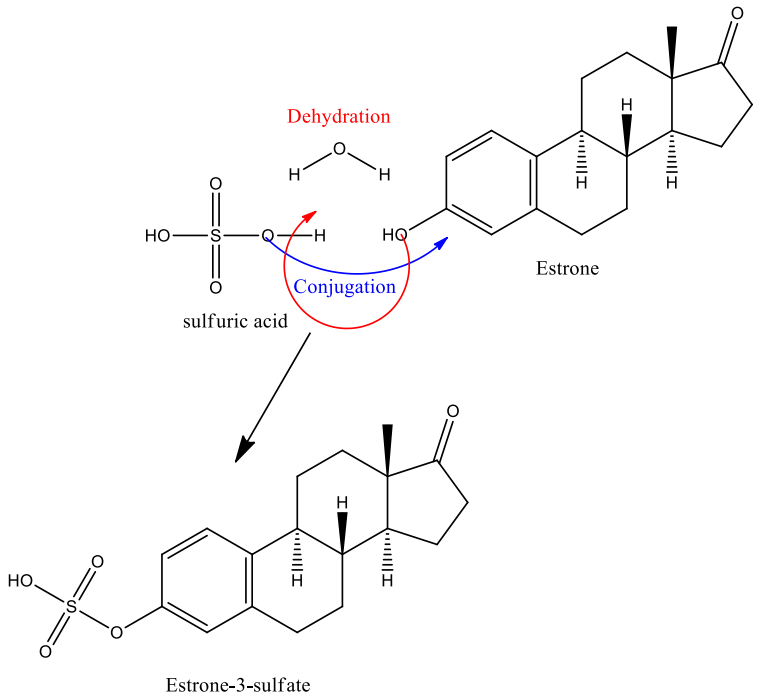


Figure 6. Coupling of E1 and Sulfuric acid (Kirilovas et al. 2007)

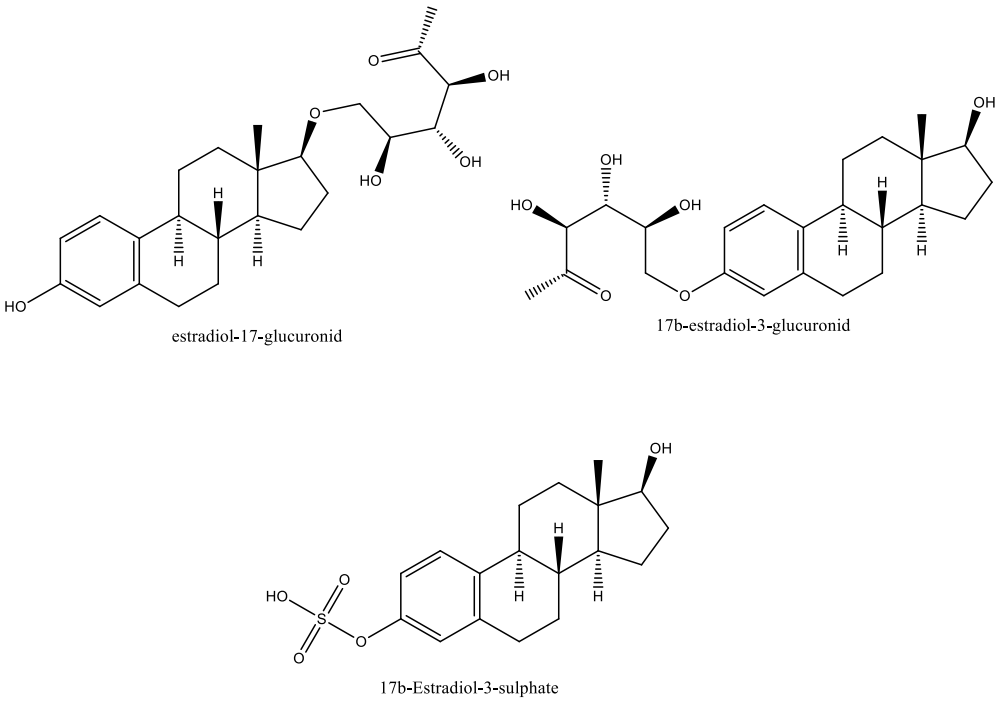


Figure 7. Conjugates of E2 (Kirilovas et al. 2007)

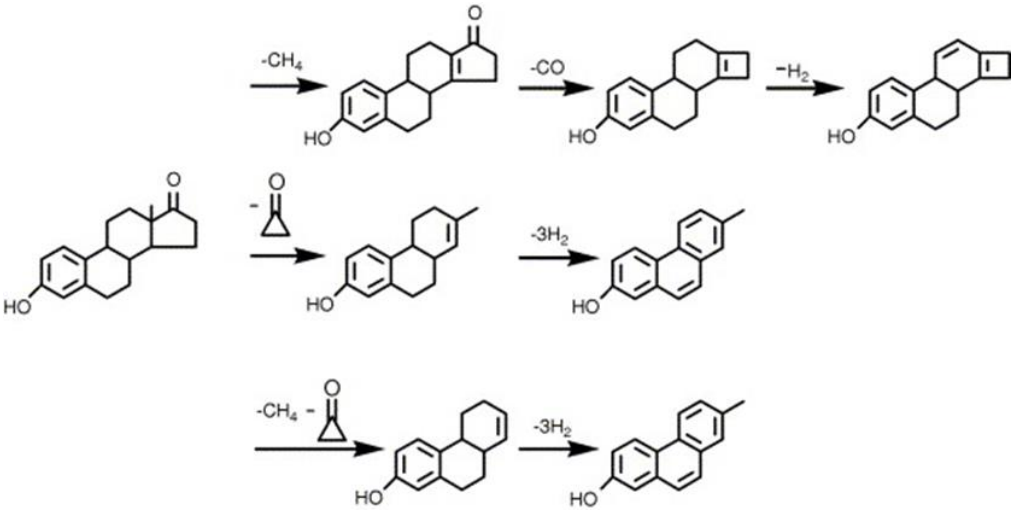


Figure 8. Degradation pathway of E1in the human body (Sun et al. 2005)

EE2 is not a naturally occurring hormone, and as discussed earlier it is introduced to the body via oral administration of contraceptives in the form of methyl-EE2, which later transforms to EE2 via demethylation reaction (Zhang 2006). Nevertheless, EE2 follows similar pathways as illustrated in Figure 5 and Figure 6 in the presence of glucoronic acid and sulfuric acid respectively.

2. Volatilization

Volatilization of estrogens is also reported in the literature (Hamid and Eskicioglu 2012). Predicted by Henry's law constant (H), Estrogens have a lower H value than that of chlorines and other aromatic compounds; which makes these compounds less susceptible to volatilization under normal pressure and temperature (Estrada-Arriaga and Mijaylova 2010; Khanal et al. 2006).

3. Photo-degradation

Research on light-induced estrogen degradation considers photolysis in association with a photocatalyst, which presents difficulty when considering this dissipation phenomenon in natural waters in the absence of the required photocatalysts. Additionally, the research conducted on the topic uses a wide range of light sources, which further complicates attempts to compare the studies. The effect of TiO2 on estrogens was studied extensively during the past 20 years. Nakashima et al. (2002), and Ohko et al. (2002), used a reactor fitted with TiO2-modified mesh sheets and black fluorescent lamps; however, no concentration attenuation occurred over the same time period when illuminated without a catalyst. On the other hand, photo-degradation of 17β-estradiol in conjunction with a titanium dioxide (TiO2) catalyst displayed 99 and 98 percent degradation after a 30-min and 1-hour UV exposure, respectively (mercury-xenon lamp, 200 W, 365-nm band-passed filter). Coleman et al. (2000) reported non-catalytic degradation of 11% and 44% over 30 and 60-minute periods of exposure to UV, respectively. Adjusting pH led to increased photolysis rates up to pH 7, followed by a decrease at pH 10 and a rapid intensification at pH 12.

Liu and Liu (2004) considered photolysis in the absence of a catalyst. Their study examined estrogen irradiation in conjunction with UV and UV-vis light sources. Half-lives generated for E1 and E2 approached 10 and 40 min, respectively, under a UV-lamp. The UV-Vis-light resulted in E1 half-life of about 50 min, but only produced minimal 17β-estradiol degradation. An absorbance peak of 288 nm by E2 may help explain this finding, as it exhibits generally weak absorption in the UV range. A modification which led to improvement to the previous study was performed by Rosenfeldt and Linden (2004), using a maximum absorbance wavelength of 280nm which led to alteration of the

concentration for both E1 and E2 from 20 to 3 mg/L and increased photolysis rates by about two-fold during UV illumination. Additionally Coleman et al. (2004) stated that photolysis occurred in the presence of UVA light with removal of the biological activity in aqueous solutions of E1, E2 and EE2 using immobilized TiO2 catalyst. Similar studies showed that temperature variations in the range of 0°C to 75°C had minimal effects on photolysis of some estrogens (Kimura et al. 2004).

Indirect photolysis may also occur when photosensitizers, such as DOC, absorb the light and generate reactive oxygenated radicals that complete the degradation step (Caupos et al. 2011). Chen et al. (2013) concluded that E3 undergoes both direct and indirect photodegradation in aqueous solutions under natural and simulated sunlight. The presence of the natural photoreactive components, NO_3^{2-} and Fe(III)-oxalate, enhanced the photodegradation of E3.

Chowdhury et al. (2011) reported on the photodegradation of E2 and the influence of some water quality parameters. They concluded that in the presence of natural water constituents, such as Nitrite, Fe^{3+} and humic acids, the photodegradation rate increased significantly, which was attributed to photosensitization by the reactive species. On the other hand, the presence of HCO_3^{2-} decreased the degradation rate due to OH^- scavenging. Chen et al. (2013) mapped the possible photodegradation mechanisms of E3 as illustrated in Figure 9. The oxidation of E3 involved attack of OH radical, and the loss of H_2O (pathway 1) or reactions of hydroxylated E3 radicals with molecular oxygen (pathway 2 and 3), followed by the elimination of OH radical to yield the final products I, II and III. Similar OH radical oxidation processes were observed in the degradation of benzene, naphthalene and naphthols (Boule and Hutzinger 1999). It should be noted that the degradation of E3 is highly affected by humic acid, nitrate and iron complexes in water

solutions. Similar studies used UV-B radiation for effluent treatment, and exposure of effluents to sunlight in holding ponds, which resulted in photodegradation of E1 (Trudeau et al. 2011). UV-B-mediated degradation leads to the photoproduction of lumiestrone, a little known 13α-epimer form of E1 with null estrogenicity as illustrated in Figure 10. According to Chen et al. (2012a), catalytic oxidation of EE2 using FeIII-TAML/H_2O_2 proved that the partial oxidation of EE2 produces two epimers as intermediates with estrogenicity greater than that of EE2 (Figure 11). Similar oxidation systems might follow such a mechanism, and the conclusion drawn is to avoid low oxidation levels when EE2 is present.

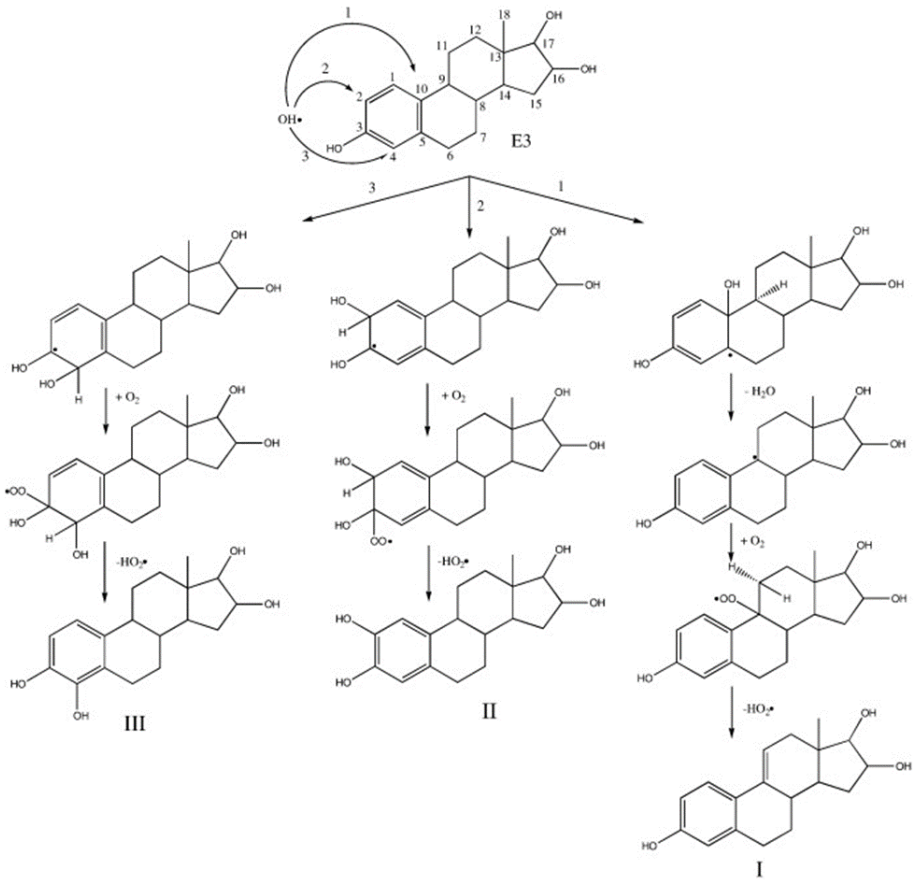


Figure 9. Photo-degradation mechanism of E3 (Chen et al. 2013)

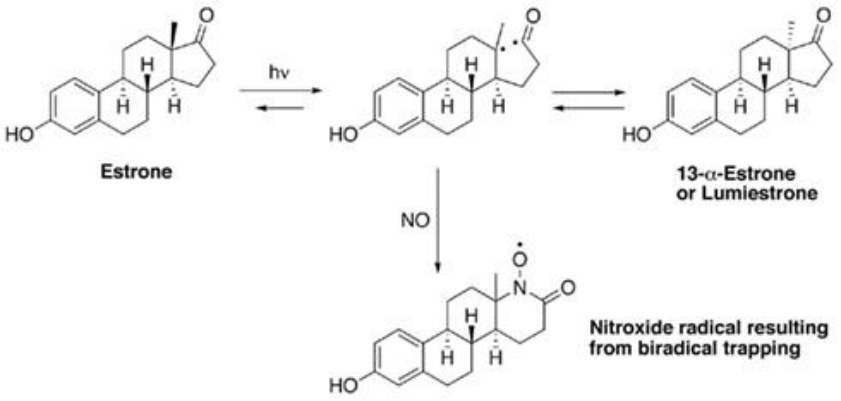


Figure 10. Mechanism of formation of lumiestrone (Trudeau et al. 2011)

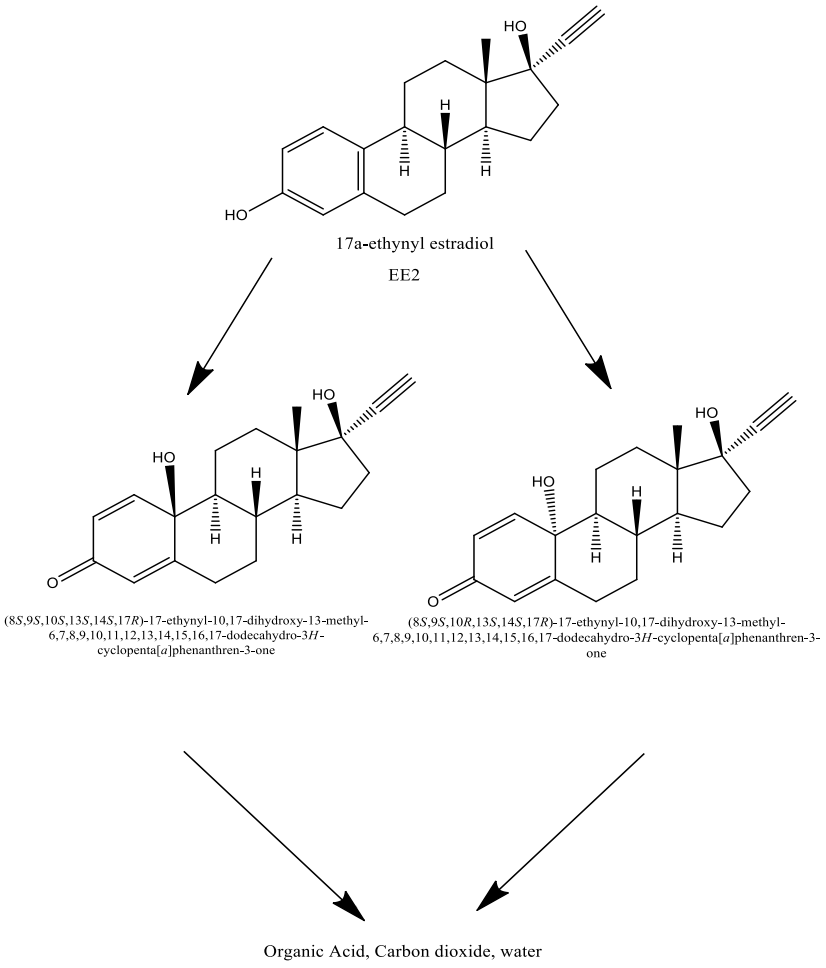


Figure 11. Catalytic oxidation of EE2 using FeIII-TAML/H_2O_2 (Chen et al. 2012)

4. Sorption

Uptake of estrogens from the aqueous phase onto a solid phase known as a sorbent is well documented (Duran-Alvarez et al. 2014; Silva et al. 2012b). Research focused on the removal of estrogens via sorption in water and wastewater treatment. Sorbent materials used were synthetic membranes (Chang et al. 2003), granular activated carbon (GAC) (Chang et al. 2004), and reverse osmosis (RO) (Schäfer et al. 2002). More recent studies focused on the sorption of estrogens from manure (Stumpe and Marschner 2010), in addition to sorption of steroids to colloids present in river sediments and wetlands (Chen et al. 2014). Fukuhara et al. (2006) assessed activated carbon as a sorbent material with resulting sorption capacities in the range of 25.6–73.5 mg/g for E1 and 21.3–67.6 mg/g for E2 at 1 μg/L in pure water. Kumar et al. (2009b) reported that higher adsorption capacities were observed for E3 concentration in the range 5–10 μg/L, with 180 min of contact time (>90% of E3 was adsorbed) and aqueous phase pH of 7. Similar studies used AC to test for EE2 sorption and concluded that the highest EE2 sorption capacity was observed at neutral conditions and with the increase in temperature up to 30 °C (Kumar and Mohan 2011). Using relatively high contact times (72-125 hr.) Zhang and Zhou (2005) assessed the adsorption capacity of multiple sorbents such as GAC, chitin, chitosan, ion exchange resin, and a carbonaceous adsorbent. The results showed high adsorption capacity for E1 and E2, with a maximum adsorption constant of 9290 mg/g for E1 and 12200 mg/g for E2. Snyder et al. (2007), used both GAC and Powdered activated carbon (PAC) to test for the sorption of E1, E2, E3, and EE2 and both sorbents removed nearly all compounds by more than 90%. Layered double hydroxides (LDH),

such as Mg–Al, was assessed as a sorption material for E2. LDH in a packed column decreased the estrogenic activity of a river water from 519 to 387 ng E2 equivalent/L (Morris et al. 2008).

Molecularly imprinted polymer (MIP) was evaluated as a sorbent for estrogens in various studies. MIP was capable to recover E2 by 100 ± 0.6% from a 2 μg/L aqueous solution (Le Noir et al. 2007). Another study concluded that maximum E2 recovery percentages were 97 ± 3% (Lai et al. 2010). A similar study investigating MIP showed recovery rates of 90 ± 5% and 96 ± 3% E2 bound onto MIP particles after 2 min incubation and after equilibrium was reached, respectively (DeMaleki et al. 2010). More recent studies used modifications of MIP sorbents, Carbon nanaotubes (CNT) functionalized with MIP that resulted in a removal efficiency range from 96.14% to 98.03% in real water samples spiked with E1 (Gao et al. 2011). Single walled CNT's removal efficiency of EE2 was between 95 and 98% (Joseph et al. 2011). On the other hand, multi-walled CNT sorption was dependent on the aqueous phase sorbate concentration and pH, showing higher sorption capacity at higher substrate load and at neutral pH conditions (Jung et al. 2013; Kumar and Mohan 2012). Poly-Amid (PA) membranes exhibited a distinct sorption capability for estrogenic compounds in water, showing sustained sorption for E1, E2, EE2, and BPA which far exceeded the sorption observed using other membranes and sorption material (Han et al. 2013). The mechanism of sorption via PA membrane is shown in Figure 12.

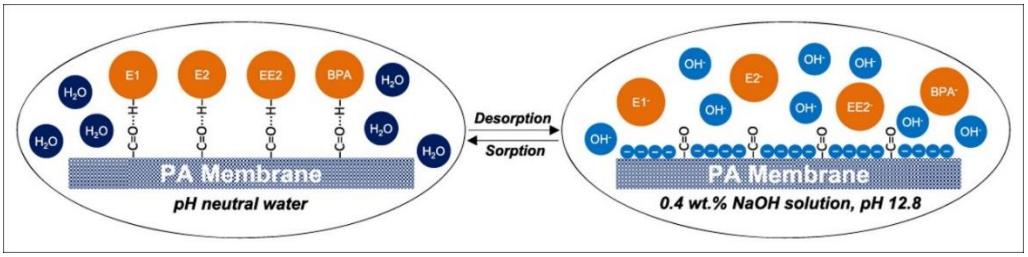


Figure 12. Distinct sorption capability of PA membranes for four estrogenic compounds in water (Han et al. 2013)

As elaborated earlier, adsorption is dependent on the membrane material used in addition to the contaminant and its properties such as hydrophobicity (Kiso et al. 2000), acid dissociation constant (Nghiem et al. 2004), and aptitude to hydrogen bond or engage in other supramolecular interactions (Comerton et al. 2007; Yoon et al. 2007). A summary of such interactions, as described in supramolecular chemistry and adapted to possible micro-pollutant interactions is shown in Figure 13 (Schäfer et al. 2011). The mechanism of possible hydrogen bonding interactions for a number of polymers is illustrated in Figure 14. In addition to H-bonding, π–π interactions are a possible mechanism that can occur. The difference between the π densities of the adsorbent and the corresponding adsorbate determines the stability of the π–π interaction. π density is determined by electron rich and deficient aromatic fragments (Davankov et al. 2003; Schäfer et al. 2011).

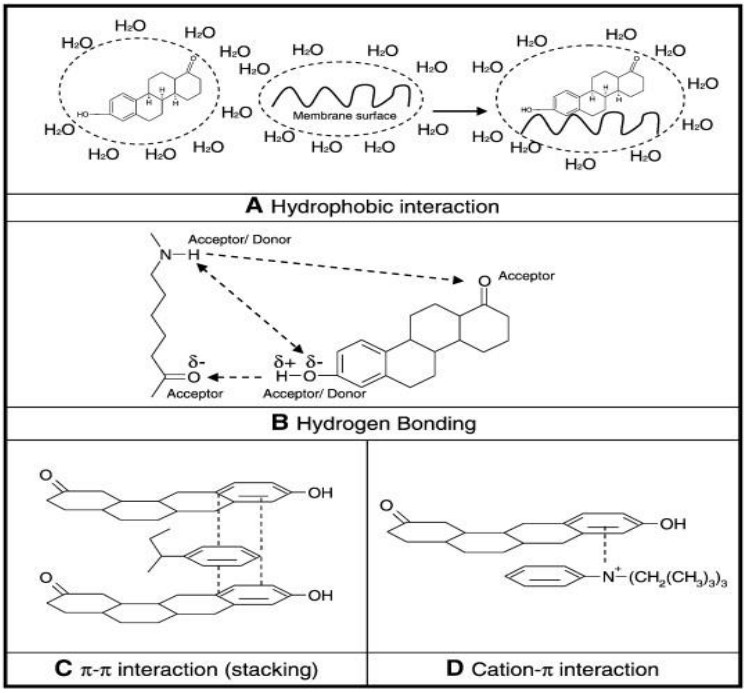


Figure 13. Selected possible polymer–micropollutant interaction mechanisms. A: hydrophobic interaction between the membrane surface and E1, B: hydrogen bonding between polyamide and E1, C: π–π interaction between aromatic rings of polystyrene and E1 (Schäfer et al. 2011)

Davankov et al. (2003) reported that π–π interactions between the hypercrosslinked polystyrene and the substances with π-systems of electrons, such as aromatic rings have a tendency to take place. Carboxyl groups govern the retention mechanisms in HPLC application with non-polar solvents (Schäfer et al. 2011). Electrodialysis was also employed to remove estrogens via the sorption mechanism (Pronk et al. 2006). The possible hydrogen bonding formation between the hormones that exhibited strongest anion-exchange membrane (AEM) (E1) and cation-exchange membrane (CEM) (Progesterone) is illustrated Figure 15. Hormones can be hydrogen-donors (contain phenolic OH groups) or hydrogen acceptors (C=O groups). The AEM functional group ($N(CH_3)_3$) can bond with molecules containing hydrogen-donor and acceptor groups (Fang et al. 2001).

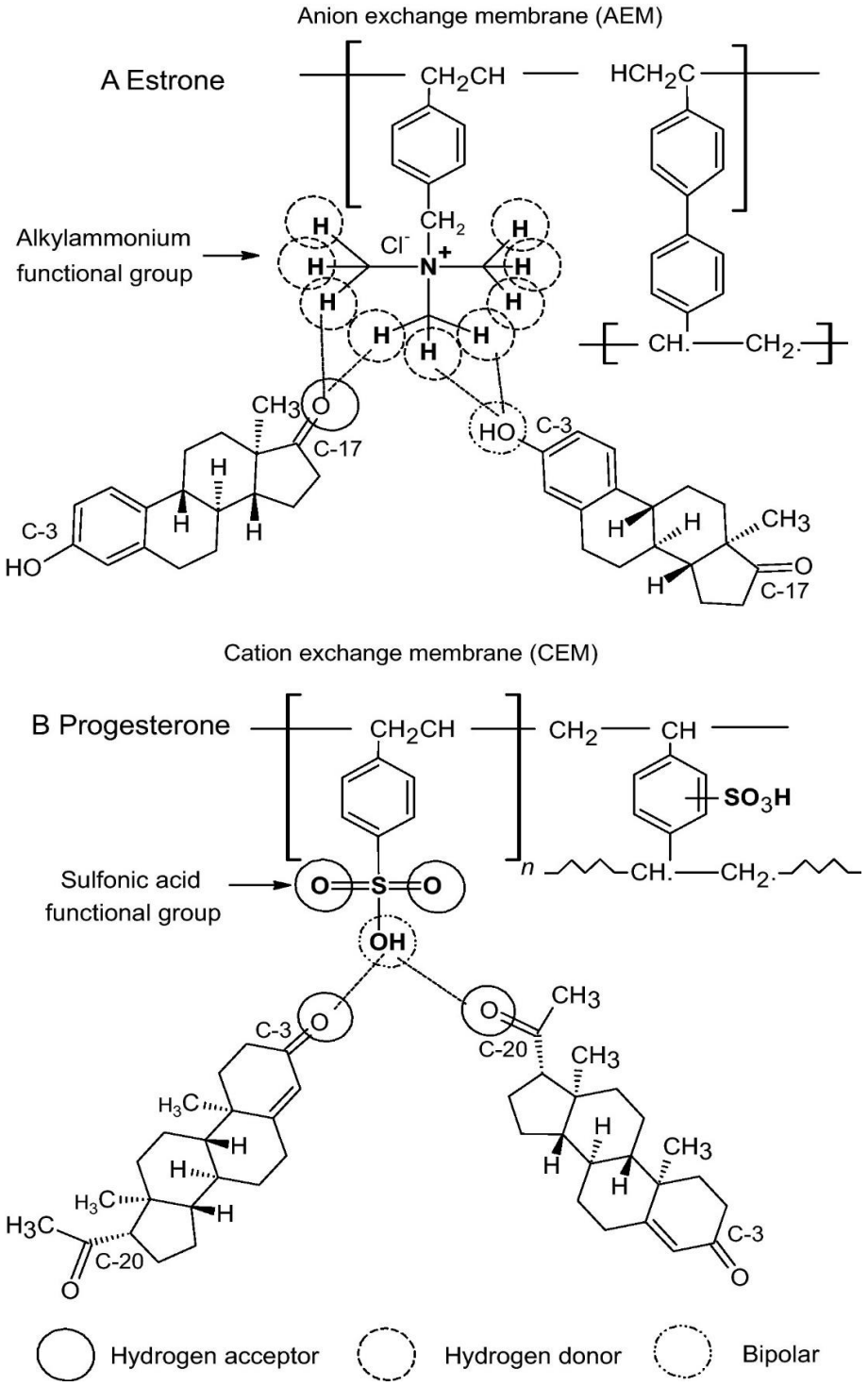


Figure 14. Schematic of possible hydrogen bonding between the hormone molecules (A) estrone and (B) progesterone and the AEM and CEM functional groups at neutral pH.(Banasiak and Schäfer 2010)

5. Membrane Treatment

The premise behind membrane treatment in general is separation based on physical and chemical properties of two phases involved within a process that uses a semi-permeable membrane (Silva et al. 2012b). Membrane processes include microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO). These processes affected very promising removal efficiencies of EDCs (Bolong et al. 2009; Schäfer et al. 2002; Silva et al. 2012b). Adsorption onto the membrane, size exclusion and charge repulsion are the most important parameters that affect removal in membrane filtration (Bolong et al. 2009). Rejection, pressure requirements, and energy consumption follow the order RO > NF > UF > MF, thus RO being the most effective in removal and conversely will require the most energy (Bolong et al. 2009; Liu et al. 2009). The vast majority of studies conducted on membrane removal of estrogens using NF and RO membranes. The rejection efficiency of such systems is dependent on estrogen's physicochemical properties, membrane operating conditions, membrane properties (for example, permeability, pore size, surface charge and hydrophobicity/hydrophilicity), membrane fouling and parameters like pH, temperature and salinity (Bolong et al. 2009; Liu et al. 2009; Schäfer et al. 2011; Schäfer et al. 2002). Rejection rates of estrogens via membrane filtration vary between studies. The assessment of UF in the removal of estrogens from drinking water resulted in efficiencies of 44%, over 95%, 10%, and 38% for E1, E2,E3, and EE2, respectively (Yoon et al. 2007). NF rejection efficiencies were much more dependent on environmental condition and parameters such as pH, temperature and salinity of water. Rejection efficiency values of 42 to 63 % were reported for the four estrogens (Bodzek and Dudziak 2006; Yoon et al. 2007). Other studies reported that in the presence of natural organic matter, hormone rejection increased when

using NF (Nghiem et al. 2004). Hu et al. (2007), and (Weber et al. 2004) concluded that with decreased pH the amount of humic acids adsorbed on the membrane increased, as well as the adsorption of E1, thus the removal of E1 was reported to increase to 95-99%. Other studies also assessed the effect of the presence of humic matter on rejection rate of hormones via NF in drinking water, and it was concluded that E1 removal increase from 80 to 98% and E2 from 65 to 97% (Koyuncu et al. 2008). This phenomenon was attributed to the enhanced size exclusion effect and the adsorption of hormones onto membranes due to the formation of macromolecular complexes, resulting from the association of hormones with functional groups of natural organic matter (Koyuncu et al. 2008). E2 removal via NF was reported in the absence of organic matter to be 44% (Bodzek and Dudziak 2006), while on the other hand, the presence of organic matter and humic substances increased the rejection of E2 to 95-99% (Hu et al. 2007; Weber et al. 2004; Yoon et al. 2007). The removal efficiency of E3 and EE2 via NF also increased in the presence of organic matter from 38% to 95% for E3 and 34 % to 99% for EE2 (Bodzek and Dudziak 2006; Weber et al. 2004; Yoon et al. 2007).

RO and combined NF/RO systems recorded the best removal efficiency of estrogens with values ranging between 97-100% recorded even in the absence of humic and organic matter (Alturki et al. 2010). Many factors can influence the performance of RO with membrane fouling being most important. Fouling results in numerous damaging effects reflected in membrane degradation and decrease of the permeate quality (Ju and Hong 2014; Ng and Elimelech 2004). Ng and Elimelech (2004) showed that the fouling of a RO membrane caused a noticeable decrease in the rejection of organic compounds, such as estrogens. Direct contact membrane distillation (DCMD) and forward osmosis (FO) were used to study the retention of two natural hormones, E1 and E2. The study

concluded that DCMD affected more than 99.5% of hormone rejection whereas FO provided 77-99% (Cartinella et al. 2006).

The mechanism of removal of estrogens via membrane filtration is dependent on size and charge of the estrogen (Figure 15). Any non-retained micropollutant will penetrate into the polymer matrix, while those retained accumulate in the boundary layer (membrane surface) (Schäfer et al. 2011).

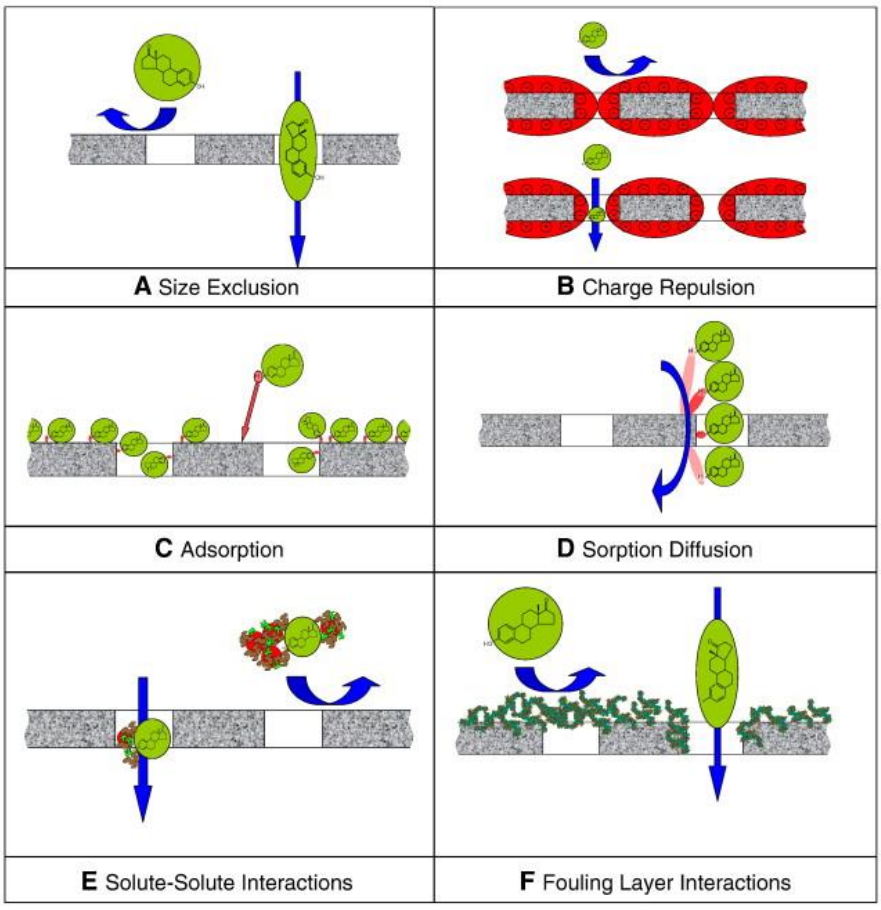


Figure 15. Micropollutant retention mechanisms in polymeric membranes A: size exclusion, B: charge repulsion, C: adsorption, D: sorption diffusion, E: solute–solute interactions, and F: fouling layer interactions (Schäfer et al. 2011)

6. Biodegradation

Biodegradation is attributed to the fact that microorganisms can utilize available compounds from the environment for various biological purposes (Zhang 2006). Many studies have been conducted to obtain information about the biodegradability of estrogens in different aquatic media, especially WWTPs. Studies monitoring the performance of WWTPs in terms of estrogen removal report wide ranges of estrogen removals, ranging from 19-94 % for E1, 76-92% for E2, and 83-87 % for EE2 (Baronti et al. 2000). Such variations are a result of different treatment procedures followed in WWTP in addition to the varied concentrations of estrogens in the wastewater influent (Liu et al. 2009; Yu et al. 2013a). The fate of estrogens in WW treatment is shown in Figure 16.

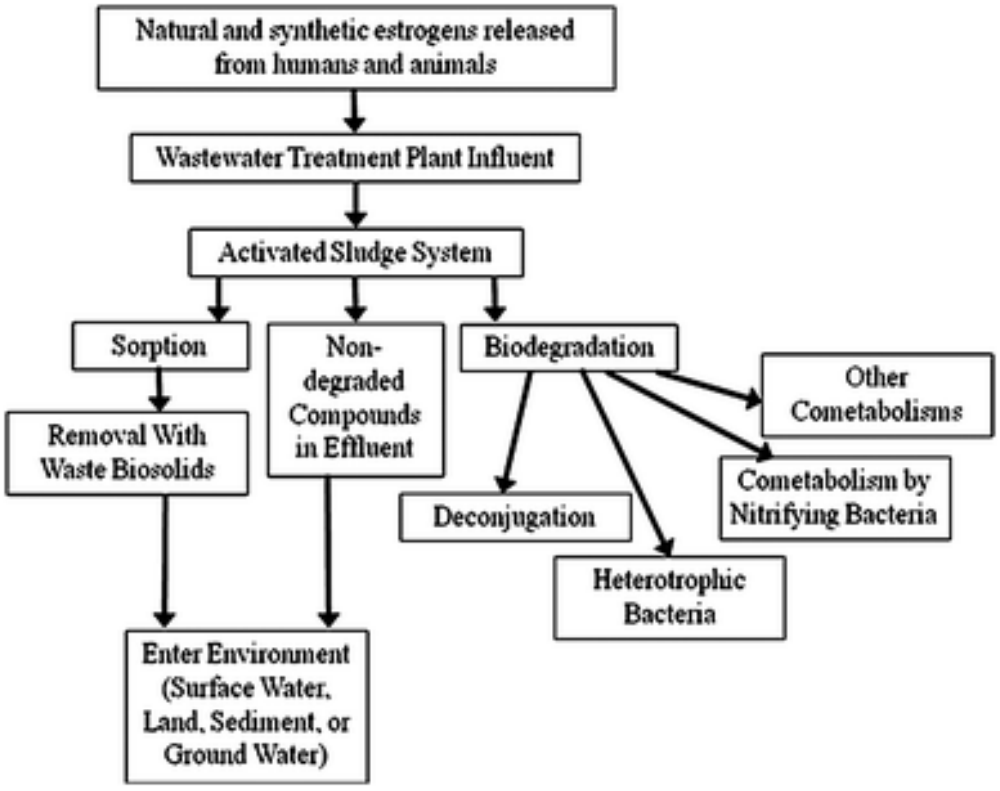


Figure 16. Fate of estrogens in wastewater treatment (Racz and Goel 2010)

According to Yu et al. (2007) Microorganisms can degrade steroidal hormone using two degradation mechanisms; in the first microorganisms utilize steroidal hormones as energy and/or carbon source for microbial growth, this is called growth linked degradation (metabolic). Metabolic pathway of degradation of EE2 via sewage bacteria is illustrated in Figure 17. The second mechanism is where bacteria utilizes existing enzymes to degrade steroidal hormones, also known as cometabolic degradation (non-linked growth). Cometabolic mechanisms yields no carbon or energy benefits to the microorganisms, therefore a primary growth substrate is needed to sustain bacterial growth (Pauwels et al. 2008; Yu et al. 2007).

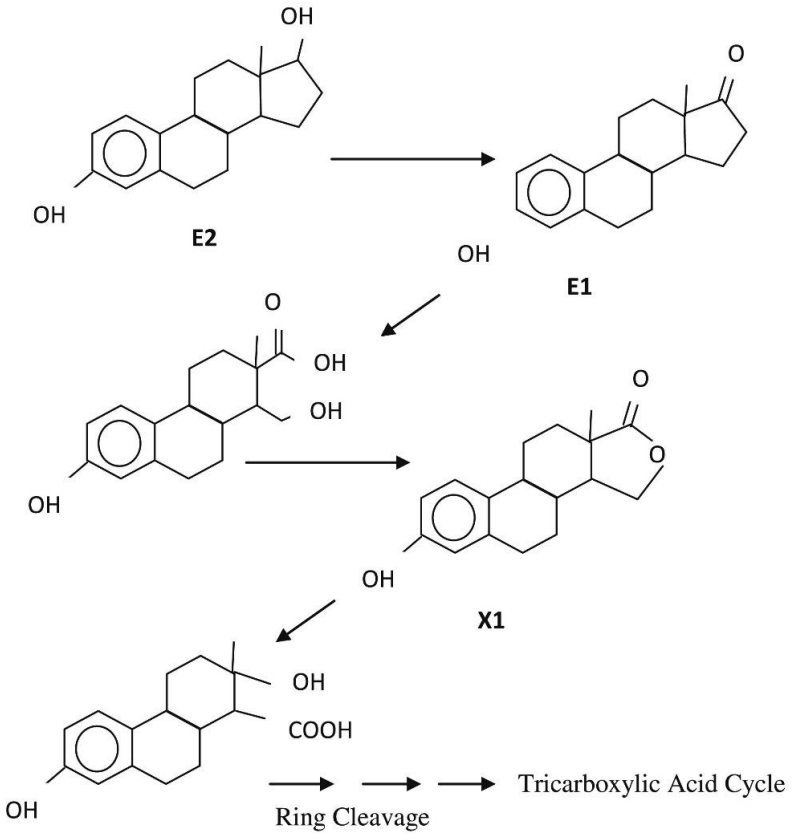


Figure 17. Proposed metabolic pathway of E2 by sewage bacteria (Glassmeyer et al. 2009; Lee and Liu 2002; Tan et al. 2007b)

An illustration of cometabolic mechanism is the Laccase oxidation of E2 which generates an oxygen radical that can delocalize to carbon-located radicals as shown in Figure 18. Subsequent coupling of these reactive intermediates produces C–C or C–O dimers, which could be further oxidized to generate oligomers and polymers (Mao et al. 2010; Nicotra et al. 2004). Lloret et al. (2013) concluded that Laccase catalyzed treatment lead to the formation of dimers and trimers of E1, E2, and EE2, as well as the decomposition of E2 into E1. Dimeric products of E2 and EE2 were found even when operating at environmental concentrations. Moreover, the reaction pathways of laccase-catalyzed transformation of E2 is summarized in Figure 19.

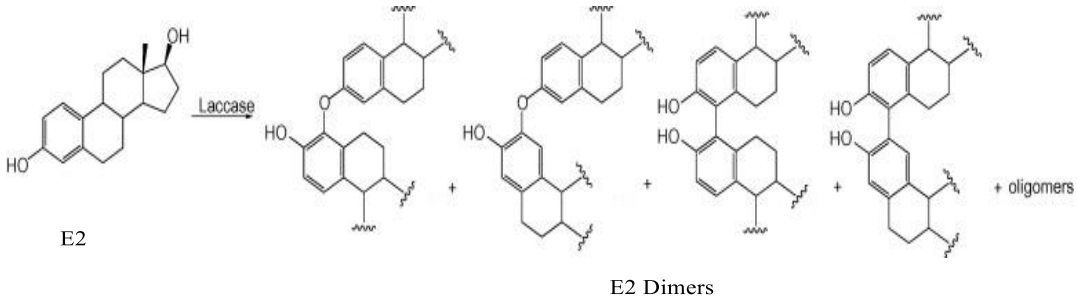


Figure 18. Cometabolic laccase mediated polymerization (Nicotra et al. 2004; Mao et al. 2010)

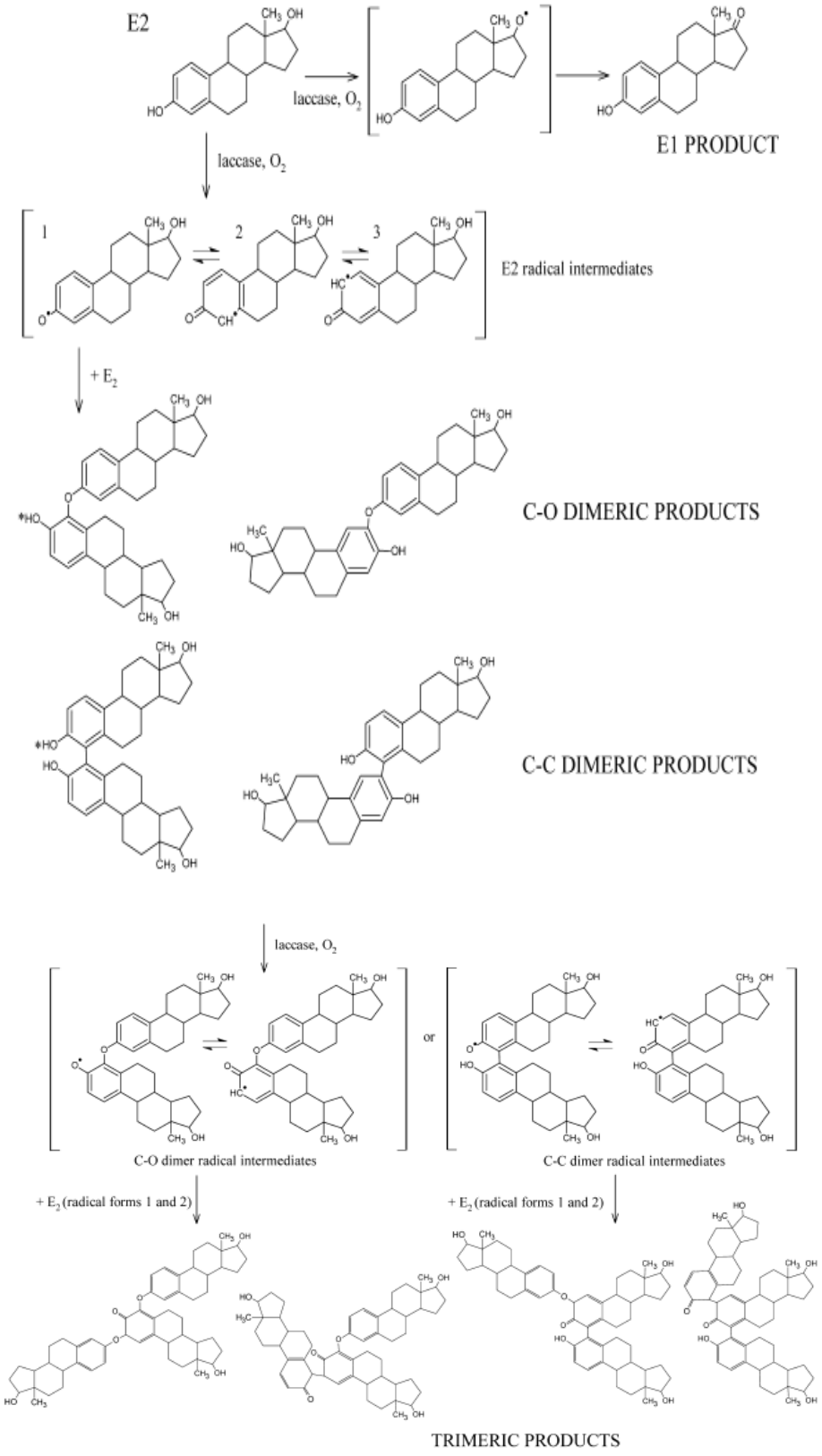


Figure 19. laccase-catalyzed transformation of E2 into trimeric products (Lloret et al. 2013)

A metabolic study was performed on the transformation of norethisterone by cephalosporium aphidicola and subsequently of EE2 by the zygomycete cunninghamella elegans in a liquid medium. The authors identified several metabolites of EE2. The transformation reactions included several hydroxylations of EE2 and in one case a subsequent methoxylation of the hydroxyl derivative as illustrated in Figure 20 (Choudhary et al. 2004).

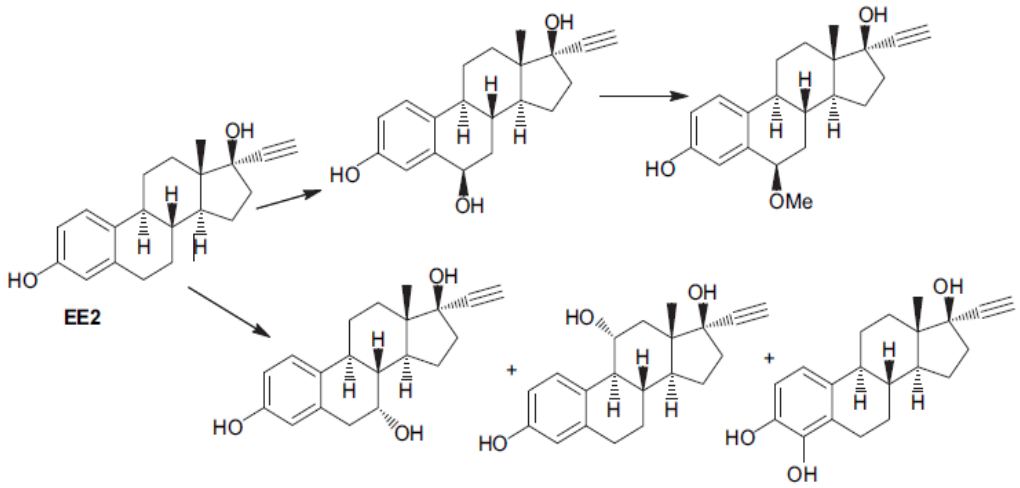


Figure 20. Transformation of EE2 in a liquid medium with zygomycete Cunninghamella elegans (Choudhary et al. 2004)

Della Greca et al. (2008) tested the biotransformation capability of 11 microalgae strains on EE2. Out of the tested strains Selenastrum capricornutum, Scenedesmus quadricauda, Scenedesmus vacuolatus, and Ankistrodesmus braunii were able to biotransform the substrate. Several transformation products were identified as illustrated in Figure 21 (Cajthaml et al. 2009; Della Greca et al. 1996). Béni et al. (2013) classified mechanisms of E2 1a transformation (Figure 22). It should be noted that 5 through 10 are

estradiol type compounds with lowered estrogenicity potential, E1 can also be degraded following a similar pathway (Modica et al. 2003).

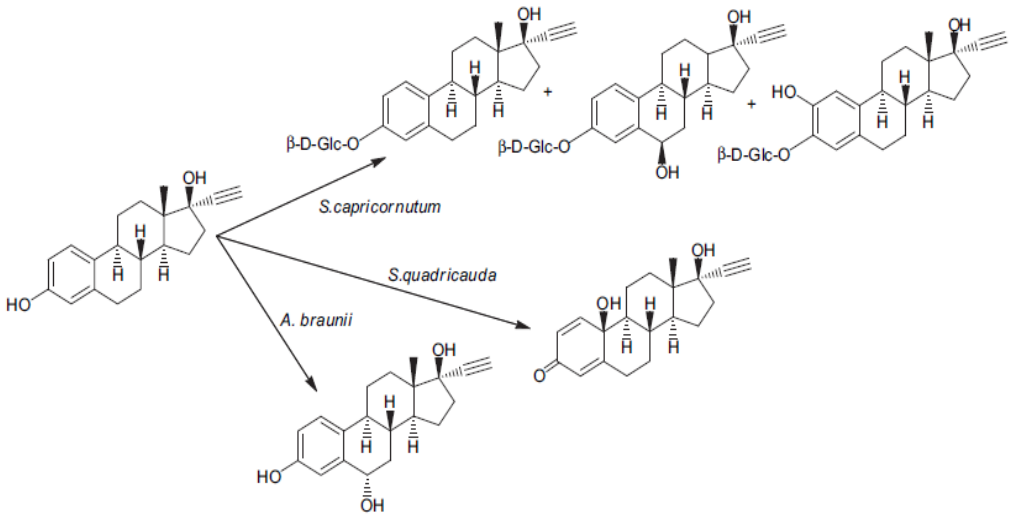


Figure 21. Biotransformation of EE2 by microalgae(Cajthaml et al. 2009)

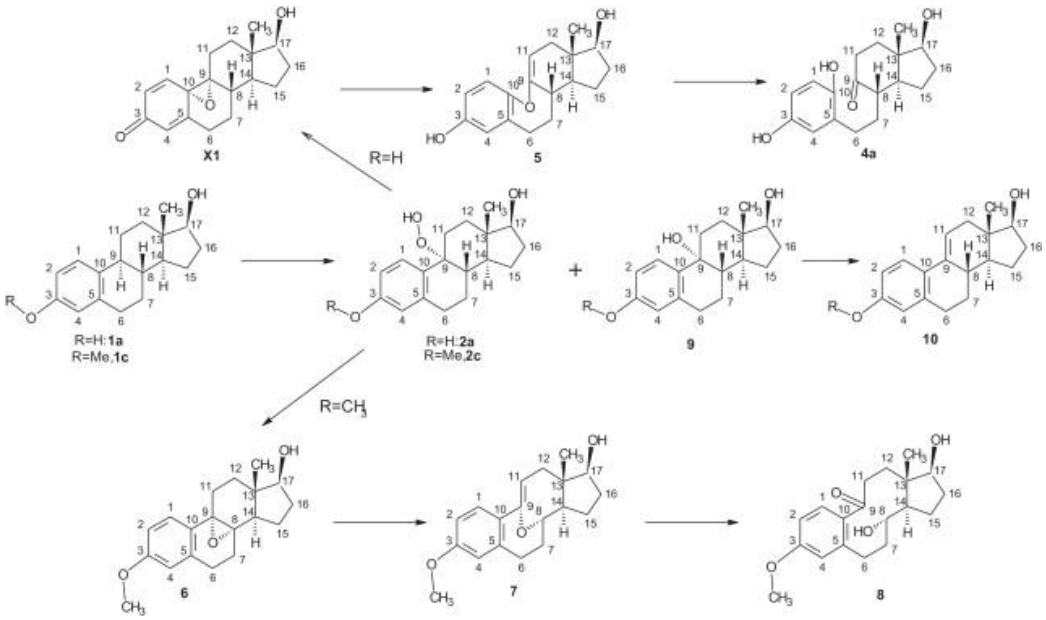


Figure 22. Microbial degredation of E2 1a (Béni et al. 2013)

Yu et al. (2013b) focused on microbial degradation of Estrogens; the research was reported in a comprehensive review of aerobic and anaerobic bacterial activity and its ability to degrade E1, E2, EE2 and E3. Coombe and co-workers (Coombe et al. 1966) were first to discuss the degradation ability of bacteria on E1. A dioxygenase was proposed to be responsible for the cleavage in the ring A of E1 under aerobic conditions. Similar pathways can be deduced for E2 and EE2 degradation as proposed in Figure 23 (Yu et al. 2013a).

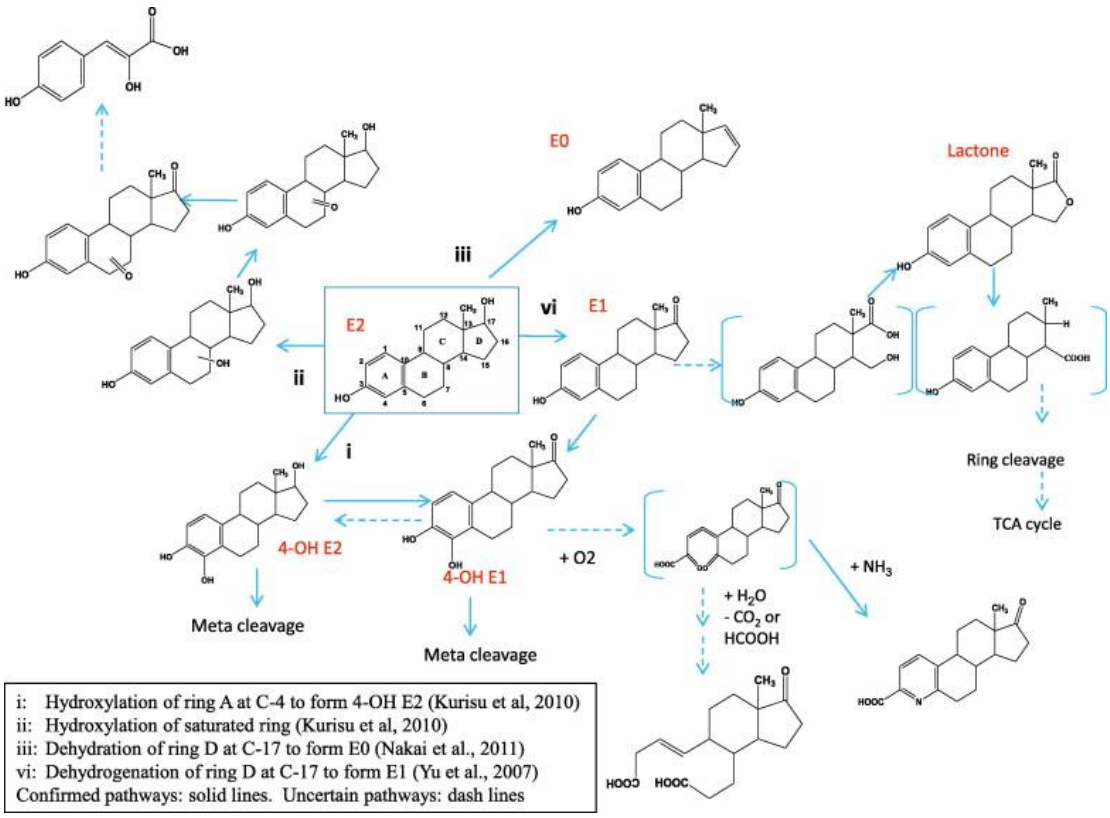


Figure 23. Degradation pathways of E2 by aerobic bacteria (Yu et al. 2013a)

Steps of mechanism:

i. Kurisu et al. (2010) attributed the detection of 4-OH-E2 to a hydroxylated E2 at C-4 of E2. In their study, they suggested that 4-OH-E2 was further degraded via meta cleavage. As such this step was defined as Hydroxylation of ring A at C-4 (Kurisu et al. 2010).

ii. Kurisu et al. (2010) also identified a degradation pathway for E2 via ring hydroxylation on different positions, based on the detection of hydroxy-E2, keto-E2, keto-E1, and 3-(4-hydroxyphenyl)-2-hydroxyprop-2-enoic acid. However, the actual ring cleavage was unclear (Kurisu et al. 2010; Yu et al. 2013a). Steps i and ii are illustrated in Figure 24.

iii. Dehydration of ring D at C-17: Nakai et al. (2010) attributed the dehydration of ring D at C-17 position of E2 and the formation of intermediate metabolite, estra-1,3,5(10),16-tetraen-3-ol (estratetraenol, E0) to the presense of Nitrosomonas europaea bacteria. Although E0 still possesses estrogenicity, N. europaea could further degrade E0 into non-estrogenic compounds

iv. Dehydrogenation of ring D at C-17: Studies have demonstrated the formation of E1 from E2 biodegradation (Yu et al. 2007).

v. During the fourth step, E1 was formed through a dehydrogenation process on the D ring C-17 position of E2. The degradation pathway of E1 in a soil isolate, Nocardia sp. E110, was first proposed by Coombe et al. (1966). Through hydroxylation at the A ring C-4 position of E1, E1 can be transformed into 4-OH E1, which can be further degraded via meta-cleavage (Kurisu et al. 2010). Another degradation pathway was suggested by Lee and Liu (2002). By studying E2

degradation in mixed sewage bacteria, they detected a new metabolite, X1, containing a lactone at ring D. X1 is believed to be further degraded by entering the TCA (tricarboxylic acid) cycle.

vi. Yu et al. (2013b) also demonstrated the pathway for the degradation of EE2 via bacteria fungi and algae (A-ring C-2 hydroxylation and ring A cleavage by nitrifying activated sludge (Yi and Harper, 2007)

a. Conversion of 3-OH into 3-keto in A ring of EE2 by an algal culture, *Scenedemus quadricauda* (Della Greca et al., 2008)

b. B-ring C-6 hydroxylation by an algal culture *Ankistrodesmus braunii* (Della Greca et al., 2008)

c. D-ring C-17 to keto by *Sphingobacterium* sp. JCR5 (Ren et al., 2007)

d. Formation of EE2 conjugation by an algal culture, *Scenedesmus capricormunam* (Della Greca et al., 2008). ETDC= (3-ethynyl-3a, 6,7-trimethyl -2,3,3a,4,5,5a, 8,9,9a 9b-decahydro-1*H*-cyclopenta[a]naphthalen-3-ol) Confirmed pathways: solid lines. Uncertain pathways: dash lines

vii. **Figure 25**), whereby EE2 is transformed to other compounds with limited estrogenicity. Figure 26 shows that the first product is ETDC without the A ring, which is expected since the electron density around the EE2 ring A was relatively high. The second, EE2-OH, is hydroxylated at the C-2, and EE2-SO_4 is conjugated at C-3, and C-3 and C-2 are also high FED carbon units (Yi and Harper 2007).

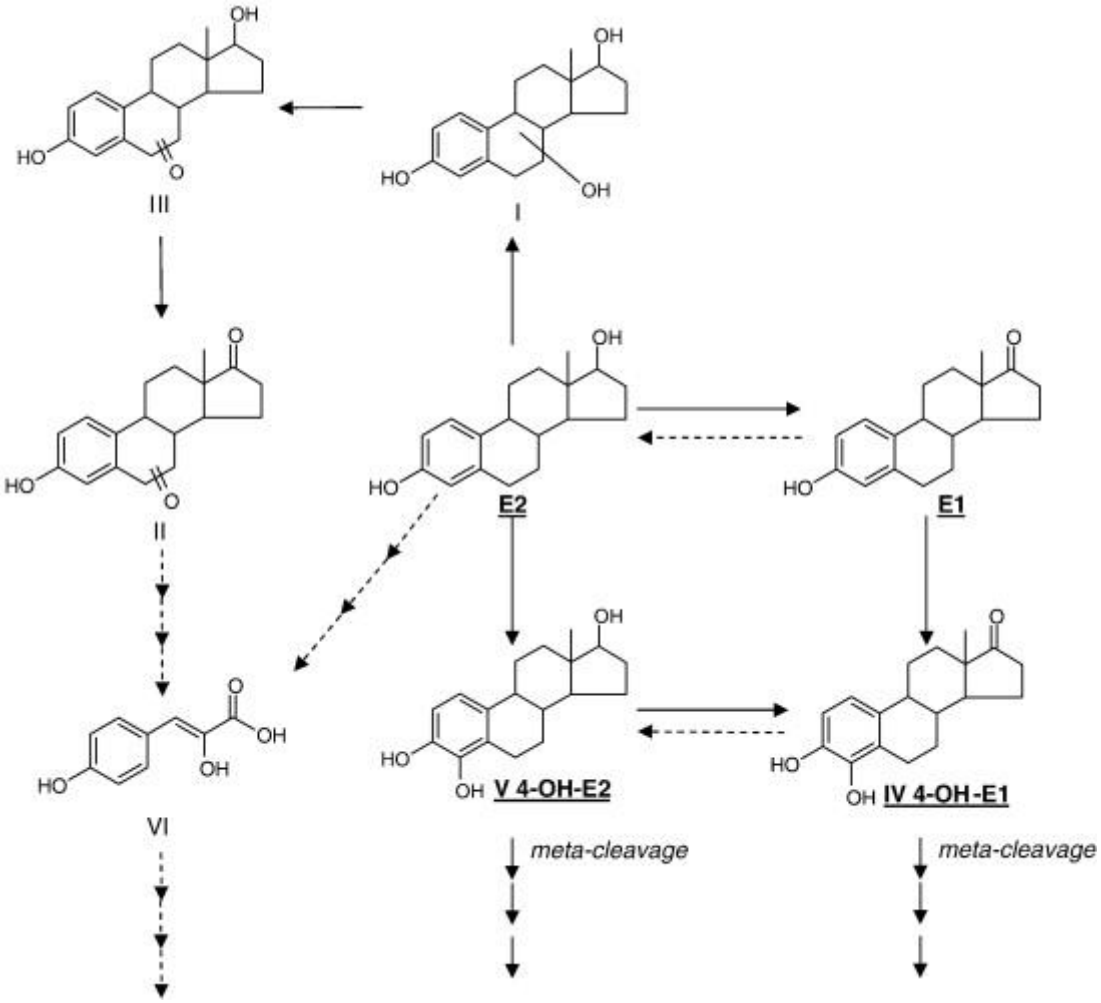
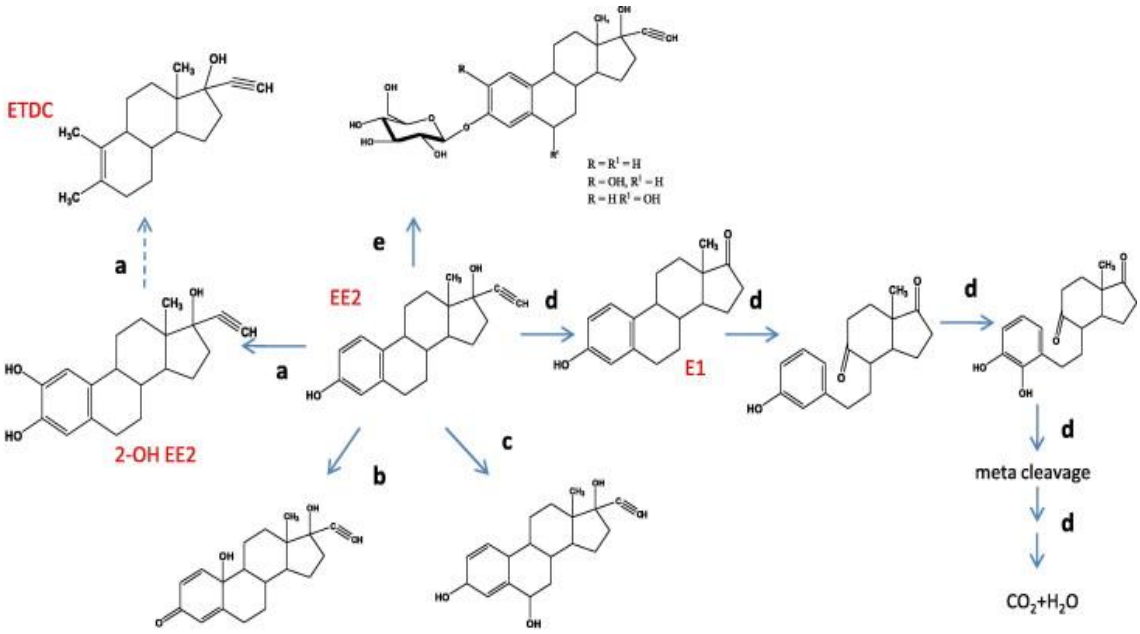


Figure 24. Hydroxylation of E2 (Kurisu et al. 2010)



e. A-ring C-2 hydroxylation and ring A cleavage by nitrifying activated sludge (Yi and Harper, 2007)

f. Conversion of 3-OH into 3-keto in A ring of EE2 by an algal culture, *Scenedemus quadricauda* (Della Greca et al., 2008)

g. B-ring C-6 hydroxylation by an algal culture *Ankistrodesmus braunii* (Della Greca et al., 2008)

h. D-ring C-17 to keto by *Sphingobacterium* sp. JCR5 (Ren et al., 2007)

i. Formation of EE2 conjugation by an algal culture, *Scenedesmus capricormunam* (Della Greca et al., 2008). ETDC= (3-ethynyl-3a, 6,7-trimethyl -2,3,3a,4,5,5a, 8,9,9a 9b-decahydro-1*H*-cyclopenta[a]naphthalen-3-ol) Confirmed pathways: solid lines. Uncertain pathways: dash lines

Figure 25. Degradation of EE2 via bacteria fungi and algae

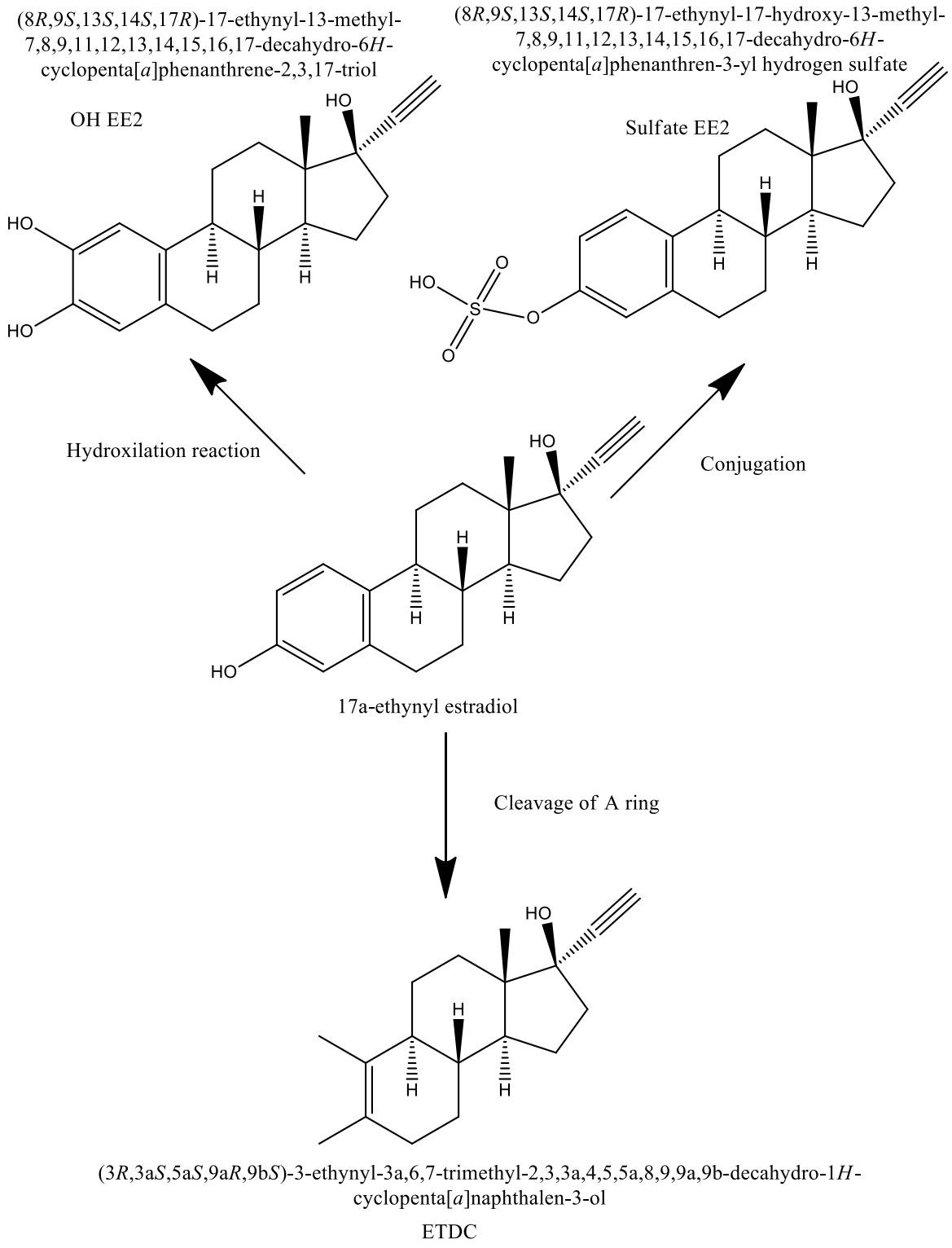


Figure 26 EE2 ring cleavage by nitrifying activated sludge (Yi and Harper 2007)

The conversion of ring 3-OH to 3-keto was also observed as shown in A-ring C-2 hydroxylation and ring A cleavage by nitrifying activated sludge (Yi and Harper, 2007)

j. Conversion of 3-OH into 3-keto in A ring of EE2 by an algal culture, *Scenedemus quadricauda* (Della Greca et al., 2008)

k. B-ring C-6 hydroxylation by an algal culture *Ankistrodesmus braunii* (Della Greca et al., 2008)

l. D-ring C-17 to keto by *Sphingobacterium* sp. JCR5 (Ren et al., 2007)

m. Formation of EE2 conjugation by an algal culture, *Scenedesmus capricormunam* (Della Greca et al., 2008). ETDC= (3-ethynyl-3a, 6,7-trimethyl -2,3,3a,4,5,5a, 8,9,9a 9b-decahydro-1*H*-cyclopenta[a]naphthalen-3-ol) Confirmed pathways: solid lines. Uncertain pathways: dash lines

Figure 25 (path b). This degradation pathway was observed in microalgae through hydroxylation and glucosylation as reported by (Della Greca et al. 1996; Della Greca et al. 2008b). Paths c and e were also reported and they include only a B ring C-6 hydroxylation, which only occurs in microalgae (Della Greca et al. 2008b). Path d of the mechanism was reported by Haiyan et al. (2007) who based their research on catabolic pathways of EE2 degradation with strain JCR5 bacteria and using characteristic biological catalysis and results obtained from mass spectrum analysis. The catabolic pathway of EE2 degradation is presented in Figure 27.

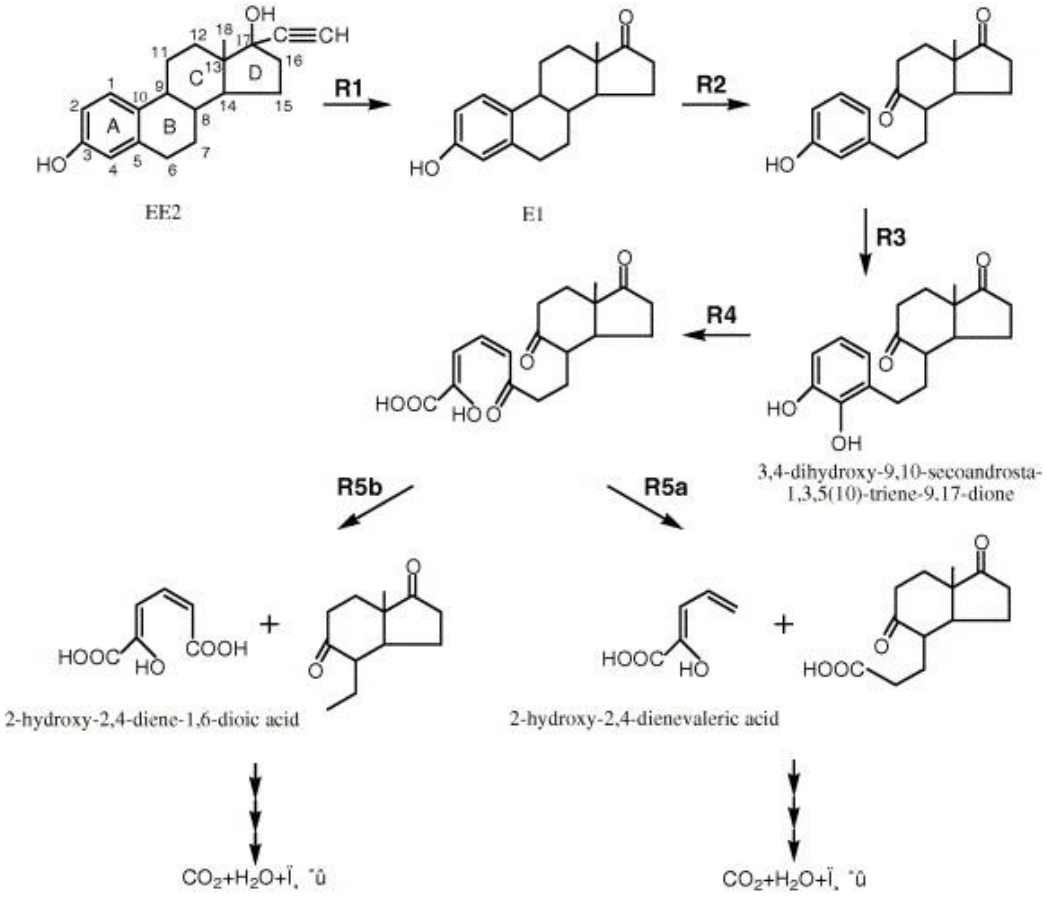


Figure 27. Catabolic pathways of EE2 degradation with strain JCR5 bacteria (Haiyan et al. 2007)

Lai et al. (2002) showed that C. vulgaris, an algae able to degrade E2 and E1, was not able to degrade EE2. In contrast, other algal cultures have shown their ability to transform EE2 into EE2 conjugates or more hydrophilic compounds as seen in the suggested mechanism below. Hence, Selenastrum capricornutum transforms EE2 into three products—EE2-glucoside, 3-β-D-glucopyranosyl-2-hydroxyethinylestradiol, and 3-β-D-glucopyranosyl-6β hydroxyethinylestradiol (Figure 28), while Scenedesmus quadricauda and Ankistrodesmus braunii transform EE2 into, respectively, 17α-ethinyl-

1,4-estradiene-10,17b-diol-3one and 6-α-hydroxy-ethinylestradiol (Della Greca et al. 2008a).

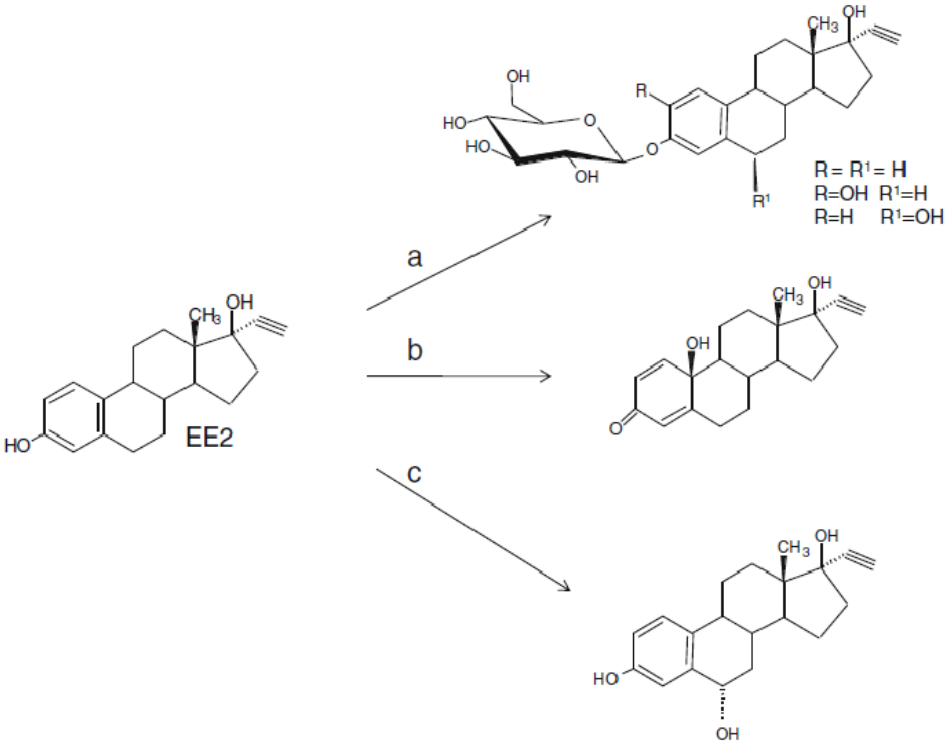


Figure 28. Selenastrum capricornutum transforming EE2 (Lai et al. 2002)

E3 is expected to follow the degradation pathways of E1 and E2 whereby conjugation can occur with similar mechanisms. A suggested pathway of biodegradation of E3 as mapped by Combalbert and Hernandez (2010) (Figure 29) includes:

1. Proposed degradation pathways of estrogens by bacteria under aerobic conditions (solid line)
2. Anoxic or anaerobic conditions (dashed line)
3. Algae (dotted line)

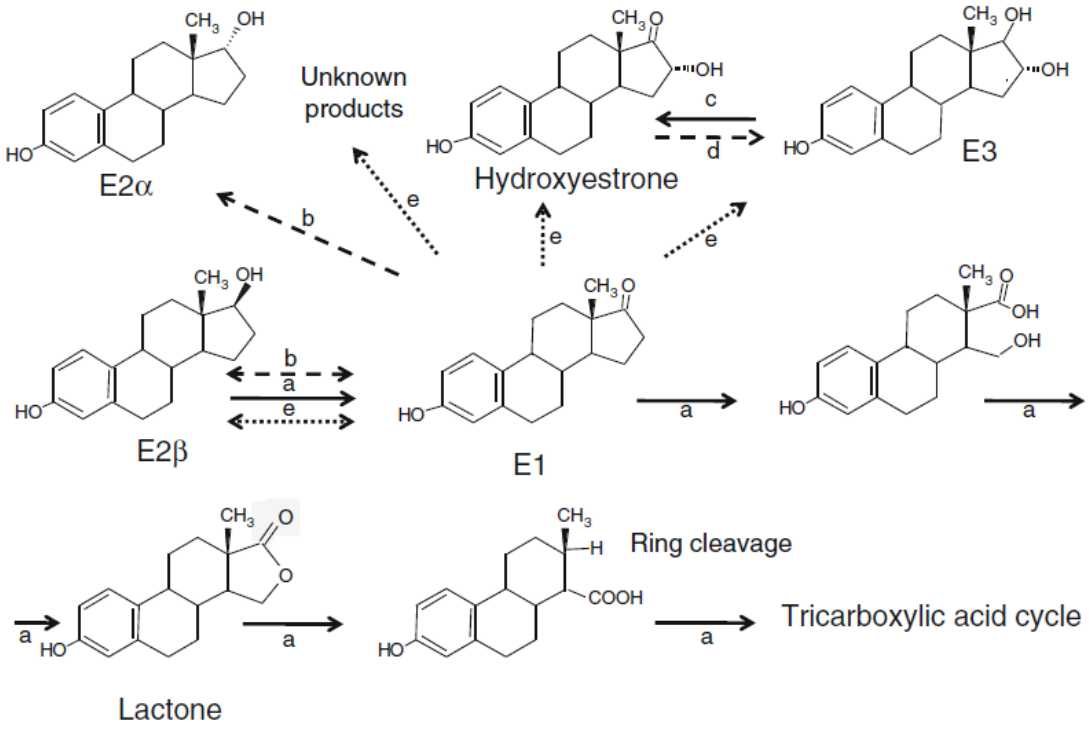


Figure 29. Biodegradation of E3(Combalbert and Hernandez-Raquet 2010b) a. (Lee and Liu 2002), b and c. (Czajka and Londry 2006; Ke et al. 2007), d. (Järvenpää et al. 1980), e. (Lai et al. 2002)

7. Chlorination

Free chlorine such as hypochlorous acid (HOCl) is commonly used for disinfection of water and wastewater effluent. HOCl readily reacts with phenolic compounds by stepwise substitution of chlorine to the aromatic ring at the 2, 4, and 6 positions (Racz and Goel 2010). The more highly chlorinated the phenolic reactant, the more likely the aromatic ring is to undergo oxidative rupture of the benzene ring. Using E1 as an example, Figure 30 illustrates the proposed pathway for the degradation of estrogens by chlorination (Lee and von Gunten 2009).

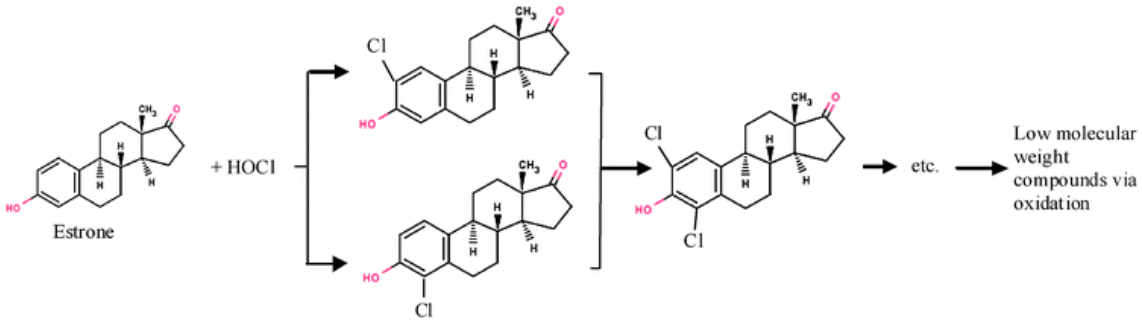


Figure 30. Degradation of estrone by chlorination (Racz and Goel 2010)

Lee and von Gunten (2009) achieved 90% conversion of EE2 with chlorine, but increased the rate of EE2 transformation by a factor of three with the addition of 0.25 µM Br. However, Br conjugates of EE2 are estrogenic of magnitude less than the parent compound and decreasing in estrogenicity with increasing bromination (Flores and Hill 2008). A bromination mechanism follows the same pathway as chlorination. In the presence of organic matter and humic substances, a competitive reaction occurs and Br is used up by humic acids faster than its utilization via estrogens thus rendering the process ineffective in the presence of such substances (Flores and Hill 2008). Even though some studies concluded that chlorination lowers the estrogenicity of WWTP effluents (Schiliro et al. 2009), others rendered it ineffective (Hu et al. 2003; Hu et al. 2002). Hu et al (2002, 2003) observed that E2 degradation by chlorination was incomplete. One explanation for these observations may be that ammonia chlorinated to form chloramines about one thousand times faster than phenol is chlorinated. Therefore, small amounts of chlorophenols or much slower rates of formation of chlorophenols would be expected in the presence of ammonia (Faust and Hunter 1967), which could be the case where a wastewater treatment plant does not include nitrification. Furthermore, there can be the

potential to form chlorinated organic intermediates, which can also pose an environmental risk (Racz and Goel 2010).

8. Oxidative coupling

Coughlin and Ezra (1968) first determined the effects of the presence of oxygen on the behavior of phenolic compounds. Since that time, multiple studies have been carried out on the sorption of phenols to GAC and soil (Vidic et al. 1993; Huang et al. 1995; Dąbrowski et al. 2005; Hamdaoui and Naffrechoux 2007; Hameed and Rahman 2008). GAC adsorptive capacity for several phenolic compounds under two different procedures, denoted as “oxic” and “anoxic” were employed in conducting adsorption isotherm tests (Vidic and Suidan 1991). That study highlighted the importance of such factors as the type of precursor of GAC, aqueous solubility of phenolic compound and oxygen availability in solution. Vidic et al. (1993) attributed the significant increase in the removal of phenolic compounds in the presence of molecular oxygen in the test environment. However, the recovery of phenols from the GAC surface was significantly diminished when the adsorption was carried out under oxic conditions. Vidic et al. (1993) attributed these findings to polymerization and the tendency of phenols to undergo oxidative coupling on the GAC surface. In the same study, three nitrophenols (2-Nitrophenol, 4-Nitrophenol, and 2,4-Dinitrophenol) exhibited no tendency to polymerize on the GAC surface in the presence of molecular oxygen. Therefore, the evidence for the occurrence of oxidative coupling as concluded by multiple studies (Terzyk 2003; Vidic et al. 1990; Vidic et al. 1997; Vidic et al. 1992):

- Sorption capacity for phenolic compounds is increased when oxygen is present
- The amount of oxygen consumed is proportional to the extent of irreversible sorption,
- The identification of some polymer products that are very difficult to desorb

The exact mechanisms governing oxidative coupling remains unclear. However, ample evidence shows that compounds with a phenolic functional group are more susceptible toward polymerization. Furthermore, the presence of acidic functional groups on the sorbent surface inhibit oxidative coupling (Juang et al. 2001; Juang et al. 1996a; Juang et al. 1996b; Bordwell and Cheng 1991; Vidic et al. 1990b). Because of oxidative coupling Vidic et al. (1993) stated that the formation of dimers, trimers, and even tetramers was possible, however only dimers were quantifiable using GC-MS.

Esperanza et al. (2007) studied the fate of seven sex hormones (E1, E2, EE2, E2, testosterone (TEST), progesterone (PROG), and androstenedione (AND)) in a synthetic wastewater (WW). In that study, RF was selected to be a representative of vegetable waste in the synthetic WW. Esperanza et al. (2007) observed that up to 90% of the estrogens were transformed under abiotic conditions. Marfil-Vega et al. (2010) studied the effect of individual constituents of the synthetic WW used by Esperanza et al. (2007) on the seven sex hormones. The study concluded that the four estrogens, E1, E2, E3, and EE2 were found to undergo abiotic transformation in the presence of (RF).

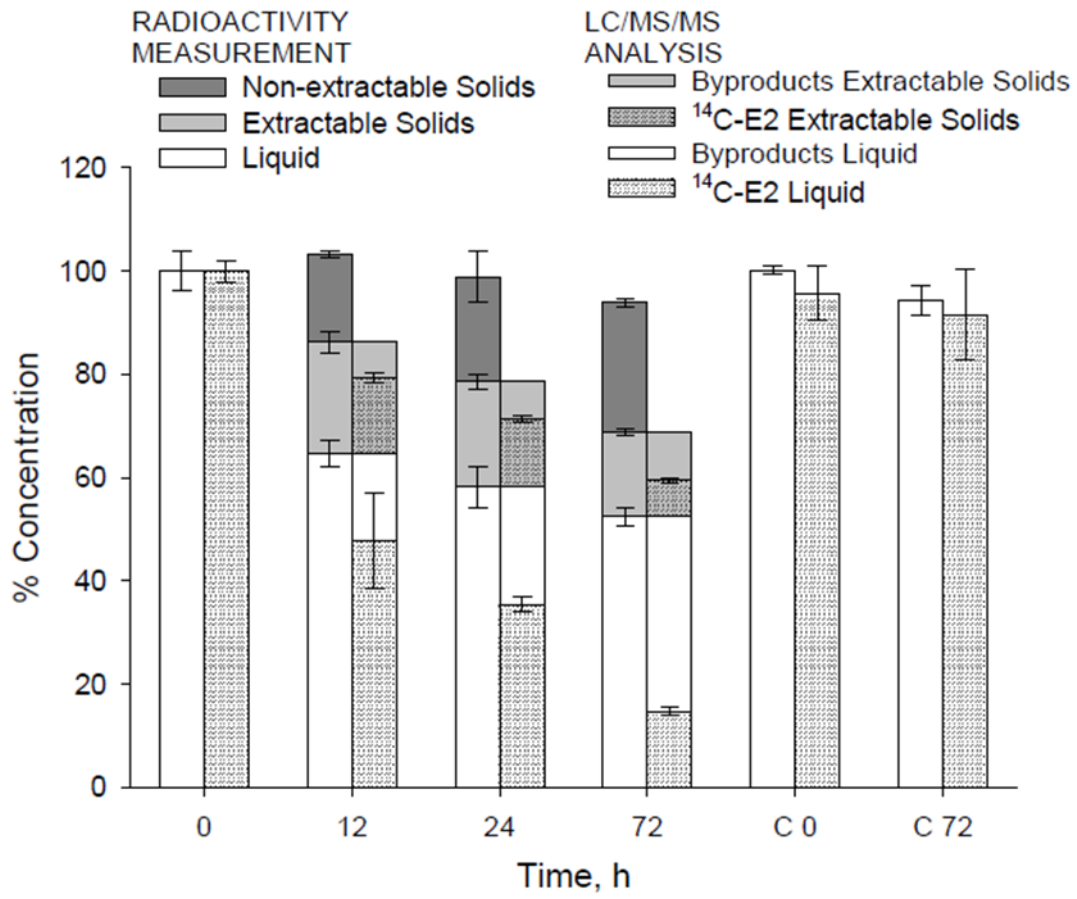


Figure 31. Percentage of concentration recovered of 17β-14C4-estradiol vs time, from radioactivity measurement (left side of bar) and LC/MS/MS analysis (right side of bar), in liquid, extractable solid, and non-extractable solid phases.
*** C0 and C72 are the control samples sacrificed at 0 and 72 h, respectively (Marfil vega et al. 2011).**

Using 14-C labeled E2, Marfil Vega et al. (2011) studied the abiotic transformation of E2 in the presence of RF and under aerobic conditions. In Figure 31the white bar on the left of every time event, represents the total radioactivity count (LSC) measured in the aqueous phase. The dotted white bar on the right represents the quantifiable mass of E2 using the LC MSMS. The difference between the white bar and the dotted white bar represents any transformation products present in the aqueous phase. The grey and dotted grey bars represent the radioactivity measurements and the

quantifiable mass of E2 using the LC MSMS extractable from the solid phase, respectively. The difference between the two bars represents the transformation byproduct extractable from the solid phase. The dark bar represents the radioactivity measured after burning the solids and capturing CO_2, which represents the mass of E2 that was not extractable from the solids. A parallel study revealed that no transformation of E2 occurred in the absence of oxygen and that E2 was completely extractable from the RF matrix (Marfil Vega et al., 2012). The total transformation of E2 was reported to be 38%, 50%, and 75% at 12, 24, and 48 h, respectively. On the other hand, the concentration measured by LC-MSMS analysis in the control samples (C0 and C72) matches the equivalent concentration calculated form the radioactivity measurement, confirming that the transformation of estrogens was abiotic and occurring exclusively in the presence of RF.

AND, TEST, and PROG with similar structure to the estrogens (Figure 32) were fully not transformed which shows that no transformation of the steroids investigated occurred when the said steroids had no phenolic functional (Marfil vega et al., 2011)

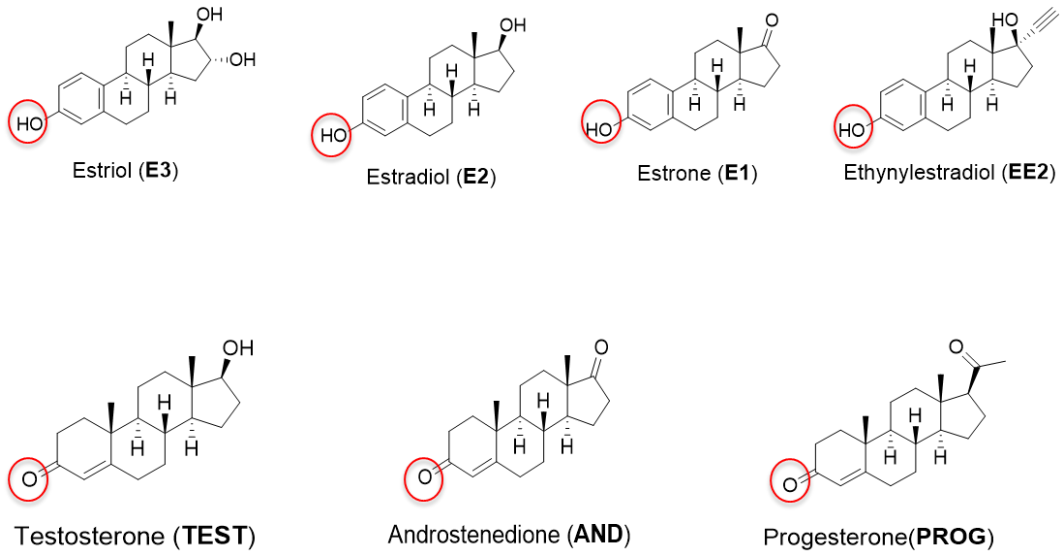


Figure 32. Structures of the seven sex hormones

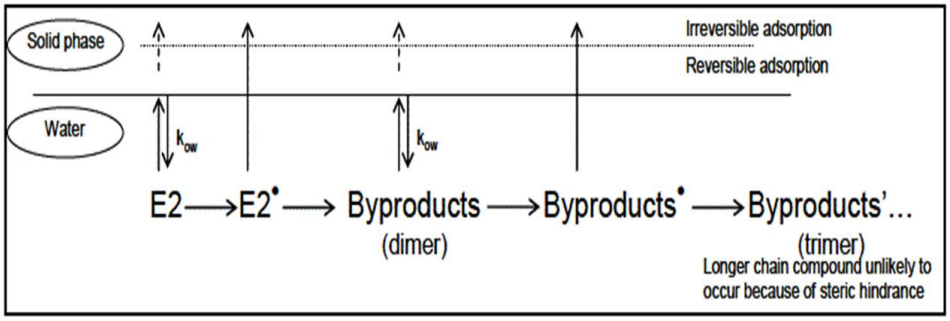


Figure 33. Proposed scheme of abiotic transformation of E2

According to Marfil Vega et al. (2011) after E2 comes in contact with the RF, it partitions reversibly to the surface of the solid material (depending upon its hydrophobicity) and, simultaneously, a semiquinone E2 radical can be formed. The radical will either undergo a coupling reaction with a similar radical forming a dimer or irreversibly incorporate into the surface of RF. The dimer can form a similar radical which can undergo the same transformation as the E2 radical.

The abiotic transformation was attributed to oxidative coupling of the estrogens, catalyzed by a still unidentified chemical. Marfil-Vega et al. (2011), hypothesized that metallic oxides in the RF might be the catalyst for the oxidative mechanism to take place. Some of these oxides, like the ones with manganese, are known to act as a true catalyst or as an oxidant, being consumed during the course of the reaction (Huang 1995).

F. Significance of Research

Human and animal excretions are the major sources of environmental estrogens (Figure 34). After excretion; these compounds enter the environment via multiple pathways (Figure 35). Sorption, photodegradation, biodegradation, and others are responsible for removal of estrogenic compounds from the environment. Existing WWTPs are not effective in the removal of emerging contaminants from effluent; most of them are not effective barriers for the retention of these contaminants discharged into the environment. Therefore, the necessity for optimizing existing wastewater treatment processes for the removal of these chemicals and the development of new cost- and energy-efficient processes is a priority. Understanding the mechanisms involved in estrogen removal from water is needed in order to help mitigate health and environmental risks resulting from exposure to EDCs. The need to develop a model for removal of estrogens from water matrices is of importance. Even though significant progress in elucidating the mechanisms of degradation of estrogens was made, abiotic transformation/degradation pathways remains a mystery. This led to inaccurate estimation of adsorption and underestimation of potential abiotic transformations that can occur. Consequently, erroneous results generated for the estimation of the potential contamination of surface and groundwater and, hence, for water recharge and reuse. As

such, understanding the abiotic pathway of estrogens is a knowledge base needed to assist the development of more effective removal processes for estrogens.

The objective of this study is to acquire a thorough understanding of the abiotic transformation of estrogens in aqueous and solid matrices. We additionally want to explore alternatives for removal of EE2, which can be implemented in current wastewater treatment practices. Experimentation conducted throughout the period of the study aimed at:

1. To demonstrate that EE2 undergoes the same abiotic transformations as was demonstrated by Marfil-vega et al. (2011) for E2
2. Demonstrate if the process is amenable to continuous flow column application for the attenuation of EE2
3. Identify natural occurring organic wastes and minerals that can affect similar transformation of EE2 to RF

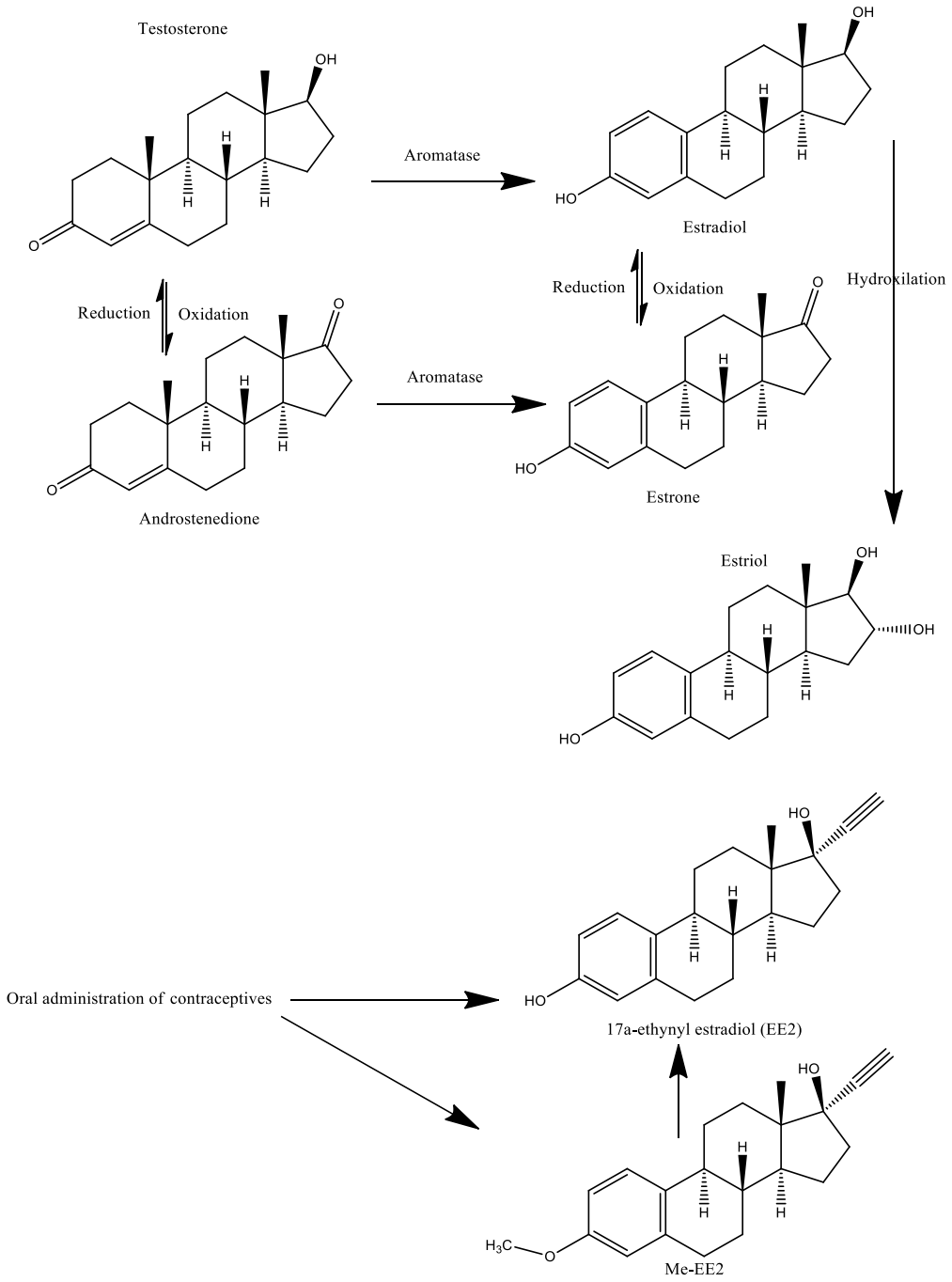


Figure 34. Estrogens in the human metabolic system

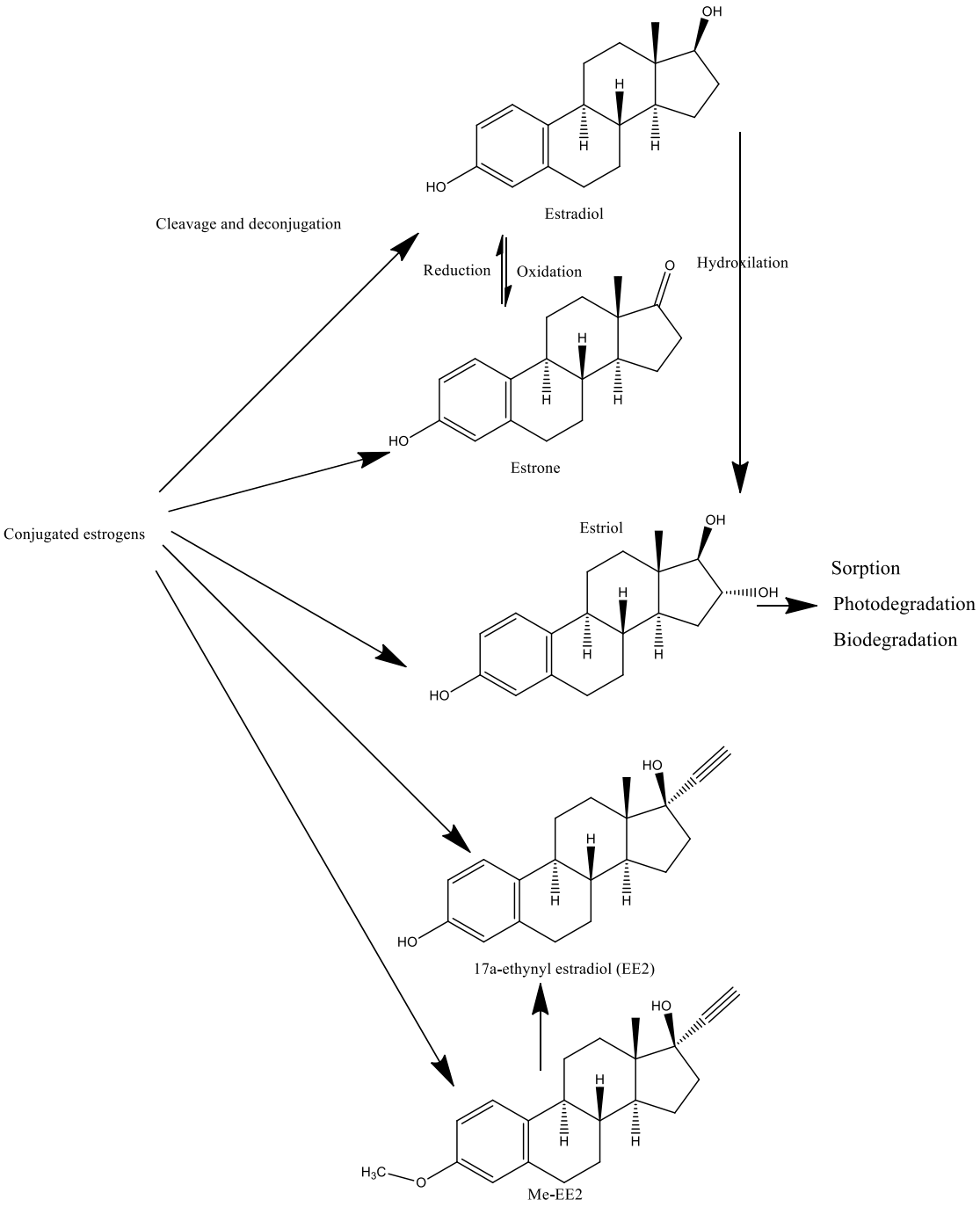


Figure 35. Estrogens in the Environment

CHAPTER III

MATERIALS AND METHODS

A. Chemicals

17α-Ethynylestradiol of 98% purity, obtained from Sigma-Aldrich (Milwaukee, WI), was spiked in milliQ water and used as primary feed. To determine the efficiency of the analytical procedure, d4-17α-Ethynylestradiol (d4-EE2 98.8 %; C/D/N Isotopes Inc., Canada) was used as a surrogate, and d4-Estrone (d4-E1 97%; Cambridge Isotope Laboratories. Inc., USA) was used as an internal standard for LC-MSMS qualification. LC-MS grade solvents Acetonitrile, Methanol, and Water (Fluka) as well as ammonium fluoride 98.0% purity (SIAL) were obtained from V.T.C s.a.l. Lebanon. Dimethyldichlorosilane (5%) in toluene was purchased from Supelco, USA. Model RF identical to the one used by Marfil-Vega et al. (2010) was used. Ottawa sand was obtained from the materials and soil laboratory at the American University of Beirut. Glass fiber syringe filters (Cronus), and Hamilton syringes with removable needle, gas tight gauge 22s borosilicate glass syringe/steel plunger/PTFE seal were supplied by V.T.C s.a.l Lebanon. Glass Fiber Filter, Pore Size 1.5 μm, Diameter 47 mm was acquired from Multilab Inc.

B. Experimental Design

A detailed description of each experiment conducted along the course of this study is available in the respective chapters.

C. Analytical Methods

In order to obtain an accurate mass balance for EE2 aqueous and solid phases were separated in all experiments by filtration with 1.2 μm pore size glass fiber filters. Each phase was processed separately. To avoid losses due to adsorption onto glass surfaces, all the glassware used was silanized (Sylon CT: 5% dimethyldichlorosilane in toluene, Supelco). Methodologies used to analyze both soluble and insoluble components for each experiment, along with the filters, were modified over the course of the study.

1. Liquid sample preparation

The filtrate was transferred to silanized bottles and spiked with the surrogate in preparation for solid phase extraction (SPE). Prior to extraction, the pH of the samples was maintained within the range of 6-8. HLB Water's Oasis (200 mg 6 cc) supplied by OASIS Inc. USA, was used to extract the EE2 from the water matrix. After being placed in a vacuum manifold, the cartridges were conditioned with methanol and LC-MS grade water and loaded at approximately 4 mL/min; after the liquid was fully loaded, the bottles were rinsed twice with 20 mL water. Before eluting the target compounds with 10 mL methanol, cartridges were washed with 10 mL of freshly prepared 10% methanol in water (v/v) and dried under vacuum for 5 minutes, followed by elution with 100% methanol. The final extract was then dried under a gentile steam of nitrogen at 40°C, and reconstitution took place in 50% methanol in water (v/v).

2. Solid sample preparation

After filtration, the solids from the batch experiments along with the grass fiber filters were placed individually in silanized glass vials. The samples are then frozen at -

80 °C and held for 24 h at that temperature. Frozen samples were later lyophilized using a freeze-dryer (Labconco Freeze-dry system/Freezone 4.5). Complete dryness of the samples was achieved after 72 h in the freeze-dryer. Afterwards the samples were collected from the freeze-dryer and spiked with the surrogate in preparation for extraction.

The analytical procedure went through major modifications. Initially, a traditional continuous extraction in an orbital shaker was used, later the solids from the column experiments were extracted using an Accelerated Solvent Extractor (ASE). Extraction experiments are described in details in Chapter 4. The samples were lyophilized prior to extraction during the initial phase of the study, and as the study progressed it was concluded that this step was not necessary when the samples were extracted in the ASE.

3. Sample cleanup

Samples requiring alumina cleanup were reconstituted in 0.2 mL DCM and 0.8 mL iso-octane. Cartridge conditioning took place with 9 mL of 30% methanol in acetone and 9 mL of 20% DCM in iso-octane. The 1 mL extract obtained after the SPE with HLB was loaded onto the cartridge, and the vials rinsed three times with 0.5 mL each of 20% DCM in iso-octane and loaded onto the cartridge. Cartridges were then washed with 9 mL hexane, and eluted with 9 mL 30% methanol in acetone. The final extracts were dried down under a gentle nitrogen stream in a water bath (at 40 °C), and reconstituted with a 50 % methanol in water mixture.

At the same time, the subsequent clean-up steps were modified: solid samples went through SPE with C-18 clean-up, and later on the HLB SPE procedure utilized for extracting the liquid samples was used for cleaning-up the extracts from the solid samples.

4. Method development

EPA method 539 is an analytical method that employs SPE and Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS) for the analysis of hormones in drinking water. The method uses analysis via internal standardization based on peak areas, and is used for the determination of seven hormones including EE2 (Smith et al. 2010). A modification of this method was developed by Yanes et al. (2011) and Wang and Cole (2009) which included replacing ammonium hydroxide with ammonium fluoride in the mobile phase of EPA 539. This modification allowed the LC-MSMS to operate at lower pH value leading to better sample preservation and improving column lifetime. The modification using Agilent 6460 triple quadrupole LC-MSMS and poroshell columns exceeded EPA LDL with 7 to 11 times more sensitivity in the negative mode and 1.7 to 2.4 times more sensitivity in the positive mode (Hindle 2013). The method requires the use of the MS/MS in Multiple reaction monitoring (MRM) mode to enhance selectivity. As such, the LC and MS conditions followed in this study are shown in Table 4. Modifications to these conditions are presented in different chapters where applicable. To quantify the target compounds (EE2, as well as the surrogate d4-EE2) using the LC-MSMS, four MRM transitions in negative mode were employed. These transitions corresponded to the pertinent precursor ion of each of the compounds at hand used as a qualifier and another less pertinent used as a quantifier. The internal standard used, d4-E1, also had two MRM transitions as shown in Table 5.

Table 4. LC and MS Conditions

LC Conditions			
Columns	Agilent Poroshell 120 Phenyl-Hexyl 2.1 x 100 mm, 2.7 um (p/n 695975-321)		
Colum Temperature	20 ºC		
Injection volume	30 uL		
Mobile phase	A: Water + 1 mM Ammonium Fluoride B: 35% Acetonitrile + 65 % Methanol		
Flow Rate	0.3 L/min		
Gradient	Time (min)	% A	% B
	0	100	0
	0.5	90	10
	12.5	0	100
	15	0	100
	22	10	90
	25	100	0
Post time	5 mins		
Total Runtime	30 mins		
MS Conditions			
Ionization Mode	ESI with negative polarity		
Drying gas temperature	350ºC		
Sheath gas temperature	375ºC		
Sheath gas flow	11 L/min		

Table 5. MRM transitions

Compound Name	Precursor Ion	Product Ion 1	Product Ion 2	Fragmentor voltage (V)	Collision Energy (V)	Polarity	Type
17α-Ethynylestradiol	295.2	145.1	159	136	36	Negative	Target
d4 17α-Ethynylestradiol	299.1	147.1	187.1	121	44	Negative	Surrogate
d4 Estrone	273.2	147	161.1	136	30	Negative	ISTD

Transitions reported in Table 5 were obtained from Agilent application notes (Hindle 2013) and were edited according to product ion scans performed using the LC-MSMS as reported in Figure 36 and Figure 37.

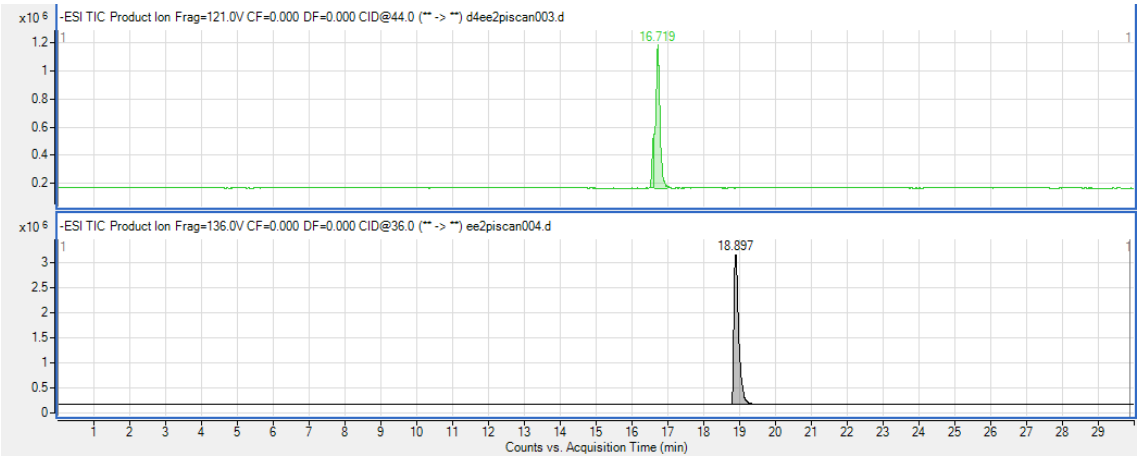


Figure 36. TIC of d4-17α-EE2 (Green) and 17α-EE2 (Black)

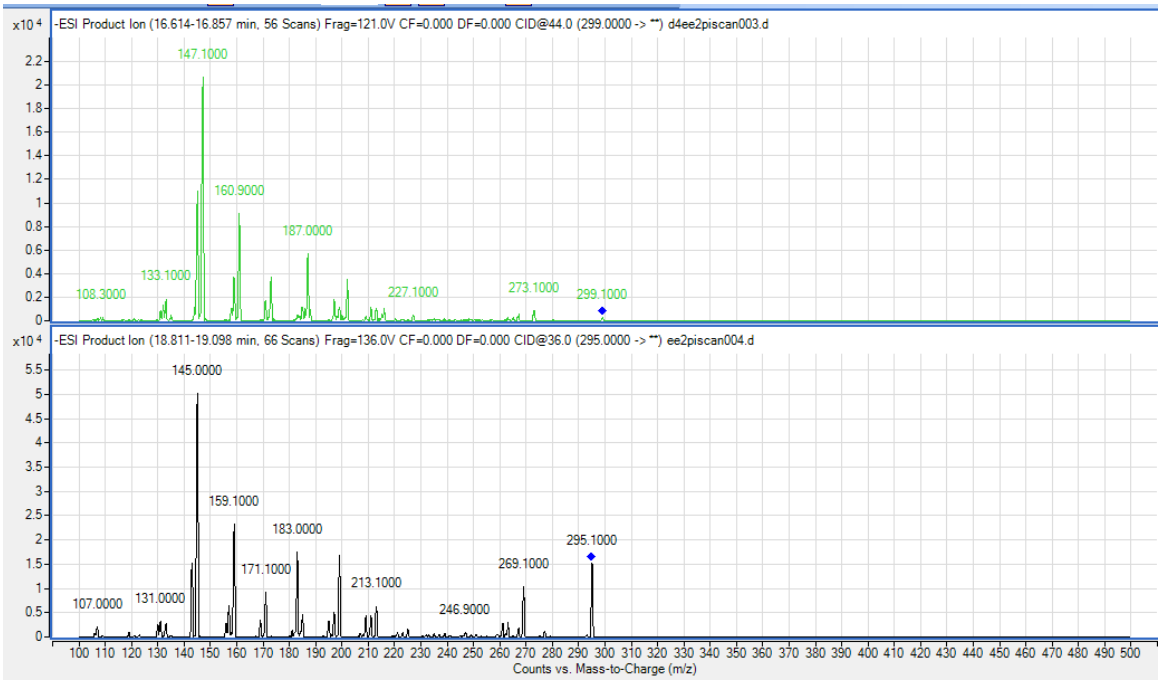


Figure 37. ESI product ions of d4-17α-EE2 (Green) and 17α-EE2 (Black)

The calibration curve, calibration checks standards, and blank samples were prepared analogously to the samples for each experiment performed, EE2 surrogate, target analyte, and ISTD were spiked into 1 mL of 50 percent methanol in water.

5. Method validation

For the LC-MSMS analysis, d4-EE2 was used as surrogate. This compound was spiked prior to extraction of the liquid and solid samples, with surrogate recovery calibration levels and quality check criteria set to 80 – 120% and 60 – 140% for liquid and solid matrices, respectively. d4-E1 was used as an internal standard and spiked at 40 ng/mL in all samples, quality checks, and calibration levels. Quantification was

performed using an external linear calibration curve, the calibration curves normally employ at least two quality checks and has an R^2 value of at least 0.95. A sample calibration curve is shown in Figure 38.

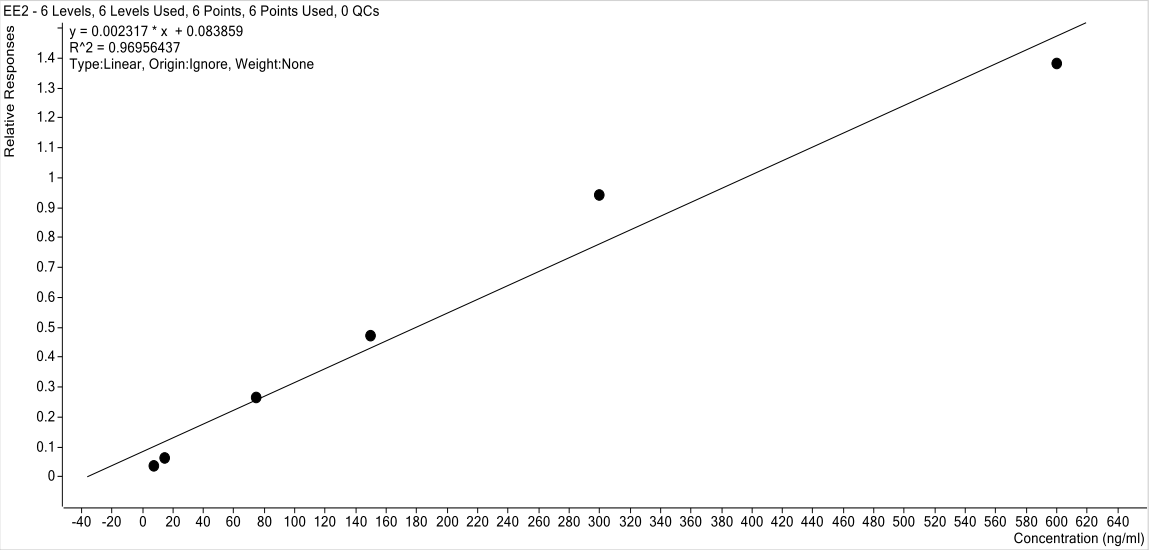


Figure 38. **Calibration curve 0 to 600 ng/L**

6. Initial demonstration of capability

In order to show method suitability for EPA 539 it is necessary to perform an initial demonstration of capability prior to demonstrating results pertaining to the LC-MSMS. Using distilled water blanks, 200 ng/mL of EE2 were spiked into five of the aforementioned samples, the samples were also spiked with the internal standard at 40 ng/mL. 200 ng/mL EE2 concentration was chosen based on proximity to the midrange of the calibration curve in Figure 38. The quantification result of these blanks was used in calculating the percent relative standard deviation as follows:

$$\%RSD = \frac{Standard\ deviation\ of\ measured\ concentrations}{Average\ concentratino} \times 100$$

$$= \frac{19.3}{214} \times 100 = 9.0\%$$

Which is less than the allowed limit of 20%. Afterwards an accuracy check was performed using the same data as the %RSD following the manner below:

$$\%Recovery = \frac{Average\ concentration}{Fortified\ concentration} \times 100 = \frac{214}{200} \times 100 = 107.1\%$$

The average percent recovery of 107.1% is acceptable the referenced value should be ±30 % of the true value. The minimum reporting level (MRL) was considered to be equivalent to 15 ng/mL being the lowest calibration. Seven replicated blanks were analyzed after spiking with 15 ng/mL. Samples showed a mean of 14.1 ng/mL and a standard deviation of 1.491 ng/mL. Therefore, the half range for the prediction interval or results (HR PIR) with its upper and lower limits are calculated as shown:

$$HR_{PIR} = 3.963 \times S = 3.963 \times 1.491 = 5.9$$

$$\frac{Mean + HR_{PIR}}{Fortified\ concentration} \times 100 = 133.1\%$$

$$\frac{Mean - HR_{PIR}}{Fortified\ concentration} \times 100 = 54.3\%$$

As such, the MRL is validated with upper and lower limits $\leqslant$150% and $\geqslant$50% respectively. Finally, the detection limit can be determined as follows:

$$DL = s \times t_{(n-1,1-\propto=0.99)} = 4.69\ ng/mL$$

S is the standard deviation of replicate analyses, and t is the Student's t value for the 99% confidence level with n − 1 degrees of freedom. For 7 replicates and at 95% confidence level t=3.143, and therefore the minimum detection limit was calculated to be 4.69 ng/mL. Experimentally, the calibration checks resulted in a minimum quantification limit of 10.2 ng/mL.

CHAPTER IV

COMPARISON OF EXTRACTION PROCEDURES OF 17α-ETHYNYLESTRADIOL (EE2) FROM VARIOUS SUBSTRATES

A. Introduction

17α-Ethynylestradiol (EE2) along with other estrogens can result in endocrine disrupting effects in humans and wildlife (Diamanti-Kandarakis et al. 2009a). Such contaminants find their way onto the environment via multiple pathways, which minimize the possibility of source control as a remediation strategy (Atkinson et al. 2012). Both growing populations and livestock-farming practices are major sources of estrogens in the environment. When present in soils, these compounds leach into ground water aquifers and as a result, find their way to water supplies. EE2 is characterized as having high resistance to degradation, more specifically biodegradation (Li et al. 2017). It has the tendency to absorb on organic matter, accumulate in sediments and concentrate in biota (Aris et al. 2014). With the aim of finding the most efficient extraction procedure, multiple extraction techniques of different compounds from various substrates were evaluated in the literature (Carro et al. 2012; Marvin et al. 1992; Shen and Shao 2005). Finding the most efficient extraction technique of a certain compound proved a challenge due to the large amount of variables involved in the process (Sadílek et al. 2016; Vazquez-Roig et al. 2010). It is necessary to develop a proper effective procedure for extracting estrogens from substrates in order to facilitate assessment of the impact of these contaminants in the environment (Priac et al. 2017). In this study, we investigated the extraction efficiency of EE2 from three different types of substrates (Ottawa sand, RF, and soil) using four different extraction techniques (Accelerated Solvent Extraction

(ASE), Sonication, Soxhlet, and conventional solvent extraction). Testes were carried out in triplicate samples, after 24 hours of spiking with EE2. The effect of having the substrate samples lyophilized (freeze-dried) prior to extraction was also investigated.

B. Materials and Methods

Red soil was obtained from farmlands near Tyre, Lebanon. Muffled Ottawa sand was purchased from VTC s.a.l. and the same RF used by Esperanza et al. (2007) and Marfil-Vega et al. (2011) was used in this study.

1. Sample preparation

Substrates used in this experiment were soil, Ottawa sand, and RF. 5 g of each substrate were weighed and placed in salinized glass vials. Samples were then spiked with 200 ng of EE2 and mixed thoroughly then held for 24 h. In case of lyophilization samples were then frozen at -80 °C and kept overnight at that temperature and lyophilized using a freeze-dryer (Labconco Freeze-dry system/Freezone 4.5) for 48 h. Afterwards, substrate samples were spiked with d4EE2 surrogate in preparation for extraction. Following EPA 1698, surrogate (d4-EE2) recovery range was set to 60 – 140%, and d4-E1 was added to the final extract to serve as an internal standard for the LC-MSMS.

2. Conventional solvent based extraction

Conventional solvent extraction of steroids from substrates is a multi-step and time-consuming procedure (Dinan et al. 2001; Schmidt et al. 1997; van Beek 2002). Following freeze-drying, aliquots of 10 mL methanol were added to the sample, with the

resulting mixture then placed in a rotary shaker operated at 200 rpm and 25 °C for 3h. Using a pasture pipette packed with glass wool and sodium sulfate, the solvent was transferred from the vial to another clean salinized vial. Another 10 mL of methanol was added and samples were placed in shaker for 3 h. This step was repeated one more time resulting in a final extract of 30 mL methanol and a total extraction time of 9 h. The 30 mL extracts were evaporated using a BUCHI rotary evaporator at 65 °C and 200 rpm, fitted with a Teflon adapter.

3. Soxhlet extraction

Using conventional Soxhlet extraction for micro-contaminants, such as POPs, PAHs, and EDCs in different substrates has been thoroughly studied (Pan et al. 2007; Picer and Picer 1995; Sadílek et al. 2016). Following EPA method 1698 (EPA 2007), samples were extracted for 16-24 hours using 200 mL of 50:50 methanol:acetone in a glass Soxhlet extractor at a rate of 2-3 cycles every 10 min . Cellulose extraction thimbles (Fisher-brand) with diameter/size 33x100 mm were used. Extracts were evaporated using a BUCHI rotary evaporator at 65 °C and 200 rpm.

4. Ultra-sonication

Samples were kept in silanized vials and extracted using four cycles of ultra-sonication. Each ultra-sonication cycle consisted of adding 15 mL of methanol:acetone (50:50) to the sample, placing the beaker into a 40 KHz 10 L ultrasonic bath (RoHS ultrasonic cleaner) for 20 min. Using a pasture pipette packed with glass wool and sodium sulfate, the solvent was transferred from the vial to a silanized 60 mL vial. The extracts

were later evaporated using a BUCHI rotary evaporator at 65 °C and 200 rpm, fitted with a Teflon adapter.

5. Accelerated solvent extraction (ASE)

ASE is widely used as a non-conventional method for the extraction of various chemical compounds from substrates (Roselló-Soto et al. 2015; Sasidharan et al. 2011). ASE places samples under pressure thus can utilize set temperatures higher than the solvent boiling point in order to achieve the best extraction efficiency (Chiron et al. 2000; Macnaughton et al. 1997). This method can improve precision of analysis of multiple compounds using less solvent and shorter extraction time (Chiron et al. 2000; Croce et al. 2003; Harb and Aldstadt III 2004). This extraction process leads to reduced cost of sample preparation (Schantz et al. 1997; Shen and Shao 2005).

Using ASE Dionex 350 (Dionex Corporation), samples were filled into a 22 mL stainless steel extraction cell. The bottom of the cell was fitted with a glass fiber filter paper (Thermo Sceintific). The remaining cell volume was filled with Ottawa sand and diatomaceous earth in order to reduce the amount of solvent used. Extraction was carried out under the following instrument conditions: 2 extraction cycles, 150 °C, 1500 psi. Solvents evaluated were 20:80 and 50:50 of methanol:acetone for RF and Ottawa sand substrates. Soil substrates were extracted using 20:80 methanol:acetone without freeze-drying. Triplicates of substrate samples were extracted consecutively. The final extracts were evaporated in preparation for sample cleanup using a BUCHI rotary evaporator at 65 °C and 200 rpm fitted with a Teflon adapter. Samples processed without freeze-drying were dried using a sodium sulfate (Na_2SO_4) and 0.2 g of DE.

6. Sample Cleanup

Samples from various extraction methods required cleanup with neutral alumina followed by HLB cleanup. The cleanup procedure was performed as described in chapter 3.

7. Analytical procedure

Analysis performed using LC-MSMS following the procedure elaborated in quantification section (chapter 3).

C. Results and Discussion

The optimum extraction process was determined based on the extraction efficiency of the target analytes from spiked substrates as determined by LC-MSMS analysis of the extract. Percent recoveries of EE2 obtained using the different extraction methods are shown in Figure 39. The highest observed recoveries of EE2 were those of Ottawa sand samples, implying minimal interactions between EE2 and the substrate.

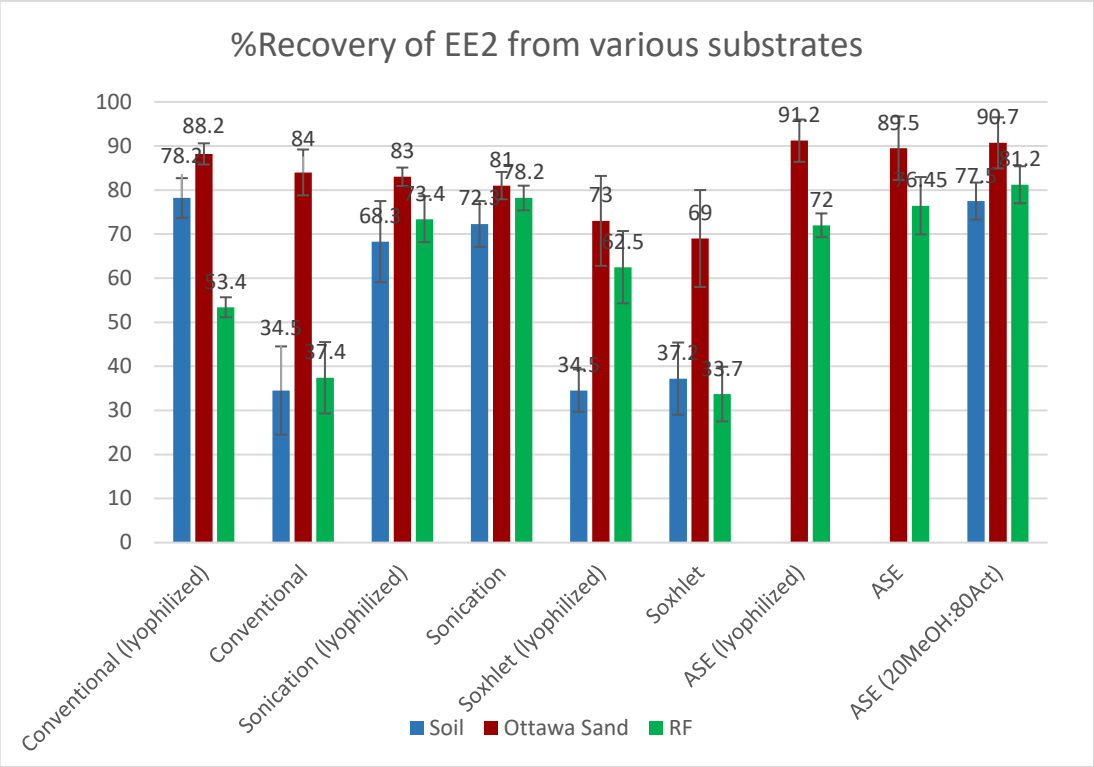


Figure 39. Percentage Recovery of EE2 from various substrates

Conventional solvent extraction results varied appreciably under different experimental conditions. Recoveries of EE2 from RF averaged at 37% and 53%, with the higher recoveries resulting from the lyophilized samples. EE2 recoveries from soil improved by almost two folds with lyophilization increasing from 34.5% to 78.2%. These figures highlight the importance of freeze-drying for conventional solvent based extraction procedure.

When comparing the results for Soxhlet extraction, recoveries from RF averaged at 33.7% and increased to 62.5% for the lyophilized samples. However, recoveries from soil substrates slightly decreased for lyophilized samples from an average of 37.2% to 34.5%. Soxhlet extraction proved to be the most time consuming of the studied extraction

procedures. Losses of EE2 can be attributed to long extraction, increased temperatures, and the use of large volume of extract (200 mL).

Sonication extraction proved to produce the highest recovery of EE2 of the described experiments with good reproducibility. Averaging 68.3 % and 72.3% for soil substrates, 78.2% and 73.4% for RF substrates, with and without lyophilizing respectively. It was deduced that lyophilization was unnecessary in this extraction procedure. It should be noted that the samples collected after sonication were the most turbid amongst the extracts from various procedures, and consequently needed further sample cleanup steps prior to injection on the LC-MSMS.

ASE proved to be the simplest, least time consuming, and an effective way of extracting EE2 from the various substrates. Observed extraction efficiency of EE2 from RF averaged at 72% and 76.45% for lyophilized and non-lyophilized samples, respectively. Using a different solvent ratio (20:80 methanol:acetone) accounted for 81.2% recovery of EE2 from RF. Soil substrates were extracted with 20:80 methanol:acetone and recoveries averaged at 77.5% without lyophilizing the samples. ASE extractions achieved a higher performance upon using a higher ratio of acetone in the extraction solvent. The lack of improvement of EE2 recovery with lyophilization suggested that the drying step was unnecessary. Furthermore, samples were cleaner than the ones extracted using sonication.

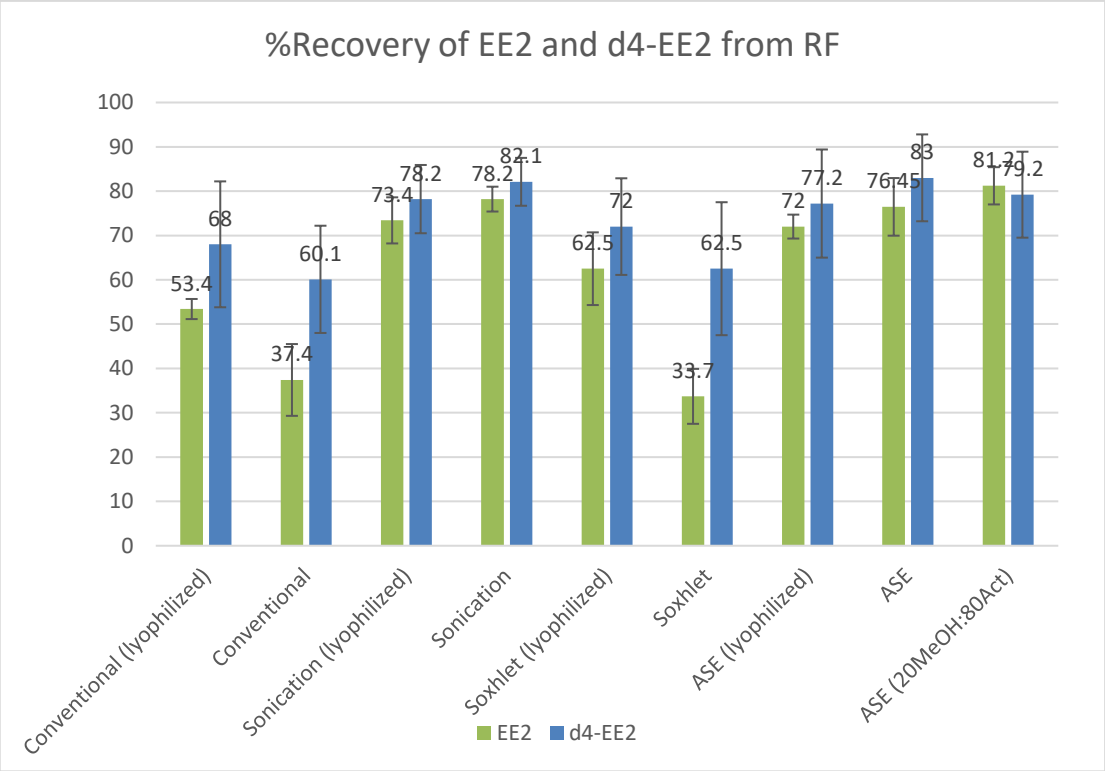


Figure 40 Percentage recovery of EE2 and d4-EE2 from RF

Comparing the Soxhlet extraction recoveries of the surrogate d4-EE2 to that of EE2 from the RF substrate (Figure 40), it was noticeable that the surrogate yielded higher recoveries averaging at 62.5 and 72 % without and with lyophilization, respectively. As explained in the experimental procedure, EE2 was spiked for 24 h prior to extraction while d4-EE2 was spiked onto the substrates directly before extraction. It was noticeable during both Soxhlet and conventional solvent based extractions, lyophilized samples returned higher recoveries of the target analyte and the surrogate. Never the less, a high percentage of EE2 was unrecoverable from the RF. The variation in recoveries of EE2 and d4-EE2 serves to show that some form of transformation of EE2 took place within the RF after being held for 24h, and was, therefore not extractable from the substrate. It

is important to point out that in earlier studies E2 exhibited similar behavior upon exposure to the same RF (Figure 31).

D. Conclusion

This chapter focused on the extraction of EE2 from various substrates, comparing four different extraction processes. The comparison was carried out using an environmentally relevant concentrations. ASE and ultra-sonication techniques proved to be the most efficient in extracting EE2 from the three substrates investigated. These techniques proved to be accurate and less time consuming producing reproducible results and eliminating the need for freeze-drying (lyophilizing). Soxhlet and conventional solvent-based extraction proved to be up to 20 times more time consuming than ASE and ultra-sonication extractions with lower recoveries. Further testing is required to provide a better comparative analysis of the different extraction procedures, the use of different solvents at multiple ratios will further improve this study. Selecting an optimum extraction technique requires a more thorough investigation of the costs, accuracy and precision, technical competence, and analysis times. Based on the results shown in this chapter, ASE was selected to be the extraction procedure of EE2 from solid matrices.

CHAPTER V

ATTENUATION OF 17 α-ETHYNYLESTRADIOL ONTO MODEL VEGETABLE WASTE

A. Introduction

E1, E2, EE2, and E3 can result in endocrine disrupting effects in human and wildlife populations, influencing reproduction and development (Ogino et al. 2007; Sone et al. 2004; Sumpter and Jobling 1995). The potency of these compounds can lead to detrimental health consequences at concentrations as low as 1 ng per liter (Caldwell et al. 2010; Irwin et al. 2001; Jobling et al. 1995). Fish species are known to be the most affected due to palpable impacts imparted by estrogens (Sumpter and Jobling 1995; Sumpter and Johnson 2005). The source of estrogens in the environment has been attributed to effluent from wastewater treatment plants (WWTPs), hospital waste, and runoff from livestock activity (Ying et al. 2002).

EE2 is a synthetic estrogen derived from the natural estrogen E2 and mostly used as an oral contraceptive (Diamanti-Kandarakis et al. 2009b). Medicinal uses of EE2 include treatment of prostatic cancer, treatment of breast cancer in postmenopausal women, and osteoporosis (Lima et al. 2011; Ying et al. 2002). EE2 has become a major problem in aquatic environments (Tomšíková et al. 2012). It is characterized as having high resistance to degradation, more specifically biodegradation (Aris et al. 2014), tendency to absorb onto organic matter, accumulate in sediments and concentrate in biota (De Wit et al. 2010). Although estrogens are typically excreted in a conjugated form, rapid conversion to unconjugated molecules can take place in the environment (Hanselman et al. 2003; Lai et al. 2000). Consequently, EE2 containing an ethynyl-group

becomes more stable against oxidation and exhibits more resistance to degradation when compared to the other estrogens (Li et al. 2013; Partridge et al. 2010). According to Johnson and Williams (2004a), 17 % of the total female population in western countries use contraceptive pills regularly. That study also reported that 4.5 and 6 μg per day of EE2 are excreted per capita in urine and feces respectively. Combalbert and Hernandez-Raquet (2010a) used these findings to estimate that a total of 4.4 kg per million inhabitants is discharged into the environment annually. Even though conventional WWTPs are considered effective in removing nutrients and solids from wastewater, estrogens on the other hand, follow a different pathway and, consequently, cannot be completely removed (Atkinson et al. 2012; Auriol et al. 2007; Mes et al. 2005). Andrew et al. (2010) studied the effect of EE2 during in vitro tests and reported that the estrogenic potency of the compound is higher than that of other estrogens (EE2>E2>E1). EE2 exhibited 11- 30 times more potency than E2 in in-vitro tests in certain species of fish (Colman et al. 2009). This made the selection of EE2 as a representative of estrogens more pertinent for this study.

In order to mitigate the risks imparted by estrogens on the aquatic environment, their elimination from water and wastewater is necessary. One of the most widely employed procedures is sorption onto a solid phase, where isotherm equations are employed to describe the sorption kinetics of target compounds onto the sorbent used (Chen et al. 2014; Silva et al. 2012b). Sorption onto activated sludge is potentially the principal process for the removal of EE2 and other estrogens in wastewater (WW) effluent (Andersen et al. 2005; Auriol et al. 2006b; Ren et al. 2007; Sumpter and Johnson 2005). With EE2 having low water solubility and poor biodegradability, sorption on suspended solids present in WW is considered to be the major factor for removal during wastewater

treatment (Andersen et al. 2005; Ren et al. 2007). Esperanza et al. (2007) reported on the degradation of estrogens in during aerobic wastewater treatment. They were first to report the abiotic transformation of estrogens in WWTPs. On the other hand, Carballa et al. (2004) noted that in conventional activated sludge systems with low sludge retention times, removal of estrogens was minimal.

Sorbents such as activated carbon (AC) have a high capacity for the estrogens E1 and E2 (Fukuhara et al. 2006). Kumar and Mohan (2011) reported on the sorption of EE2 onto AC in batch and column studies with up to 78 percent removal. Other studies suggested that the removal of estrogens using AC is highly dependent on the presence of organic matter and other substances present in water that can compete for sorption sites (Fukuhara et al. 2006; Kumar et al. 2009a; Snyder et al. 2007). The presence of these substances can affect the frequency of regeneration/replacement of AC beds in order to maintain removal effectiveness (Zhang and Zhou 2005).

Marfil-Vega et al. (2010) conducted batch experiments to model the catalytic conversion of E1, E2, E3 and EE2 in synthetic wastewater using RF as surrogate material for vegetable wastes found in wastewater. Up to 80% removal of estrogens was reported, and this removal was attributed to abiotic transformation under aerobic conditions and in the presence of RF. Forrez et al. (2009) also assessed the occurrence of catalytic reactions mediated by chemical agents such as metals or metal oxides resulting in abiotic transformation of estrogens, without being able to define a clear mechanism for this process.

The importance of abiotic transformation of estrogens as representative compounds of phenolic EDCs lies in the ability to extrapolate such transformation to other micropollutants with similar chemical structure (BPA and alkylphenol surfactants)

(Omar et al. 2016). To demonstrate that EE2 undergoes the same abiotic transformations as demonstrated by Marfil-vega et al. (2011) for E2, batch experiments were carried out using the similar experimental conditions. Upon completion of the batch runs, this study aimed to demonstrate the potential for continuous flow column application for the attenuation of EE2. The set objective was achieved by exploring the sorption of EE2 onto model vegetable waste (RF) by running breakthrough experiments in columns packed with a mixture of Ottawa sand and ground RF.

B. Materials and Methods

d4-EE2 and d4-E1 were used as surrogate and internal standard, respectively for LC-MSMS quantification. LC-MS grade solvents acetonitrile, methanol, and water (Fluka, LC-MS Chromasolv) as well as ammonium fluoride of greater than 98.0% purity (SIAL), obtained from V.T.C sal. Lebanon were used in the LC mobile phase. Quartz glass columns with outer diameter of 3 cm, inner diameter of 2.76 cm, and total length of 30.5 cm and Teflon fittings were used to insure minimal interference with attenuation of estrogens. All glassware used in this experiment was salinized with 5% Dimethyldichlorosilane in toluene.

1. Batch experiment

In this study, we investigate the fate of EE2 in the presence of RF. Multiple sets of batch experiments were performed over the course of 192 h (8 days), where 50 ng of EE2 was spiked in 100 mL distilled water and placed in serum bottles containing 1.4 g/L of RF. Another batch run was conducted using 2000 ng/L EE2 and 3.3 g/L of RF.

Samples were continuously shaken at 200 rpm and 25 °C until they were sacrificed at preset time internals. Throughout the experiment, liquid and solid phases were analyzed independently at each sampling time using LC-MSMS. Solid and liquid phases were separate using a 1.2-micron glass microfiber filter. Control runs with 0 g of RF were carried out in parallel with each experiment.

2. Column experiment

Column experiments were carried out in triplicates. Quartz columns were packed with a mixture of sand and ground RF and run alongside blank columns containing only Ottawa sand as packing material. Columns containing RF were packed in three layers, an active middle section comprised of a mixture of equal size Ottawa sand and RF, sandwiched between two Ottawa sand layers. Metallic screens placed at both ends of the column with similar opening size to the packing materials insured that column packing remained intact throughout the study. Flow rate was controlled using low flow pumps (Cerampump FMI QG 50) with a maximum flow of 5 mL/min, and a "CSC-W" stainless steel pump head fitted and PTFE tubing. A schematic of the column experiments along with the control column run (0 g RF) is illustrated in Figure 41.

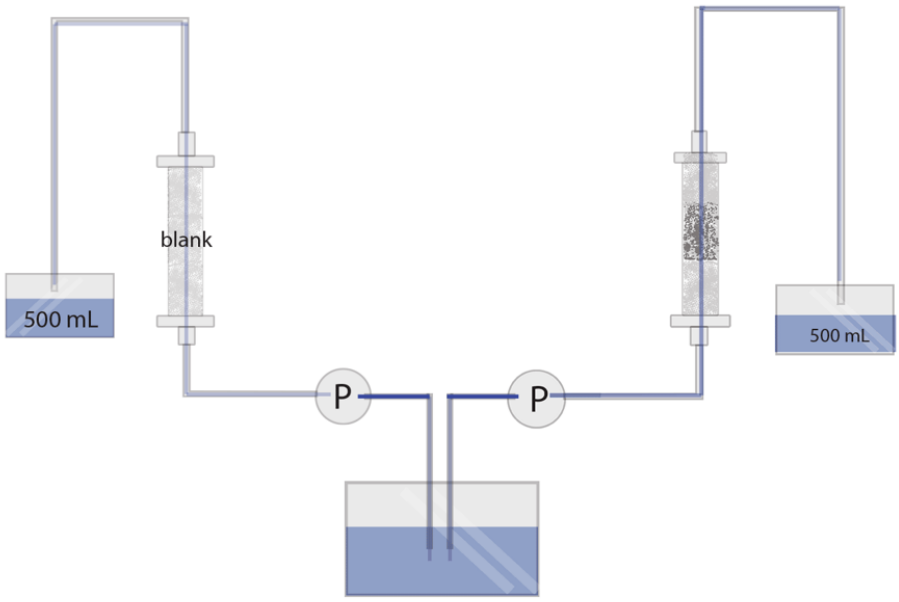


Figure 41 Schematic of the column experiments

Influent consisted of EE2 spiked at 200 ng/L in distilled water with sodium azide (20 mg/L) acting as an inhibitor of aerobic biological activity. The experiments were operated in upflow mode at room temperature 22-24 °C. Flow rates and the mass of RF employed were varied for different sets of experiments. Flow rates of 0.17, 0.35, and 0.7 mL/min and masses of RF of 3.2, 5.0, and 7.5 g were used with each flow rate. Effluent was collected in a collection flask and held until a final volume of 500 mL was reached prior to sample analysis. To avoid losses due to sorption onto glass surfaces, all the glassware used was silanized with 5% dimethyldichlorosilane in toluene (Supelco, USA). An Agilent 6460 LC-MSMS was used to measure the concentration of EE2 in both the aqueous and solid phases.

a. Sample preparation

Following effluent collection, the pH was adjusted in the range of 6-8. d4-EE2 was then added to serve as a surrogate. Samples were then extracted by means of solid phase extraciton (SPE) using HLB Water's Oasis (200 mg 6 cc) cartridges (OASIS Inc. USA) (Fang et al. 2016). The cartridges were conditioned with methanol and LC-MS grade water before being loaded with the column effluents at approximately 4 mL/min. Finally, the cartridges were washed with 10 mL of 10% methanol in water, and dried under vacuum for 5 minutes. Elution was with 10 mL methanol, and the eluent was filtered using a PTFE syringe filter, and latex and silicone-oil-free 5 mL inert syringes (NORM-JECT). The filtrates were dried under a gentle nitrogen stream at 40 °C and reconstituted in 0.5 mL of 50% methanol in water.

Following completion of the experiments, columns were emptied and the sandwiched active part was isolated and left to dry in open air for extraction using Dionex ASE 350 Accelerated Solvent Extractor (ASE). The samples were spiked with d4-EE2 surrogate and mixed with approximately 4 g of diatomaceous earth to remove the remaining traces of water trapped in the solids. ASE cell void volume was filled with Ottawa sand in order to reduce the amount of solvent used, and was extracted under the following conditions: 2 extraction cycles, 150 °C, 1500 psi, and methanol: acetone 20:80 (v/v). The final extracts were collected in silanized vials. The extracts were dried under a gentle nitrogen stream at 40 °C and reconstituted in 0.2 mL dichloromethane (DCM) and 0.8 mL iso-octane for further cleanup with neutral alumina. Supelclean 3 mL LC-Alumina-N cartridges were conditioned with 9 mL of 30% methanol in acetone followed by 9 mL of 20% DCM in iso-octane. The 1 mL extracts were transferred to the cartridge, and the vials were rinsed three times with 0.5 mL of 20% DCM in iso-octane. Samples

combined with the rinses were loaded by gravity and washed with 9 mL hexane. Samples were eluted with 9 mL 30% methanol in acetone, then filtered through a syringe filter. The final extracts were dried under a gentle nitrogen stream at 40 °C and reconstituted with 0.5 mL of 50% methanol in water.

3. Quantification

A calibration curve, calibration check standards, and blank samples were prepared for every experiment performed. EE2 surrogate and target analyte were spiked in 1 mL of 50 percent methanol in water. The calibration standards covered a concentration range of 6.5 to 200 ng/L. LC and MS conditions were adopted from EPA standard method 539 and Agilent application notes (Hindle 2013; Smith et al. 2010). Following EPA 539, and 1698 standard methods, surrogate (d4-EE2) recovery quality check criteria were set to 70 – 120% and 60 – 140% for liquid and solid matrices, respectively (Smith et al. 2010). d4-E1 was used as an internal standard and spiked into the concentrated extract immediately prior to injection into the LC-MSMS. LC and MS conditions used for this experiment are shown in Table 6.

Table 6 LC and MS conditions (column experiment)

LC Conditions			
Columns	Agilent Poroshell 120 Phenyl-Hexyl 2.1 x 100 mm, 2.7 um (p/n 695975-321)		
Colum Temperature	20 °C		
Injection volume	5 uL		
Mobile phase	A: Water + 2 mM Ammonium fluoride		
	B: 35% Acetonitrile + 65 % Methanol + 2mM Ammonium fluoride		
Flow Rate	0.3 L/min		
Gradient	Time (min)	% A	% B
	0	100	0
	6	90	10
	12.5	0	100
	15	0	100
	22	10	90
	25	100	0
Post time	5 mins		
Total Runtime	30 mins		
MS Conditions			
Ionization Mode	ESI with negative polarity		
Drying gas temperature	350°C		
Drying gas Pressure	40 psig		
Sheath gas temperature	375°C		
Sheath gas flow	11 L/min		

C. Results and Discussion

1. Batch experiment

Two sets of batch experiments were performed to model the behavior of EE2 upon exposure to RF. The first batch experiment had an initial concentration of 500 ng/L EE2 and 0.14 g RF; the second had an initial concentration of 2000 ng/L EE2 and 0.1 g RF. During the first batch experiment (Figure 42) the data reported by Marfil Vega et al. (2011) for E2 proved to be similar for EE2. With EE2 recovered from the liquid phase dropping to half the initial concentration after 48 h, and to 10 percent of initial concentration after 120 h (5 days). EE2 extractable from the RF also dropped with time, the recovered EE2 from the solid phase was 16, 11, 9.5, and 5 percent at 12, 24, 48, and 72 h respectively. On the other hand, the concentration measured by LC-MSMS analysis in the control samples (Figure 43) confirmed that the transformation of the estrogen was abiotic occurring exclusively in the presence of RF.

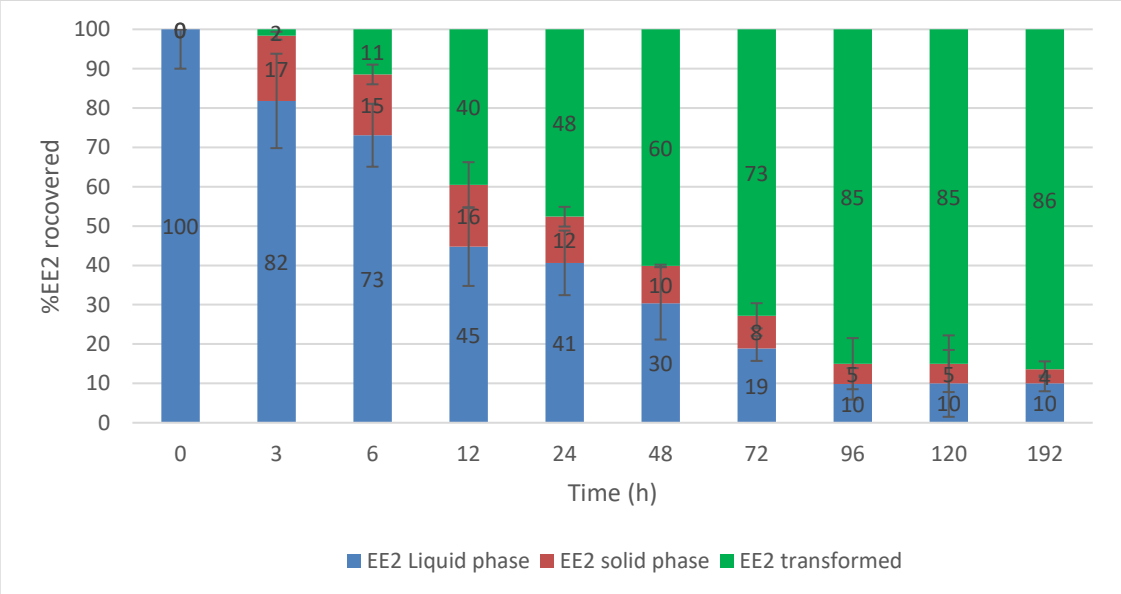


Figure 42. Percentage recovery of EE2 from liquid and solid phases with time (500 ng/L EE2, 0.14 g/L RF)

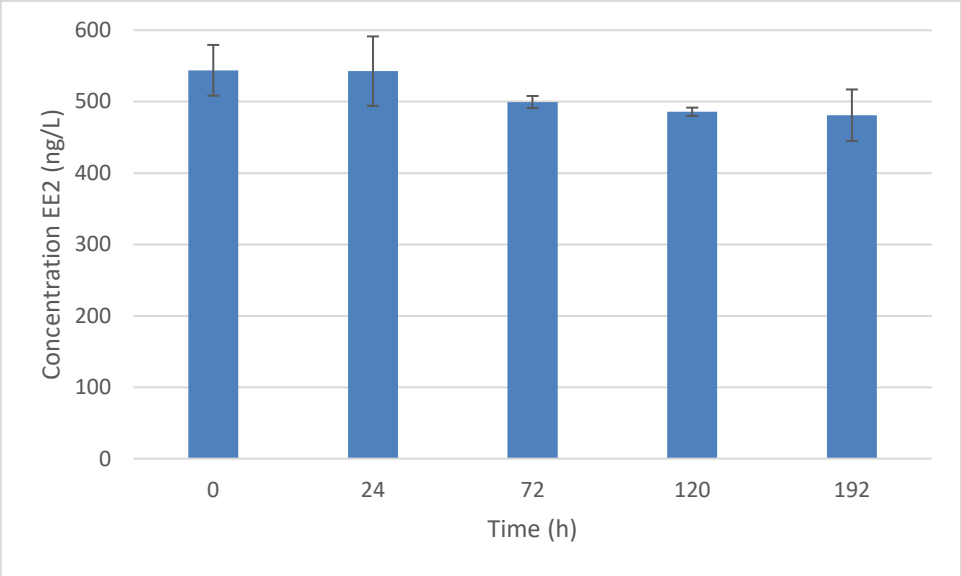


Figure 43. Concentrations of EE2 recovered from control samples

Transformation of EE2 was computed as follows: initial concentration minus the sum of concentration measured in liquid and solid phases, respectively. The maximum decrease in the concentration of EE2, as measured by LC-MSMS, occurred between 48 and 72 h (2-3 d) into the experiment where 73 percent of the initial concentration was transformed to unknown byproducts formed during the experiment as a consequence of catalytic/enzymatic transformation. Up to 85 percent EE2 was transformed after 180 h (Figure 44).

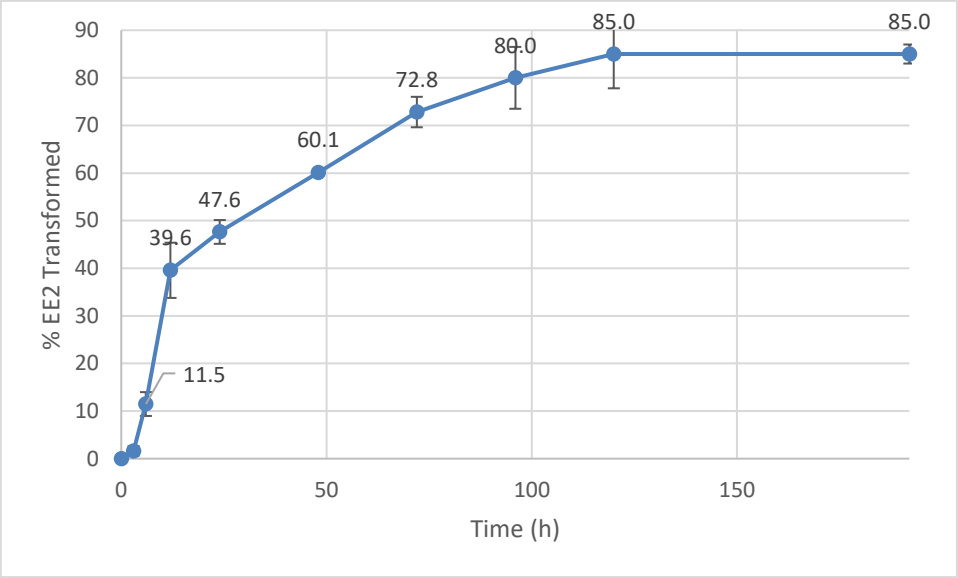


Figure 44. Percent EE2 transformed with time (500 ng/L EE2 and 0.14 g RF)

EE2 recoveries during the second batch experiment are shown in Figure 45. Compared to the first batch, a lower mass of RF was used with a higher concentration of EE2. With EE2 recovered from the liquid phase dropping to half the initial concentration after 96 h. EE2 extractable from the RF reached a maximum of 28 percent after 48 h followed by a decrease during the next three sampling events. The percent recoveries of EE2 from the RF were from 24, 22, and 21 at 72, 96, and 120 h respectively. Control samples showed negligible removal of EE2 in the absence of RF. Total transformation of EE2 was calculated similar to the first batch experiment and are represented in Figure 46.

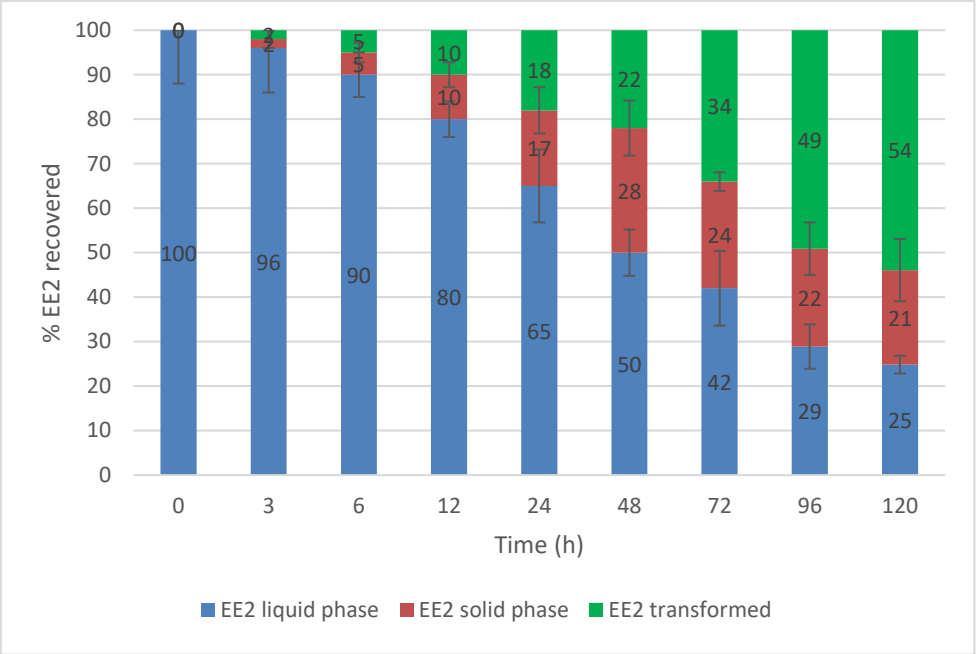


Figure 45. Percentage recovery of EE2 from liquid and solid phases with time (2000 ng/L EE2, 0.1 g/L RF)

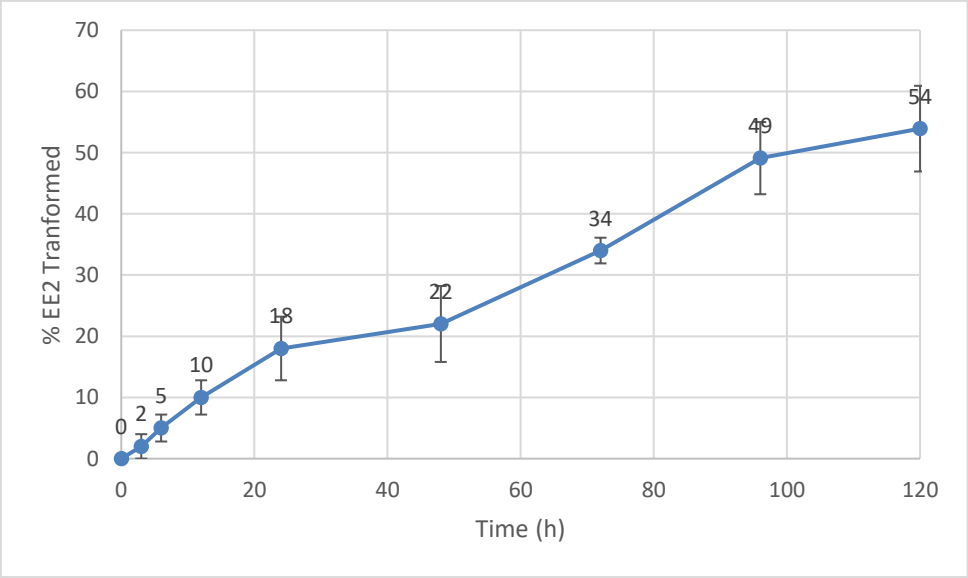


Figure 46. Percent EE2 transformed with time (2000 ng/L EE2 and 0.1 g RF)

The results from the batch experiments indicate that adsorption onto the surface does play a role in the elimination of EE2. The increase of percentage EE2 extractable

from the surface of the RF clearly indicates that adsorption onto the surface took place. This increase is followed by a decrease in the percentage recovered in both liquid and solid phase, which is an indication that abiotic transformation took place.

For a better comparison of the batch runs, the rate of transformation of EE2 was computed using the linear form of the pseudo-first and second order kinetic models. The pseudo-first-order kinetic model is represented as (Olivella et al. 2012):

$$\frac{dq_t}{dt} = k_1(q_e - q_t)$$

Where q_e is the amount of EE2 transformed at equilibrium (ug/g), q_t is the amount of EE2 transformed at time t (ug/g), and k_1 is the pseudo-first order rate constant in h^{-1}. The equation can be rearranged as follows:

$$\log(q_e - q_t) = \log q_e - \left(\frac{k_1}{2.303}\right)t$$

Plotting Log (q_e-q_t) vs. t will return the value for k_1 from the slope of the fitted line.

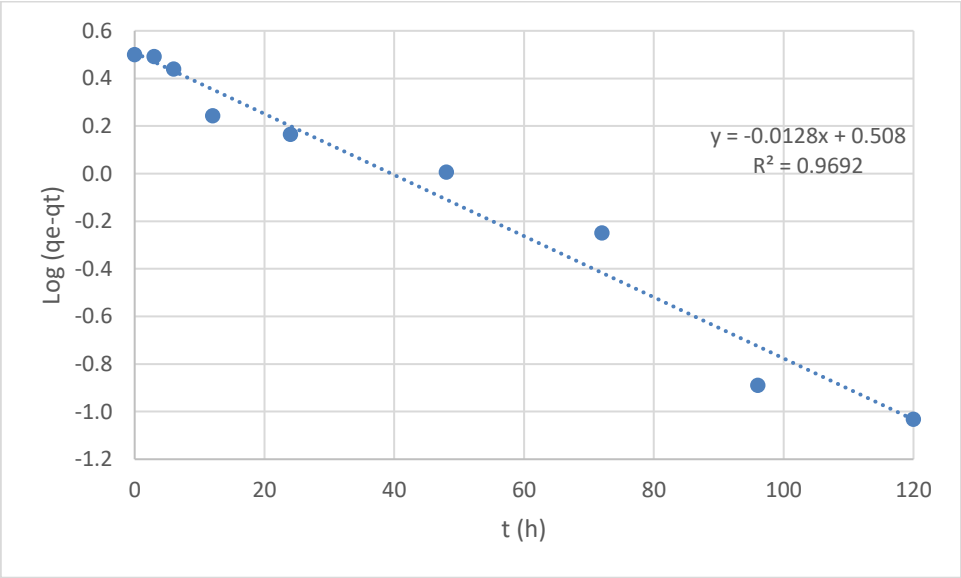


Figure 47. Pseudo-first-order kinetics (500 ng/L EE2 0.14 g/L RF)

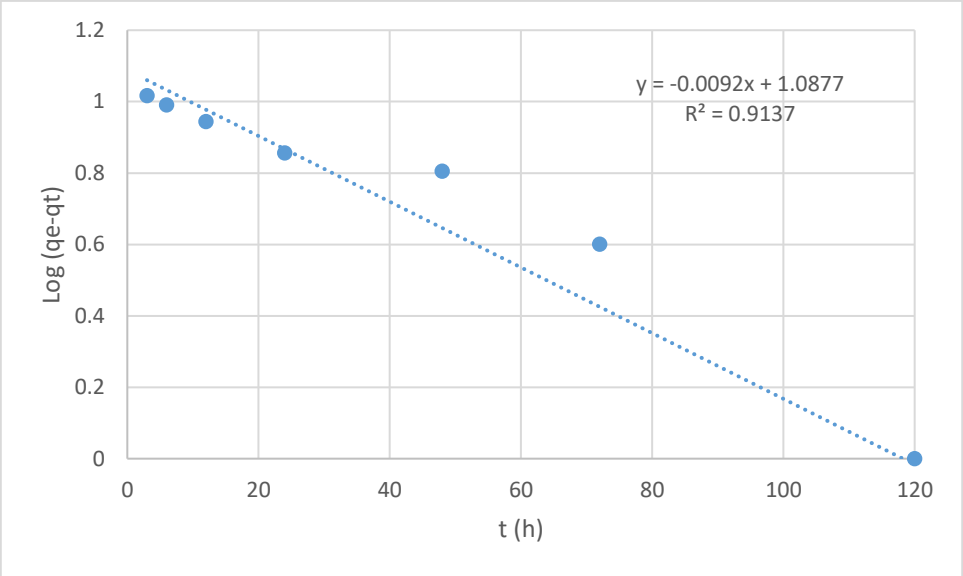


Figure 48 Pseudo-first-order kinetics (2000 ng/L EE2 0.1 g RF)

Pseudo-second order kinetic model is represented as (Venkateswarlu et al. 2007):

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \left(\frac{1}{q_e}\right) t$$

Where q_e is the amount of EE2 transformed at equilibrium in (ug/g), q_t is the amount of EE2 transformed at time t (ug/g), and k_2 is the pseudo-second-order rate constant (g/ug.min). Plotting of t/q_t versus t will return values of q_e and k_2 from the slope and the intercept of the straight line graph.

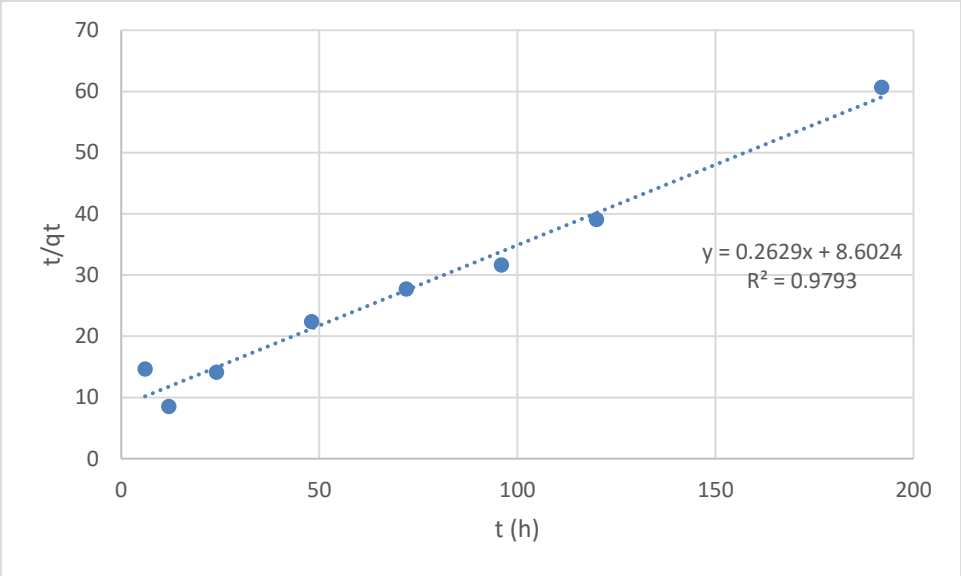


Figure 49. Pseudo-second-order kinetics (500 ng/L EE2 0.14 g/L RF)

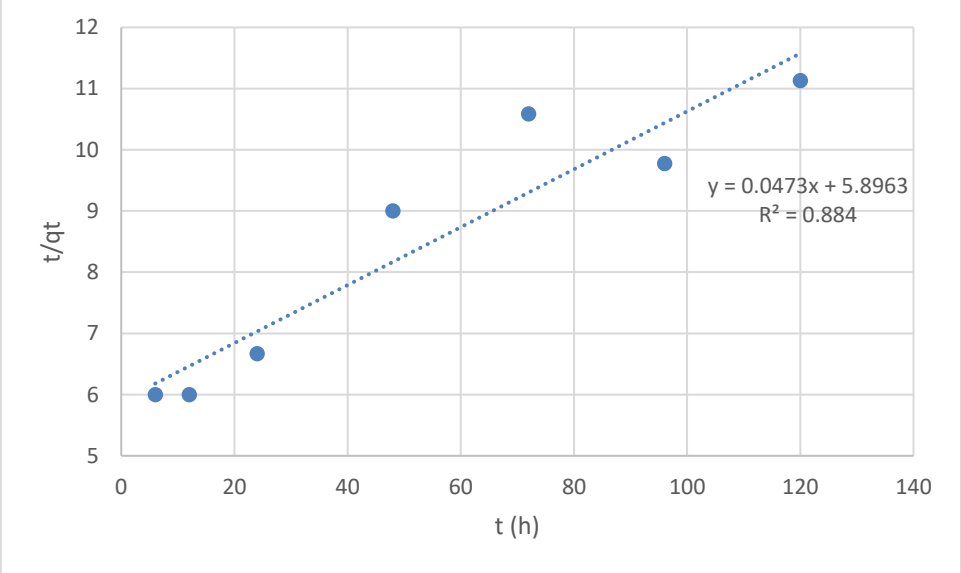


Figure 50. Pseudo-second-order kinetics (2000 ng/L EE2 0.1 g RF)

Table 7. Pseudo-First-Order and Pseudo-Second-Order Constants and Correlation Coefficients

		Pseudo-first-order kinetics				Pseudo-second-order kinetics			
Experiment	q_e(exp)	q_e(cal)	Error on q_e %	R-sqr	k_1	q_e(cal)	Error on q_e %	R-sqr	k_2
1	3.16	1.66	47	0.97	0.029	3.8	19	0.98	0.0088
2	10.78	2.97	73	0.91	0.021	21.1	96	0.84	0.0004

The kinetic constants and correlation coefficients are reported in Table 7. R-square values for both pseudo fist, and second order kinetic models ranged between 0.84 and 0.98 indicating good fits with the experimental data. For the first batch experiment, calculated q_e values fit well with the experimental q_e values using the pseudo-second-order kinetic model. The error on q_e calculated for the first and second order kinetic models were 47 and 19%, respectively. On the other hand, error in the q_e values calculated for the first and second order kinetic models were 73 and 96 %, respectively. In addition, the overall transformation rates k_1 and k_2 were higher during the first batch experiment with lower starting concentration of EE2. Two hypothesis might explain this observation; the first can be attributed to the consumption of the catalytic agents mediating the transformation of EE2, and the second hypothesis is that RF reached self-inhibition at high concentrations. In summary, our findings demonstrated a viable process for the control of EE2 in wastewater. The batch tests experiments confirm that reversible adsorption of EE2 onto the RF surface occurs in parallel to the occurrence of a catalytic transformation in both the liquid and solid phases.

2. Column experiment

Starting with an EE2 concentration of 200 ng/L in 10 L distilled water at a neutral pH 7, columns were run in triplicates, using different flow rates with different masses of RF. Detailed conditions for each experiment are presented in

Table **8**. Effluent was collected and samples were analyzed when the collected volume reached 500 mL.

Table 8. Experimental conditions

Exp #	Flow (mL/min)	Mass of RF (g)	Run time (day)
1	0.17	3.2	24
2	0.17	5.0	24
3	0.17	7.5	24
4	0.35	3.2	12
5	0.35	5.0	12
6	0.35	7.5	12
7	0.7	3.2	6
8	0.7	5.0	6
9	0.7	7.5	6

Breakthrough curves showing EE2 effluent concentration profile for 3.2, 5.0, and 7.5 g of RF are shown in Figure 51. Using 3.2 g of RF in the column was sufficient to affect complete attenuation of EE2 during the first two sampling events with full breakthrough occurring after passing 4.5, and 5.5 L of spiked feed for 0.35, and 0.17 mL/min flow rates, respectively. Increasing the mass of the sandwiched RF to 5.0 g under

the same conditions caused a further delay to breakthrough whereby 100 % removal of EE2 occurred during the first three sampling events with full breakthrough occurring after passing 5, and 5.5 L of spiked feed for 0.35, and 0.17 mL/min flow rates, respectively. Further improvement in the breakthrough curve occurred when 7.5 g of RF were sandwiched in the column. In this instance 100 % removal of EE2 occurred during the first four sampling events at flows of 0.17 and 0.35 mL/min with full breakthrough occurring after passing 6 L of spiked feed. Experiments operated under a flow rate of 0.7 mL/min only showed a 100 percent removal of EE2 for the first sampling event with 5.0 g RF sandwiched in the active part, with full breakthrough occurring prior to the lower flow rates upon passing 4 L of spiked feed. On the other hand, blank columns with zero concentration of RF showed full breakthrough occurring on the first sampling event of each experiment.

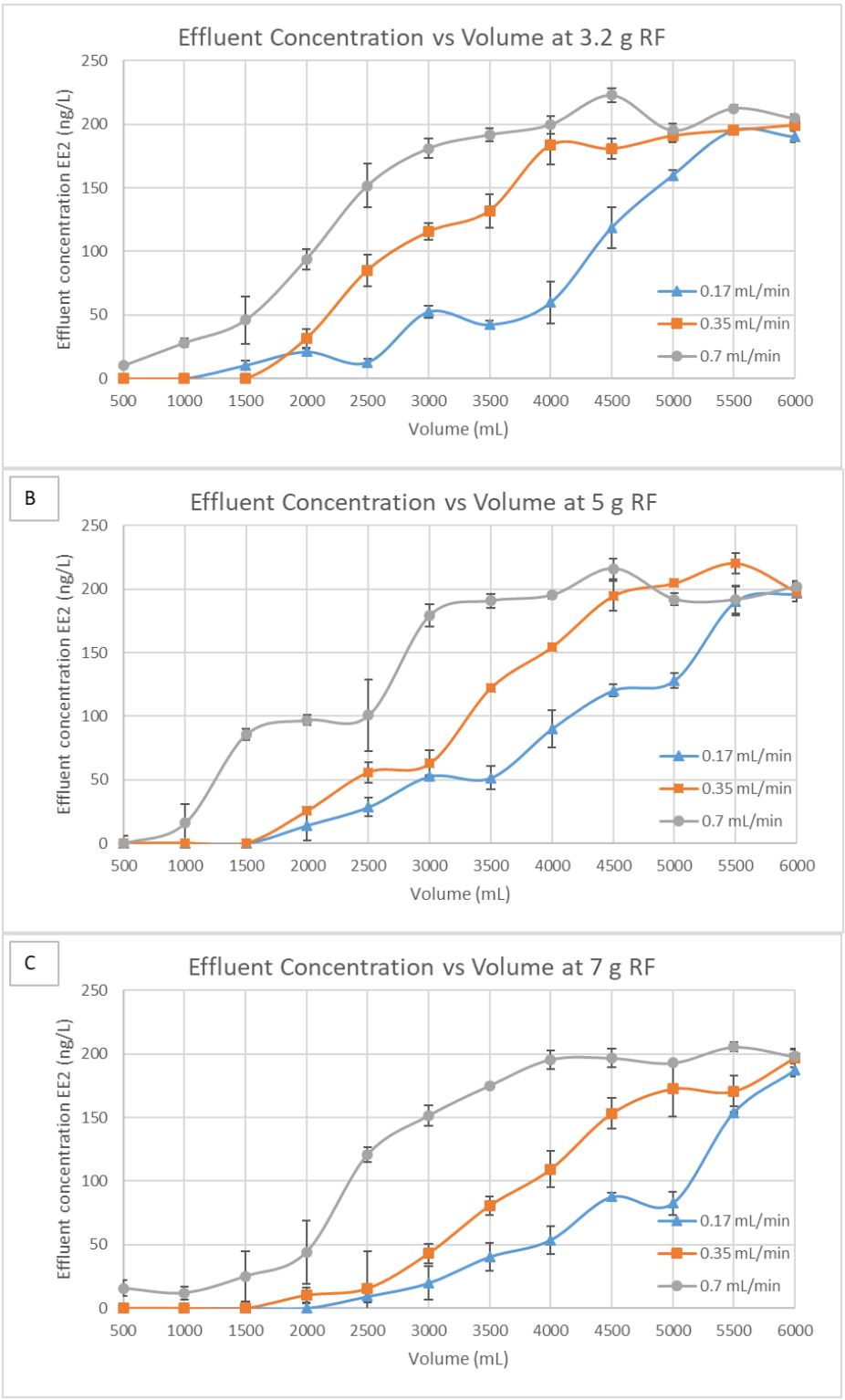


Figure 51. Breakthrough curves showing EE2 effluent concentration profile at 3.2, 5, and 7.5 g RF under different flow rates

Upon completion of individual experiments, the EE2 remaining in the sandwiched active part of each column was extracted, and a mass balance of the EE2 in solid and aqueous phases was performed as illustrated in Figure 52. The unquantifiable mass of EE2 during the runs was assumed to be the mass of EE2 transformed. Experimental results showed an increasing fractional transformation with increasing mass of RF and decreasing flow rate. Two-way ANOVA was performed to evaluate the separate impact of flow and mass of RF. At 95% confidence the effect of mass of RF and Flow, proved to be significant (p value <0.05) with R-Sq = 99.41% and R-Sq(adj) = 98.81%. Total mass adsorbed vs time is reported in Figure 53.

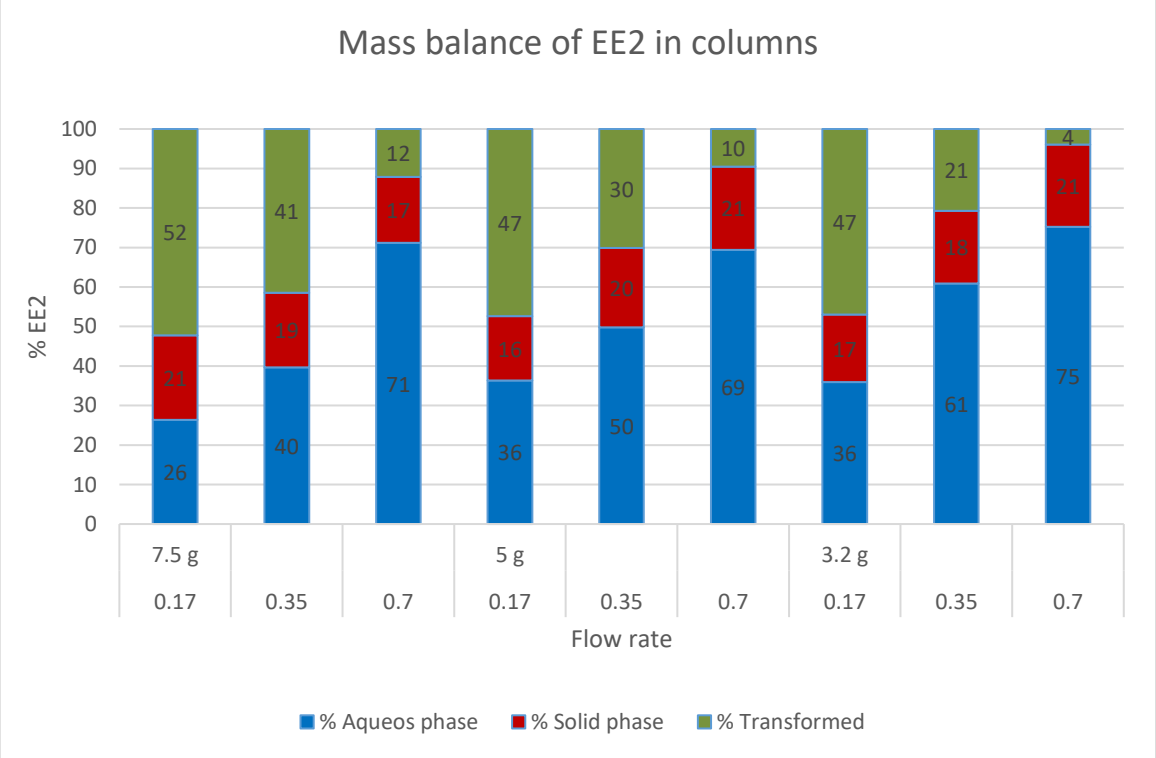


Figure 52. Mass balance of EE2

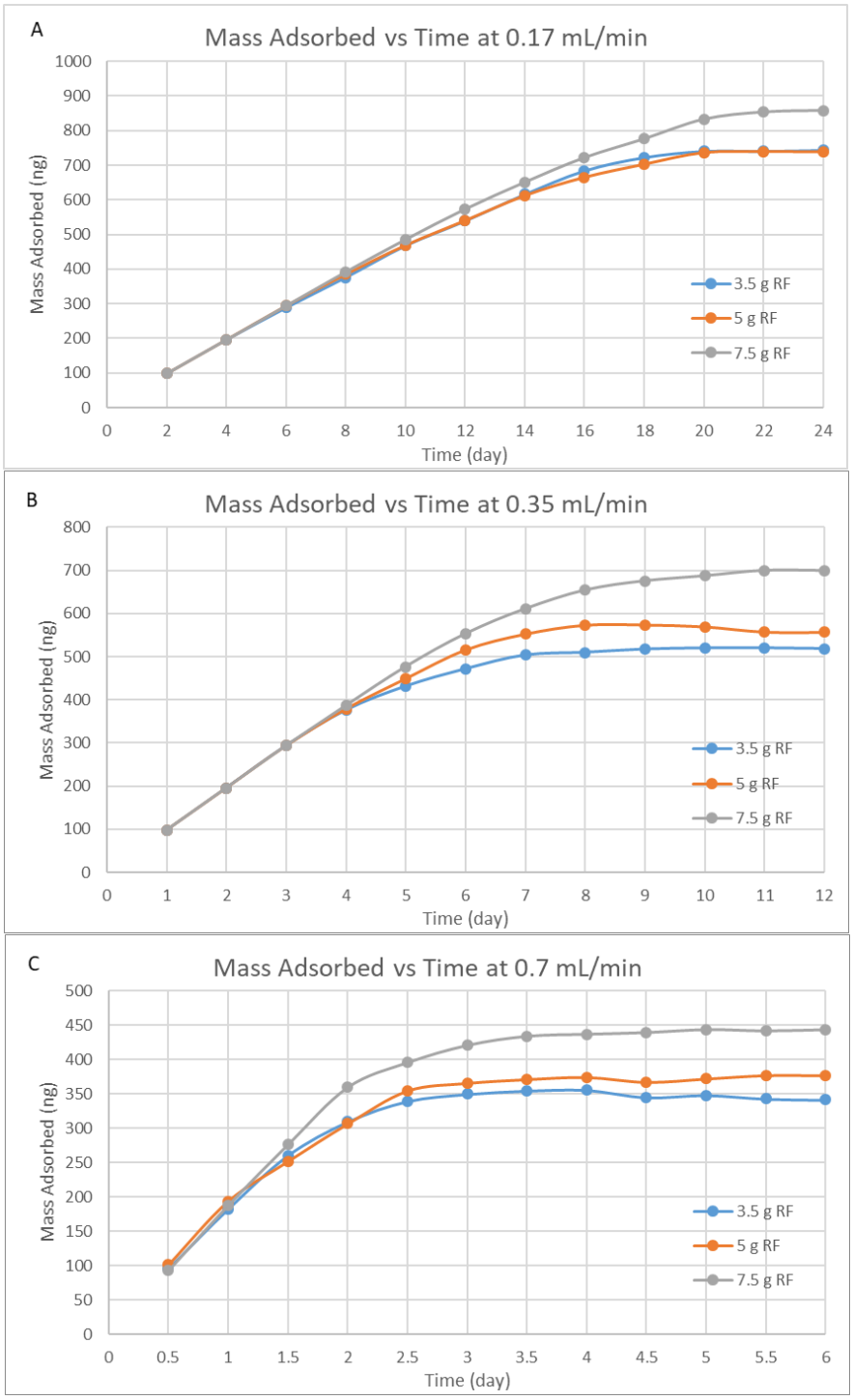


Figure 53. Mass adsorbed of EE2 vs Time

3. Modeling Breakthrough curves

Marfil-Vega et al. (2011) evaluated the adsorption and transformation of estrogens using 14C E2. They reported the absence of any transformation products in the aqueous phase. Drawing on the oxidative coupling of phenolic compounds in soils (Huang 1995) and activated carbon (Andersen et al. 2005; Chang et al. 2004; Vidic et al. 1993a; Vidic et al. 1993b), Marfil-Vega et al. (2010) proposed a mechanism for the abiotic transformation of E2 in the presence of RF as illustrated in Figure 33. Assuming that their observation also holds true for EE2, the breakthrough curves for EE2 are analyzed using traditional adsorption behavior. Thomas, Bohart-Adams, and Yoon-Nelson models were used to analyze the behavior of EE2 sorption onto RF.

a. Thomas Model

According to Rozada et al. (2007) the Thomas model for column performances is the most general and widely used theoretical treatment. The linearized form of the Thomas model is expressed as (Karunarathne and Amarasinghe 2013; Secor Robert 1956):

$$\ln\left(\frac{C_0}{C_t}-1\right) = \frac{K_{Th}q_0m}{Q} - K_{Th}C_0t$$

Q is the volumetric flow rate, K_{Th} Thomas kinetic coefficient, t is the total flow time (min), q_0 and m are the Adsorption capacity and mass of the adsorbent, respectively. The values for K_{Th} and q_0 were determined by plotting $\ln\left(\frac{C_0}{C_t}-1\right)$ vs. t (Chen et al. 2012b) (Figure 54). R^2 ranged between 0.88 and 0.96 indicating good agreement between the experimental data and the model. Results for different runs are summarized in Table 9 , showing that an increase in flow rate lead to an increase in K_{Th} and a decrease in q_0.

On the other hand, increasing the mass of RF led to a decrease in both K_{Th} and q_0. Starting with a constant concentration of EE2, and increasing the mass of RF in the columns led to the RF being exposed to lower concentrations EE2, thus lowering the attenuation capacity of the column. The variation in K_{Th} shows that the optimal conditions for sorption of EE2 on RF occurred at low flow and higher RF masses.

Table 9. Thomas model parameters

Flow (mL/min)	RF mass (g)	C_o (µg/L)	K_{Th} (L/µg.d)	q_0 (µg/g)	R^2
0.17	3.2	0.2	2.05	232.3	0.89
0.17	5.0	0.2	1.90	153.4	0.92
0.17	7.5	0.2	1.81	120.8	0.94
0.35	3.2	0.2	4.00	182.2	0.96
0.35	5.0	0.2	4.26	126.7	0.95
0.35	7.5	0.2	4.11	104.2	0.96
0.7	3.2	0.2	6.80	130.5	0.93
0.7	5.0	0.2	7.44	82.64	0.95
0.7	7.5	0.2	7.72	64.49	0.94

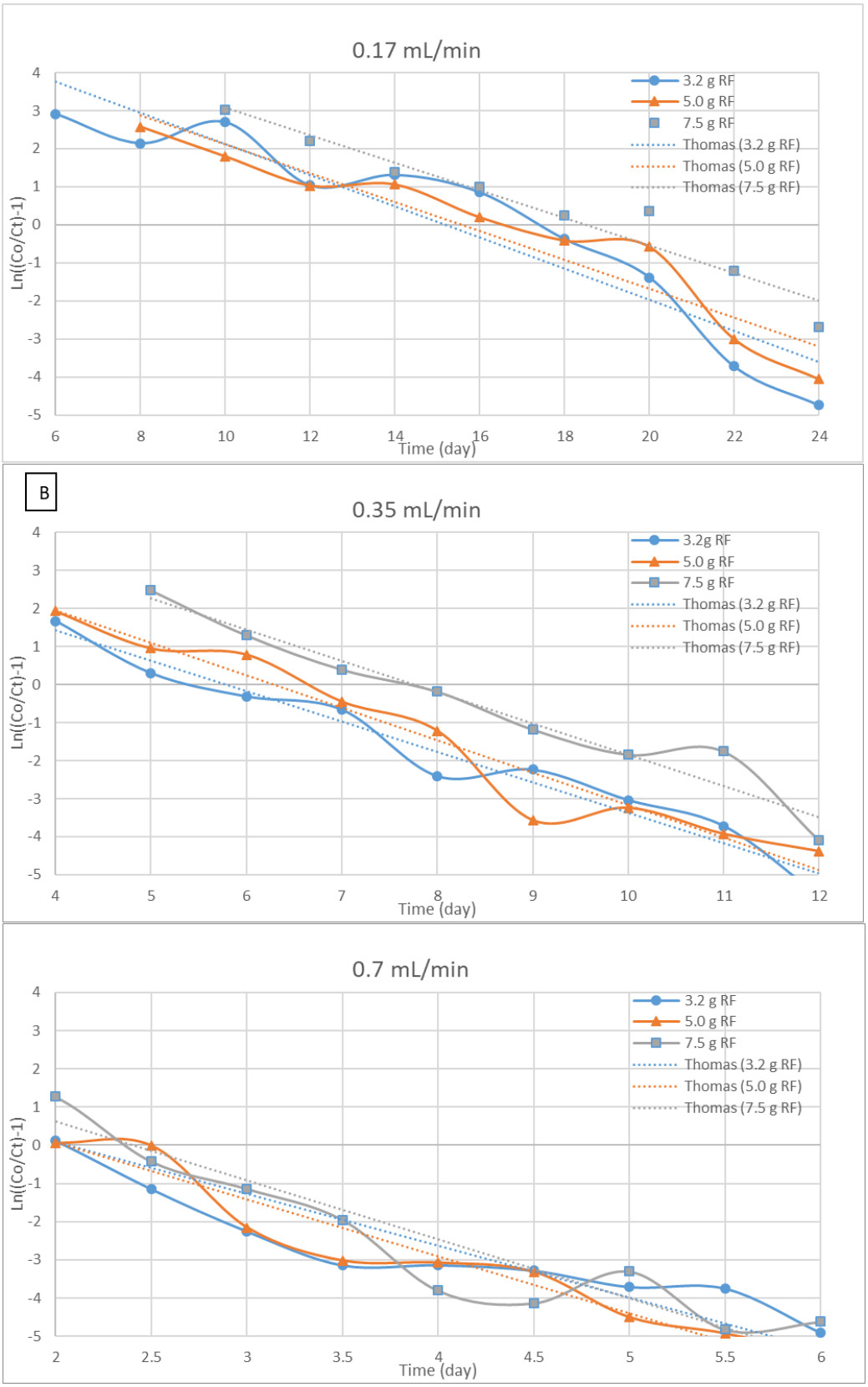


Figure 54 Thomas model kinetic plots

b. Yoon-Nelson Model

A far simpler model for single component systems is the one offered by Yoon-Nelson (Ayoob et al. 2007). This model assumes that the rate of decrease in the probability of adsorption for each adsorbate molecule is proportional to the probability of adsorbate adsorption and the probability of adsorbate breakthrough on the adsorbent (Ayoob et al. 2007; Yoon and Nelson 1984)

$$\ln \frac{C_t}{C_0 - C_t} = K_{YN} t - \tau K_{YN}$$

Where, K_{YN} is the rate constant τ is time required for 50 % adsorbate breakthrough, and t is time of the run. K_{YN} and τ can be determined by plotting the graph $ln \frac{C_t}{C_0 - C_t}$ versus time as shown in Figure 55. Model parameter values are given in Table 10 with K_{YN} increasing with higher flow and less mass of RF which further proves the correlation between flow and mass of RF and their combined effect on removal of EE2 from the studied matrix.

Table 10. Yoon-Nelson model parameters

Flow (mL/min)	RF mass (g)	K_{YN} (L/day)	τ(day)	R^2
0.17	3.2	0.41	15.18	0.89
0.17	5.0	0.38	15.59	0.92
0.17	7.5	0.37	18.35	0.94
0.35	3.2	0.80	5.78	0.96
0.35	5.0	0.81	6.56	0.95
0.35	7.5	0.82	7.76	0.96
0.7	3.2	1.19	1.73	0.93
0.7	5.0	1.49	2.05	0.95
0.7	7.5	1.54	2.40	0.94

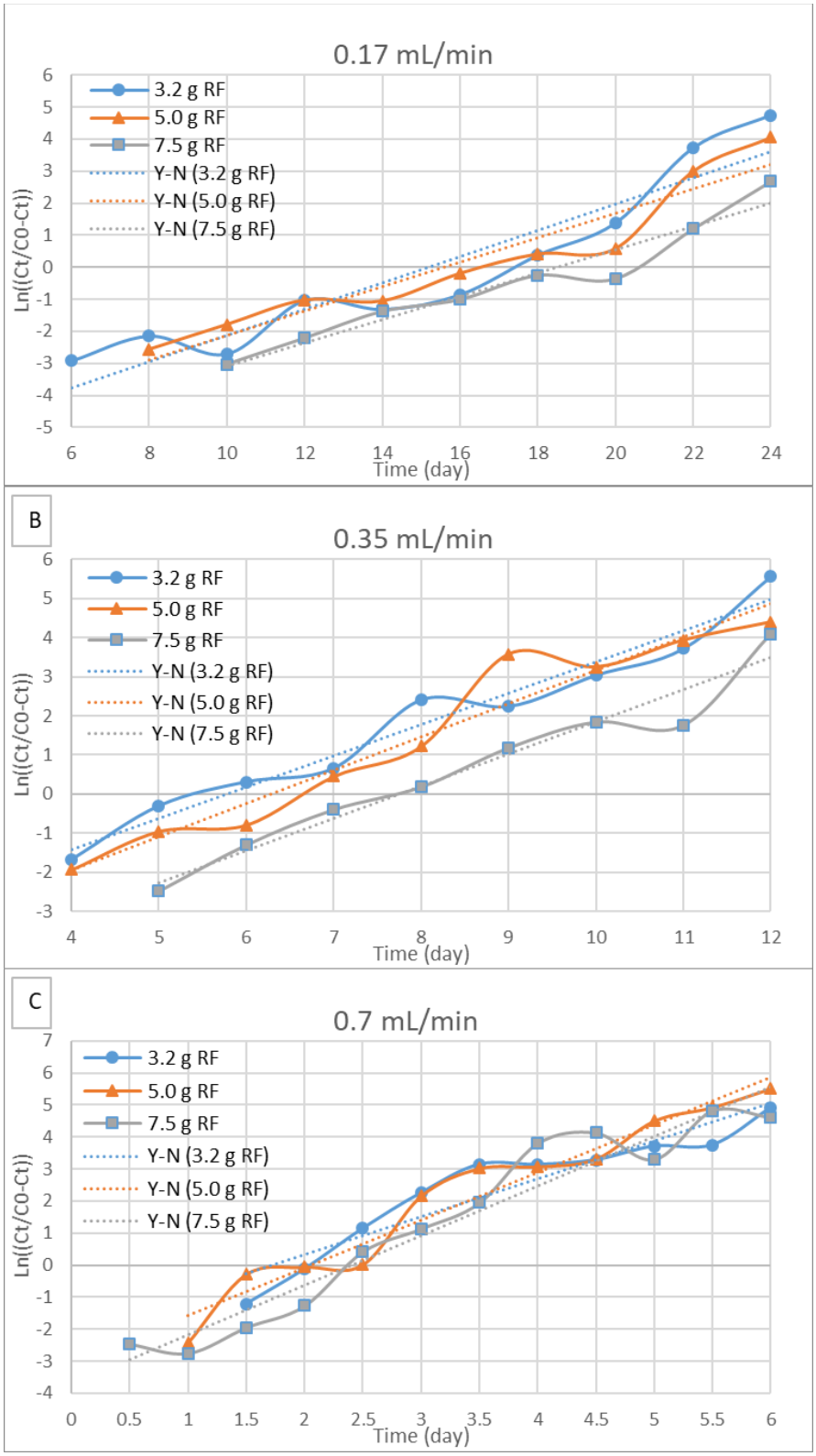


Figure 55 Yoon-Nelson model kinetic plots

c. Bohart-Adams model

Bohart and Adams (1920) model is based on the surface reaction theory and is considered to best describe the initial part of sorption breakthrough curve(Chu 2010). The ultimate equation for this model as expressed by Trgo et al. (2011)

$$\ln\left(\frac{C_t}{C_o}\right) = k_{AB}C_0 t - k_{AB}N_0\frac{Z}{u_0}$$

k_{AB} (L /ug.d) is the mass transfer coefficient, t (d) is the sample time, N_0 (mg/L) is the saturation concentration, U_0 (cm/min) is the linear velocity calculated by dividing the flow rate by the column cross-sectional area, and Z (cm) is the bed depth (Ahmad and Hameed 2010; Marzbali and Esmaieli 2017). As shown in Table 11, k_{AB} increases and N_0 decreases with increased flow and mass of RF respectively. The low R-sqr value proved that this model is not as descriptive as the previous two models. Figure 56 illustrates the kinetics plot of the Bohart-Adams model.

Table 11. Bohart-Adams model parameters

Flow (mL/min)	RF mass (g)	k_{AB} (L/ug.d)	N_0 (ug/L)	R^2
0.17	3.2	0.94	0.0134	0.92
0.17	5.0	0.78	0.0128	0.94
0.17	7.5	1.01	0.0122	0.94
0.35	3.2	0.93	0.0121	0.71
0.35	5.0	1.22	0.0122	0.82
0.35	7.5	1.87	0.0126	0.87
0.7	3.2	2.26	0.0109	0.69
0.7	5.0	1.77	0.0101	0.60
0.7	7.5	2.68	0.0112	0.78

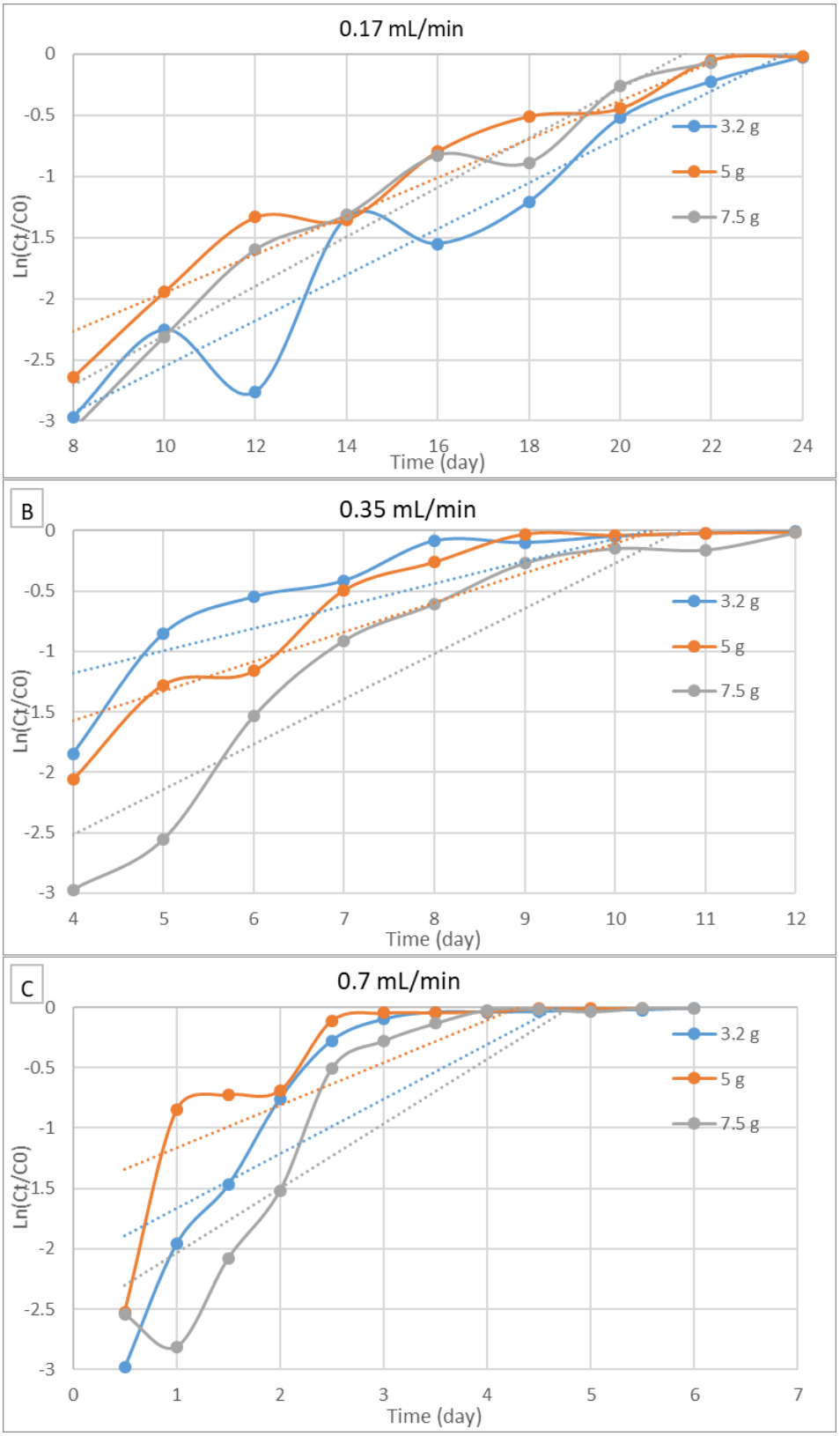


Figure 56. Bohart-Adams kinetics plot

d. Experimental vs Theoretical data

The deduction reached from comparing the models at hand is that Thomas and Yoon-Nelson correctly smooth the experimental curve of opening. On the other hand, Bohart and Adams describes only the initial part of the curve of opening at later stages the plots tend to deviate from the experimental data. A Comparison of predicted models curve and the experimental curve for flow of 0.17 mL/min and RF mass of 7.5g is illustrated in , the plots show that predicted Thomas and Yoon-Nelson curves are extremely close to the experimental data curve and to one another as opposed to Bohart-Adams.

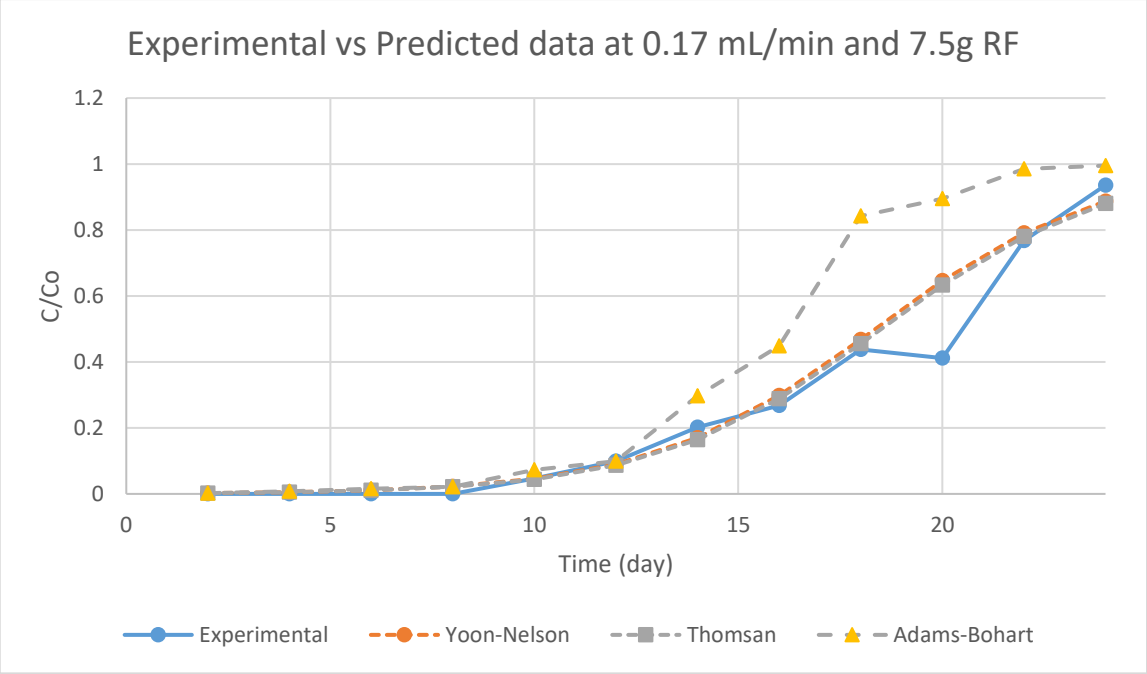


Figure 57. Predictive vs Experimental data plots

e. Error analysis

R-Sqr values calculated from the models indicate that both Thomas and Yoon-Nelson models described the data well. The squares of the differences between model and experimental data were determined using the following formula (Ghribi and Chlendi 2011):

$$\delta = \sqrt{\frac{\sum \left(\frac{C}{Co}\right)_{cal} - \left(\frac{C}{Co}\right)_{exp}}{N}}$$

Where N is the number of observations, (C/Co)cal is the ratio of effluent to influent EE2 concentrations, as obtained from the models, and(C/Co)exp is the ratio of effluent to influent EE2 concentrations obtained from experimental data. **Table 12** elaborates the absolute error function on the used models.

Table 12 Absolute error functions for different models

Flow (mL/min)	Mass of RF (g)	Thomas (δ)	Yoon-Nelson (δ)	Bohar-Adams (δ)
0.17	3.2	0.0298	0.0824	0.1921
	5.0	0.0325	0.0541	0.1842
	7.5	0.0375	0.0549	0.1928
0.35	3.2	0.0325	0.0421	0.9520
	5.0	0.0388	0.0489	0.9881
	7.5	0.0299	0.0512	1.8905
0.7	3.2	0.0425	0.0528	4.5240
	5.0	0.0322	0.0541	4.9804
	7.5	0.0301	0.0429	6.2883

D. Conclusion

EE2 undergoes abiotic transformation in the presence of RF. Mass balance of EE2 was reported based on an independent analysis of liquid and solid phases. Results obtained from two batch experiments provided evidence that sorption plays a role in the removal of EE2 from the liquid phase, however abiotic transformations in the solid phase represent the major driver for the removal of EE2.

Column experiments demonstrated the effectiveness of RF as a sorbent for the removal of EE2 from aqueous and solid phases. Being the most recalcitrant estrogen with high resistance to biodegradation, understanding the mechanisms involved in EE2 removal from water is needed in order to help mitigate the health and environmental risks of exposure to EE2 and other EDCs. The Behavior of breakthrough curves and RF adsorption capacity is strongly influenced by flow rate. Breakthrough time increased with a lower flow rates and higher mass of RF. Breakthrough curves were analyzed using the Thomas, Bohart-Adams, and Yoon-Nelson models. Thomas, and Yoon-Nelson models agreed with the experimental data, absolute error analysis proved that Thomas model produced the most accurate predictive results. Bohart-Adams is not as descriptive as the other models due to a significantly higher absolute error values.

CHAPTER VI

FATE OF 17α-ETHYNYLESTRADIOL (EE2) IN THE PRESENCE OF VITAMINS AND VARIOUS VEGETABLE MATERIALS

A. Introduction

The previous chapters focused on conducting batch and column experiments to model the transformation of EE2 in wastewater using RF as surrogate material for vegetable wastes found in wastewater. Up to 80% transformation of EE2 was reported, and this transformation was attributed to abiotic transformation. Huang et al. (1995) reported the major metals involved in the polymerization of phenolic compounds were copper, manganese, and iron oxides. Marfil Vega et al. (2011) conducted inductively coupled plasma (ICP) analysis of the RF (Figure 58) and the final metals concentration were calculated taking into consideration that 1.4g/L RF is present in the WW. Copper, manganese, and iron concentrations were reported to be 0.019, 0.193, and 0.466 mg/L respectively. Additionally, the same study calculated the concentration leached into the liquid phase of the synthetic WW with time. Iron was not found to leach, while both manganese and copper concentration in the solution increased with time.

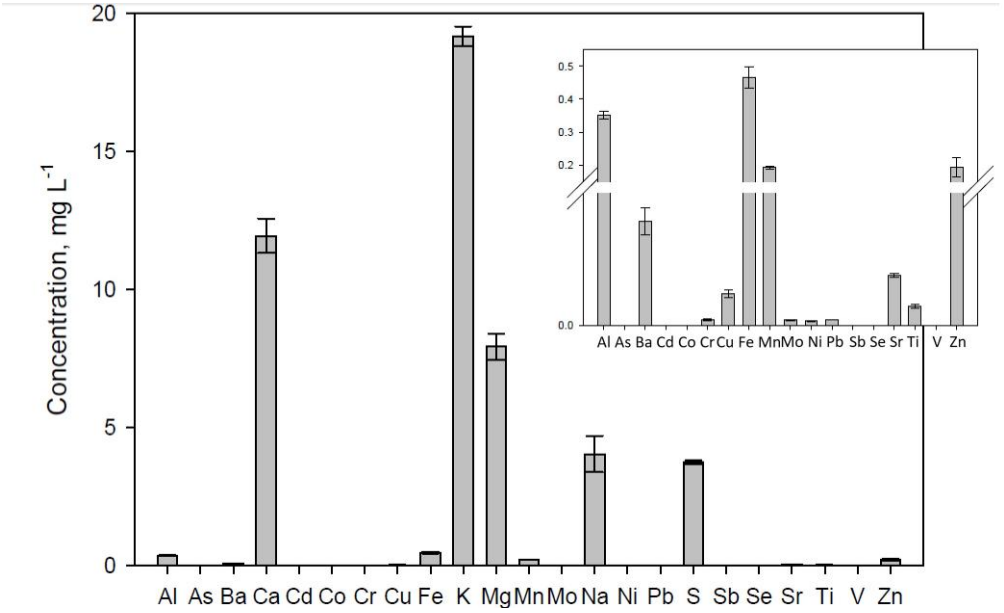


Figure 58. Concentration of metals in raw rabbit food (Marfil Vega et al. 2010)

The ultimate driver for abiotic transformation has not been identified. However, the hypothesis based on the work conducted by Huang et al. (1995), and Vidic et al. (1993) was that metallic oxides present in RF promoted the catalytic transformation of phenolic compounds. Unsuccessful attempts were made to obtain a more detailed characterization of RF from the supplier. For this reason, several materials were evaluated as sorbents for the transformation of EE2. The removal of recalcitrant compounds in the presence of vegetable wastes such as radishes, tomato, lettuce, and cabbage has been reported (Carter et al. 2014; Carter et al. 2018; Malchi et al. 2014; Tames and Hance 1969). In addition to its organic content, RF is known to contain a combination of vitamins and minerals. The goal of this chapter is to determine the fate of EE2 in the presence of various vitamins, minerals, and vegetables wastes

B. Materials and Methods

1. Chemicals and materials

Chemicals used were described in chemicals section in chapter 3. Vegetables, purchased from a local supermarket, were; spinach, carrots, carrot leaves, radish, radish leaves, lettuce, corn, and cabbage. Vitamins used in this study are shown in Table 13 and were purchased from Amazon.com. Laboratory grade minerals used were; calcium sulfate, zinc sulfate, magnesium sulfate, and manganese sulfate purchased from V.T.C s.a.l. Dietary fiber optimal nutrition was purchased from "First Nutrition Inc.". Concentrated vitamin premix "Vita-Land" obtained from a local poultry farm with the characterization shown in Table 14. The insoluble part of the premix consisted of ground corn.

Table 13. Vitamins used, concentrations, and manufacturer

Vitamin	Concentration	Manufacturer
Vitamin A	8000 I.U	Nature Made
Vitamin B6	100 mg	Nature's bounty
Vitamin B7	10000 mcg	Nature's bounty
Vitamin B12	500 mcg	Nature Made
Vitamin B Complex	(refer to Table 16)	Nature Made
Vitamin C	500 mg	Nature's bounty
Vitamin D3	5000 I.U	Nature Made
Folic acid	800 mcg	Nature's bounty
Omega 3 6 9	1000 mg	Now foods

Table 14 Composition of 1kg of "Vita-Land" premix

Mineral	Concentration
Vitamin A	25*10^6 I.U
Vitamin D3	5*10^6 I.U
Vitamin E	2500 I.U
Vitamin B1	4500 mg
Vitamin B2	2500 mg
Vitamin B6	750 mg
Vitamin B12	5 mg
Vitamin K3	2500 mg
Copper sulfate	1200 mg
Manganese sulfate	250 mg
Iron sulfate	2000 mg
Zinc sulfate	300 mg
Cobalt sulfate	25 mg
Ca-Pantothenate	5500 mg
Folic Acid	500 mg
Nicotinic Acid	100 mg

Table 15. Composition of B complex

Vitamin	Concentration
Vitamin C	60 mg
Vitamin B1	25 mg
Vitamin B2	20 mg
Niacin	25 mg
Vitamin B6	5 mg
Folate	400 mcg
Vitamin B12	100 mcg
Vitamin B7	1000 mcg
Pantothenic acid	5.5 mg

2. Experimental Design

Three sets of experiments were conducted in this study. During the first two sets EE2 removal was monitored in the presence of various vegetable wastes, and micronutrients (vitamins and minerals). Vegetables were cut into small pieces and later

lyophilized using a freeze-dryer (Labconco Freeze-dry system/Freezone 4.5) for 24 h. Lyophilized vegetables were ground separately and stored in a refrigerator at 6 °C. Vitamin tablets were ground using a pestle and mortar, and used immediately after grinding. Vitamins enclosed in soft gel tablets were directly emptied into vials before starting the experiment. Stock solution of 2000 ng/L EE2 in distilled water were prepared using a 4 L silanized glass beaker. Sodium azide (20 mg/L) was added to act as an inhibitor of aerobic biological activity. 0.1 grams of each material was added to 40 mL silanized glass vials, followed by the addition of 30 mL of the stock solution. Batch experiments were conducted in triplicates, in an orbital shaker at 200 rpm and temperature of 25 °C for 5 days. Mixtures of different minerals contained 0.1 g of calcium sulfate, zinc sulfate, magnesium sulfate, and manganese sulfate, amounting to 0.4 g. Fiber, vitamins, and minerals were mixed by adding 0.1 g of crushed multivitamins, 0.1 g of fiber, and 0.1 g of the mineral mixture to a beaker. Later 0.1 g of the resulting mixture was used in the experiment.

The final set of experiments was designed to estimate EE2 disappearance rate and model the estrogen's behavior upon exposure to a combination of vitamins minerals and substrates "premix" (Table 14). To serve that purpose, EE2 (2000 ng/L) was spiked into silanized vials containing 30 mL of Distilled water and 0.1 g of premix. Multiple sets of batch experiments were run. Vials were continuously shaken at 200 rpm until they were sacrificed at preset time internals over a 5-day period (120 h). During the course of this study, we encountered a failed attempt to show the behavior of EE2 in the presence of chicken feed. Samples with chicken feed exhibited high viscosity consequently SPE was not possible.

3. Extraction and cleanup

Throughout the experiments, liquid and solid phases were analyzed separately at each sampling event using LC-MSMS. Liquid and solid phases were separated by vacuum filtration using glass fiber filters of effective opening of 1.5 μm and 47 mm in diameter. Following filtration, d4-EE2 was then added to the liquid filtrate to serve as a surrogate. Samples were then extracted by means of solid phase extraction (SPE) using HLB Water's Oasis (30 mg 1 cc) cartridges. The cartridges were conditioned with methanol and then washed with LC-MS grade water before being loaded with the filtrate at an application rate of approximately 4 mL/min. Finally, the cartridges were washed with 2 mL of 10% methanol in water, dried under vacuum for 5 minutes. Elution was conducted with 2 mL methanol, and the eluent was filtered using a PTFE syringe filter (Kinesis 13 mm 0.22 μm) and latex and silicone-oil-free 5 mL inert syringes (NORM-JECT). The filtrates were dried under a gentle nitrogen stream at 40 °C and reconstituted in 0.3 mL of 50% methanol in water (v/v).

Solids along with the filter paper were dried using sodium sulfate (Na_2SO_4) and 0.2 g of diatomaceous earth (DE) in preparation for extraction. Samples were filled into a 10 mL stainless steel extraction cell, and spiked with d4-EE2. The remaining cell volume was filled with Ottawa sand and diatomaceous earth in order to reduce the amount of solvent used. Using an ASE Dionex 350, extraction was carried out under the following instrument conditions: two extraction cycles, 150 °C, 1500 psi, and methanol: acetone 20:80 (v/v). The extracts were dried under a gentle nitrogen stream at 40 °C and reconstituted in 0.2 mL dichloromethane (DCM) and 0.8 mL iso-octane for further cleanup with neutral alumina. The HLB cleanup procedure was performed as described

in chapter 3. The final volume of the solids extract was 0.3 mL of 50 % methanol in water (v/v).

4. Analytical procedure

Analysis performed using LC-MSMS following the procedure described in the quantification section (chapter 5). Calibration curves covering a concentration range of EE2 6.25-200 ng/mL, and quality checks were used as shown in Figure 59.

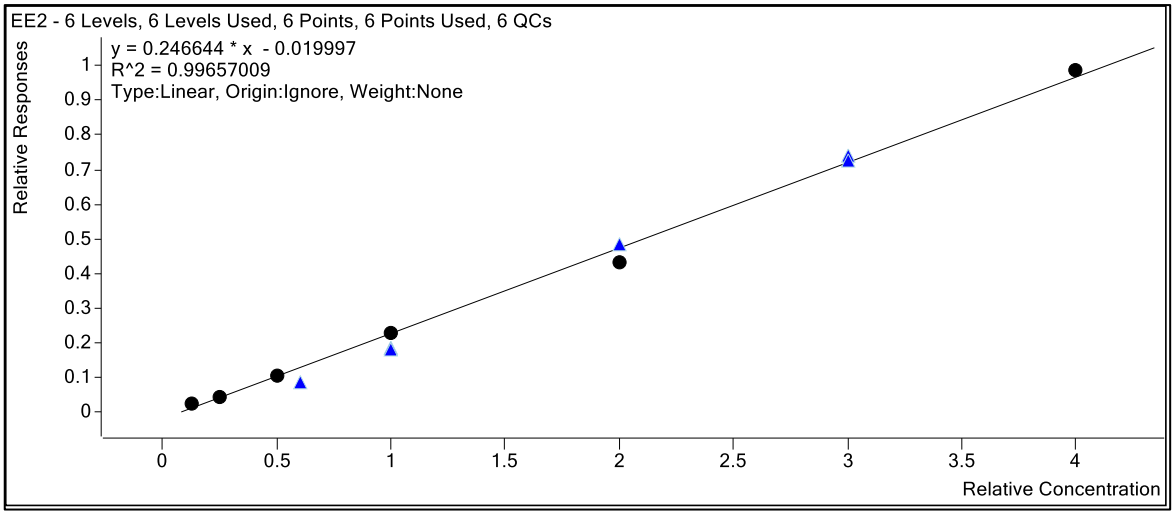


Figure 59 Calibration curve and QCs

C. Results and Discussion

Three sets of experiments were carried out to monitor the transformation of EE2 under different conditions. In all experiments, aerobic biological activity was suppressed using NaN3. The first and second experiments were designed to assess losses of EE2 attributable to exposure to different vegetables and micronutrients. Losses due to volatilization and adsorption onto glass surfaces were non-existent, blank samples showed full recovery of EE2 after the 5-day period of the experimentation. Various

vitamins and minerals were tested individually to ascertain their potential to affect the observed transformation of EE2. The mass of EE2 removed from the aqueous phase, m_r (ng/g) on different materials used was determined using the following equation:

$$m_r = [(C_0 - C_t) \times V] \div M$$

Where Co is the concentration of EE2 at time 0 (ng/L), Ct is the concentration of EE2 at time t=5 days (ng/L), V is the volume of the sample, and M is the mass of material used (0.1 g). m_r represents the sum of the mass of EE2 that was adsorbed or transformed.

1. Vegetables batch run

Resulting m_r values for each vegetable waste are shown in Table 16. Lowest m_r values were observed for mint. On the other hand, carrot leaves, corn, and radish leaves had comparable m_r values to those of RF with radish leaves exceeding the values of RF by approximately 75 ng/g.

Table 16 m_r, q, and m_t of different vegetable wastes

Vegetable waste	m_r (ng/g)	q (ng/g)	m_t(ng/g)
Carrot Leaves	441.7	164.7	277.0
Carrot	335.7	189.9	145.8
Radish Leaves	525.4	115.6	409.8
Radish	314.2	186.1	128.0
Mint	72.0	72.0	0.0
Lettuce	229.5	80.8	148.7
Corn	361.1	309.3	51.7
Cabbage	244.3	234.9	9.4
Spinach	181.8	164.7	17.1
RF	450.9	127.4	323.5

q is defined as the mass of EE2 desorbed from RF, ng/g, while the mass of EE2 transformed, m_t (ng/g) is given as:

$$m_t = m_r - q$$

q is calculated from the mass of EE2 extracted from the solids. Figure 52 shows m_r, q and m_t for the different materials studied. The results obtained show that transformation of EE2 was significantly enhanced upon exposure to radish leaves. EE2 percent recoveries were observed to be 12.4 and 24.9 in the liquid phase 19.3 and 21.2 in solid phase of radish leaves and RF, respectively. The fraction of EE2 transformed was 68.3 and 58.9 for radish leaves and RF, respectively. Figure 60 reports the mass balance of EE2 in the solid and liquid and the mass transformed.

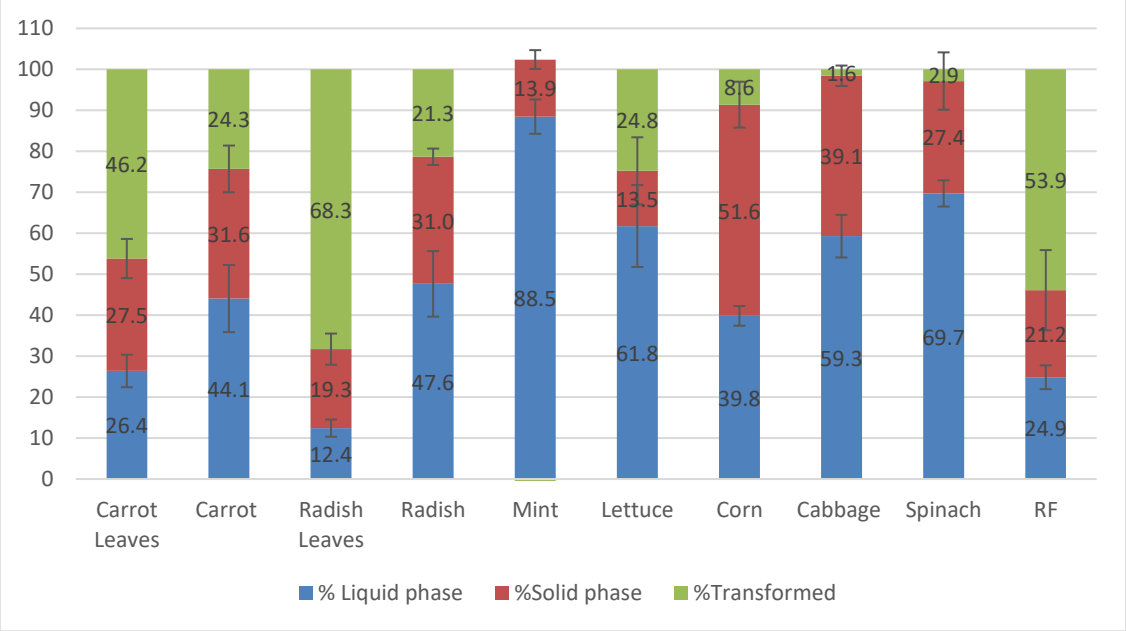


Figure 60. Mass balance of EE2 with different vegetable wastes

Radishes contain several enzymes including cysteine synthase and multiple transferase type enzymes (Dahlbender and Strack 1986). In addition to these enzymes, radish leaves contain catalase and glutathione reductase, which are believed to be the main driver for the removal of metals such as cadmium from aqueous solutions (Vitoria et al. 2001). Glucuronic, oxalic, and malonic acids as well as sulfates are also known to be present in radish leaves (Lee and Kim 1994). Behavior of estrogens in the presence of glucuronic acid and sulfates was illustrated in Figure 7, the formation of byproducts such as estrogen sulfates and estrogen glucuronates might be responsible for the transformation of EE2 upon exposure to radish leaves.

Gutierrez and Perez (2004) also reported the presence of phenolic compounds in radishes and radish leaves among which anthocyanins, pelargonidin, and cyanidin were considered responsible for the color of the root vegetable. In theory, these phenolic compounds can from semiquinone radicals capable of coupling to EE2 in a pathway similar to the one demonstrated in Figure 33. Creason et al. (1985) concluded that radish leaves contain sulfonium diateroisomer S-adenosylmethionine (AdoMet). Alongside the enzymatic function, AdoMet is known to be methyl group donor (Gutiérrez and Perez 2004). The possible interaction of AdoMet with EE2 might lead to the formation of byproduct methyl-EE2, which possesses similar estrogenic effects to those of EE2 (Zhang 2006).

Spinach and cabbage showed comparable m_r, and q values with transformations of 2.9 and 1.6 percent for each, respectively. Mint runs reported full recovery of EE2 from the liquid and solid phases indicating that no transformation took place. The behavior of EE2 upon exposure to vegetables and vegetable wastes, led to the conclusion that leaves (carrot leaves and radish leaves) had a much higher impact on transformation of EE2 than

that of the edible part of the root vegetable. Corn also showed promise in removing EE2 from the liquid phase; however, reversible sorption onto the vegetable waste was more dominant than the observed transformation. After 5 days EE2 recovered from the liquid phase was 39.8 percent, and 51.6 percent was extractable from the solid phase with only 8.6 percent transformed into unknown byproduct. Examination of the effect on EE2 of a far more abundant agricultural waste from cornfields such as the cornhusk is necessary for future work.

2. Vitamins and minerals batch run

Batch experiments were carried out in an effort to determine the effect of different vitamins and minerals on EE2 transformation. A constant mass of 0.1 g of individual vitamins was added to separate vials, while for soft-gel vitamins an entire capsule was emptied in the vial. Mixtures of different minerals contained 0.4 g total mass was used instead of 0.1 g. Fiber, vitamins, and minerals were mixed by adding 0.1 g of crushed multivitamins, 0.1 g of fiber, and 0.1 g of the mineral mixture to a beaker. Later 0.1 g of the resulting mixture was used in the experiment.

The removal of EE2 upon exposure to vitamins A, B6, B7, B12, B complex, D3, E, and C is shown in Figure 61. Almost all vitamins resulted in some transformation of EE2, the highest of which was that of B complex where 23 % of initial EE2 transformed into unknown byproduct. In the presence of a solid phase, EE2 extractable upon exposure to vitamins D3 and C were 21.3 and 18.6 % respectively. Runs with vitamins D3 and C returned transformations of 10 % and 11 % EE2 respectively.

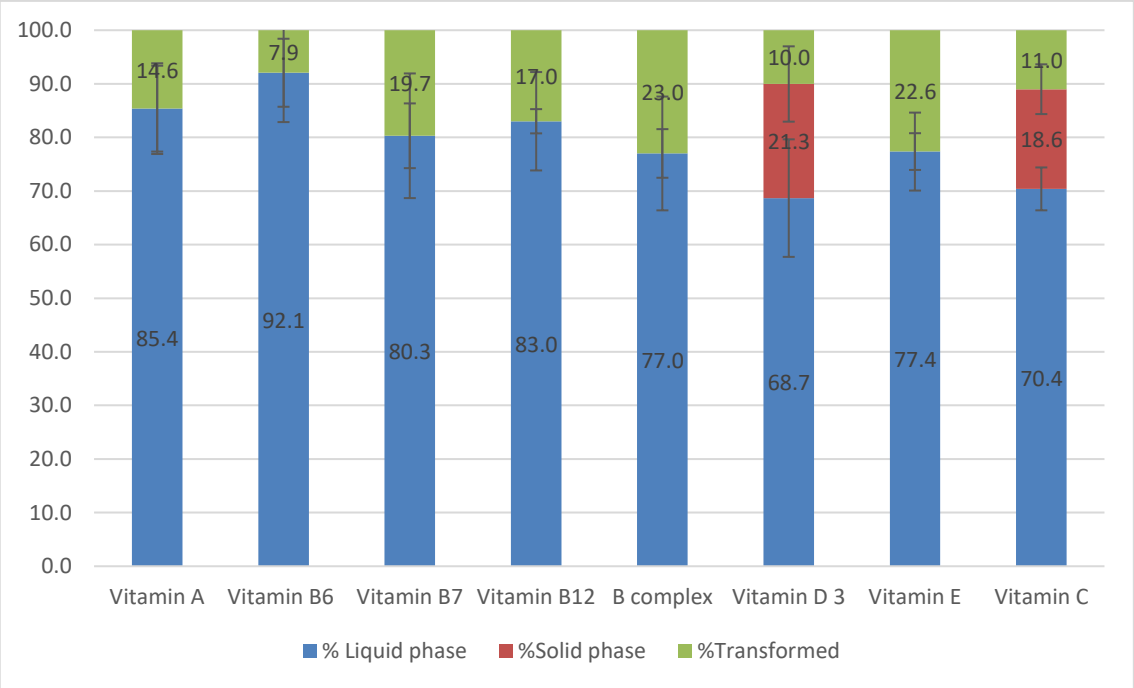


Figure 61. Mass balance of EE2 with different vitamins

Figure 62 shows the removal of EE2 with different minerals. 10.5, 7.4 and 18.8 % EE2 was transformed in the presence of calcium sulfate, manganese sulfate, and magnesium sulfate respectively. Dietary fiber runs resulted in an average of 57.8, and 36.3 % EE2 in liquid and solid phases respectively, with 5.9 % of EE2 transformed. The resulting transformations of EE2 agree with data from previous studies on abiotic transformation taking place as a consequence of catalytic reactions mediated by chemical agents such as metals or metal oxides (magnesium and manganese oxides) (Hanselman et al. 2003; Lee and von Gunten 2009).

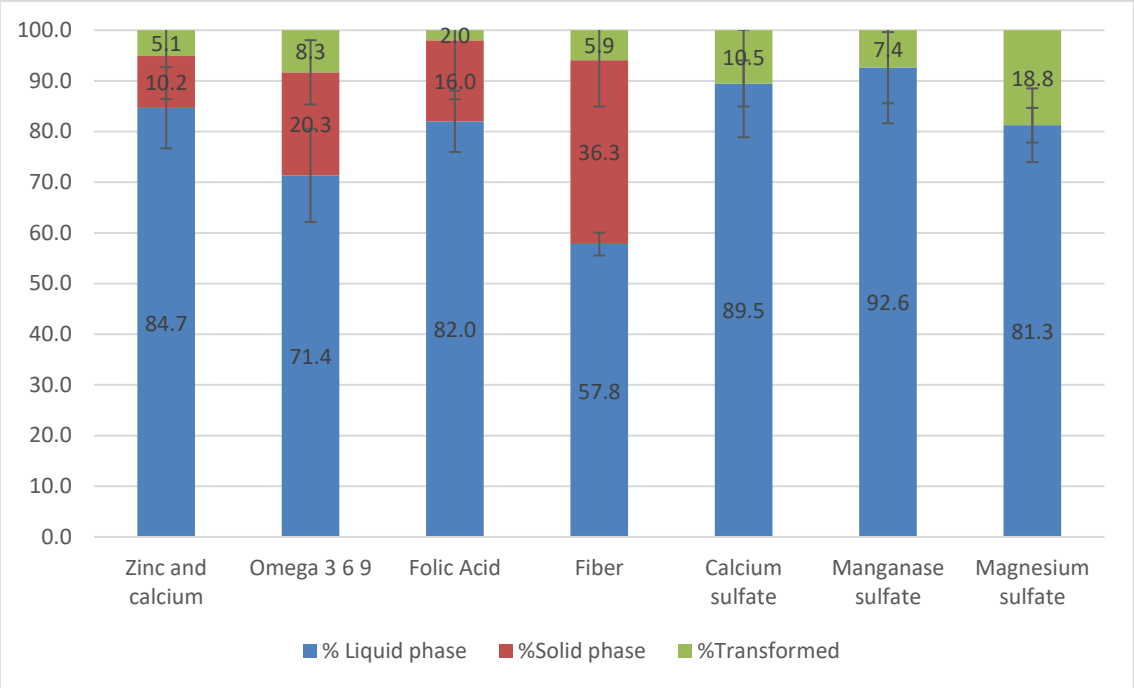


Figure 62. Mass balance of EE2 with different minerals

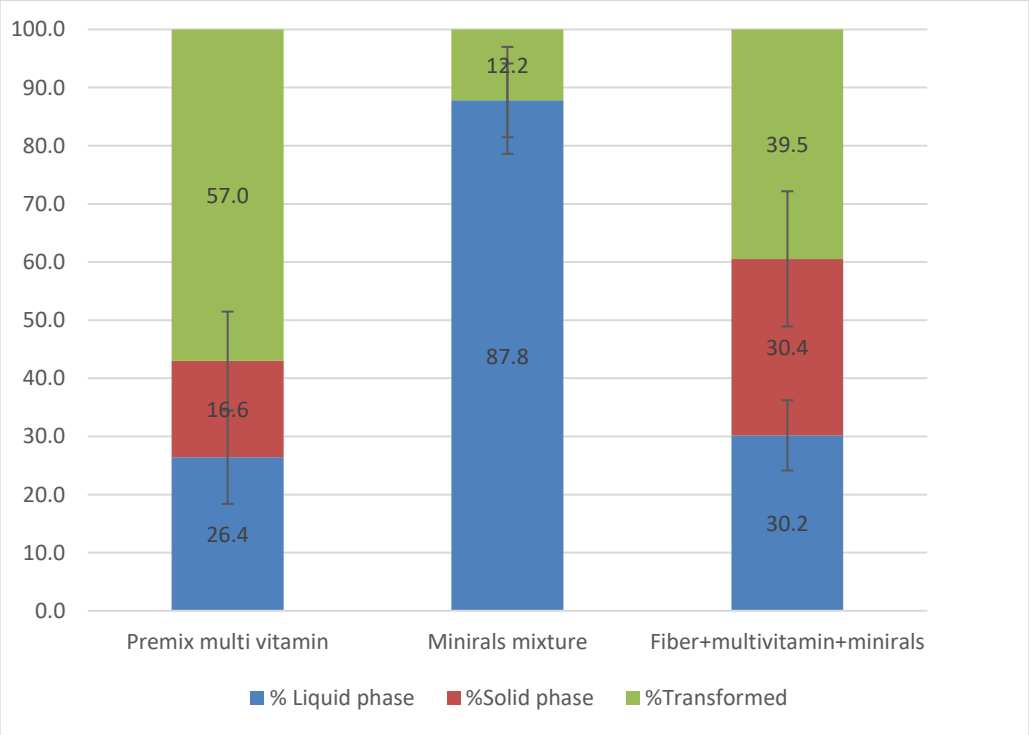


Figure 63. Mass balance of EE2 with different mixtures

Figure 63 shows that the mixture of minerals resulted in the transformation of 12.2 % EE2. The presence of fiber provided a surface for sorption to take place and the percentage transformed of EE2 using fiber, multivitamins and minerals reached 39.5. Premix resulted in a greater transformation of EE2 whereby 57 % of the estrogen was transformed. Since biotic transformations were eliminated based on previous studies and the addition of sodium azide, the observed decrease in mass of EE2 recovered in the matrix is due to abiotic transformation. The improved transformation results in the presence of insoluble solids suggests that sorption on a surface occurs followed by abiotic transformation.

It was necessary to unify the mass units for vitamins in order to calculate the mass transformed of EE2 (m_t) for each individual vitamin and compare that to the value returned from 0.1 g premix. Using Robert Forbes and Associates (RFA) conversion chart m_t for vitamins used is reported in Table 17.

Table 17. m_t for vitamins and premix

Component	m_t (ng/g)	Percent in premix
Premix	342	-
Vitamin A	182.5	320
Vitamin B6	94.8	13
Vitamin B7	1182	-
Vitamin B12	207.6	10
Vitamin C	13.2	-
Vitamin D3	4800	500

Vitamins B6 and B12 were tested at a lower concentrations than those present in 0.1 g premix. 13 and 10 percent of B6 and B12 used in individual testing experiments is contained in 0.1g premix, respectively. These results led to the conclusion that vitamins

had a high impact on transformation of EE2. The lowest m_t values were those of vitamin C. B7 and D3 showed promise in transforming EE2, further experimentation is needed to explain the high m_t values for those vitamins.

3. Premix batch run

Results from the premix batch run are given in Figure 64. The concentration of EE2 in the solid phase increased initially and reached 21.5% after 24 h. Subsequently, the extractable EE2 from the solid phase decreases steadily during the remaining duration of the experiment reaching 15.6 at 120 h. This indicates that adsorption onto the premix solid surface (mainly content of ground corn) plays a role is the transformation of EE2 from the matrix. The initial increase in % EE2 in the solid phase after 24 h indicates that adsorption is taking place, the decrease in the aforementioned concentration for the remainder of the run in both the solid and liquid phases can be considered as an indicator for the occurrence of abiotic transformation in both phases. Figure 65 reports the highest decrease in the concentration of EE2 as measured by LC-MSMS occurred between 48 and 72 h (2-3 d) into the experiment where 56 percent of the initial concentration was transformed to most likely unknown byproducts formed during the experiment as a consequence of catalytic/enzymatic transformation. The total transformed percentage of EE2 after 120 h was reported to be 56. Control samples with zero mass of premix as measured by the LC-MSMS confirm that no transformation of EE2 occurred in the absence of the catalyzing agent.

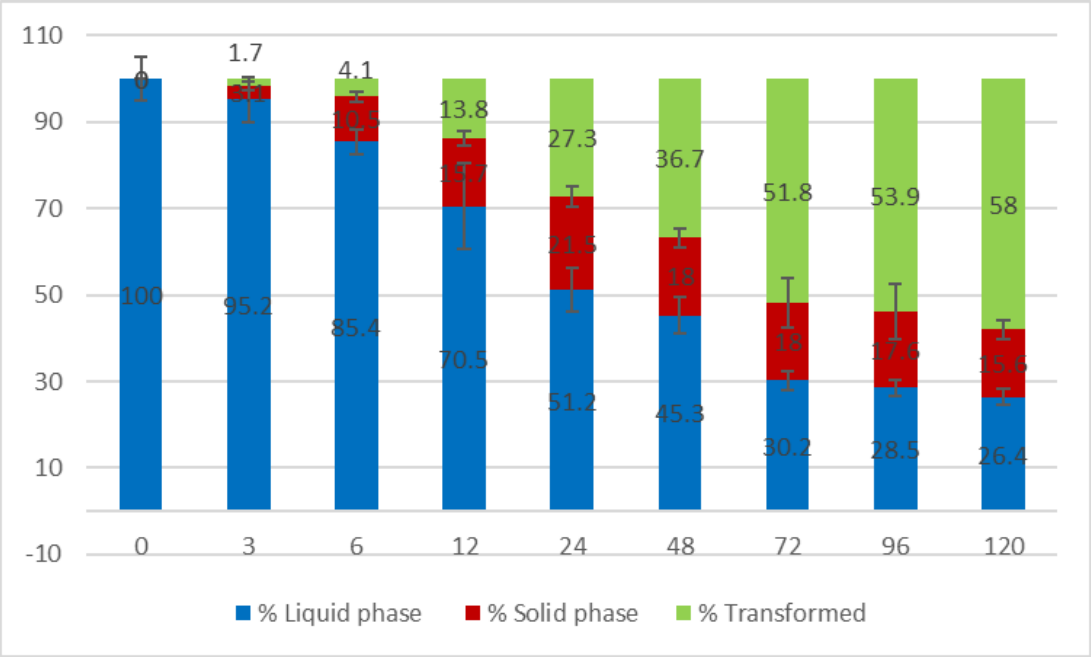


Figure 64. Percentage EE2 from liquid and solid phases with time (premix)

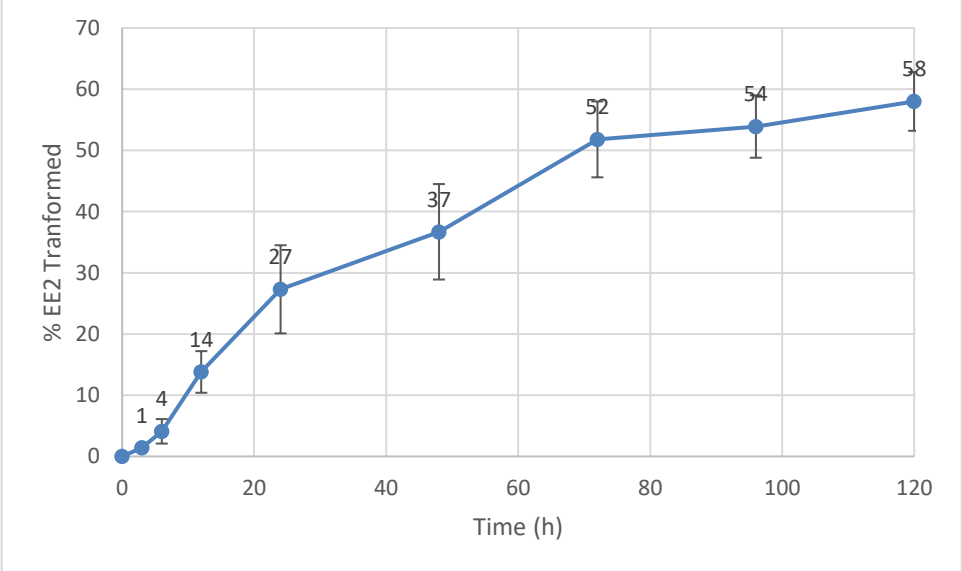


Figure 65. Percentage EE2 transformed with time (premix)

In this study, the rate of transformation of EE2 was computed using the linear form of the pseudo-first order kinetic model:

$$\log (q_e - q_t) = \log q_e - \left(\frac{k_1}{2.303}\right) t$$

Plotting log (q_e-q_t) vs t. (Figure 66) returned the value for k_1 as 0.0253 h^{-1}, with an R-square of 96.18%. However, the experimental q_e and the calculated one were 11.6 and 2.91 ug/g respectively.

Pseudo-second order kinetic model is represented as (Chapter 5):

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \left(\frac{1}{q_e}\right) t$$

Plotting (t/q_t) vs t as shown in Figure 67 returns the values for the kinetics coefficients k_2, and q_e calculated as 0.00055 g/ug.min, and 17.6 ug/g, respectively. An R-square of 97.6 % indicates a good fit with the experimental data. q_e experimental was closer to q_e calculated from the second order kinetic models which indicates that the pseudo-second order kinetic model better described the data of the premix experiment. Comparing the kinetics coefficients calculated for premix to those of RF presented in Table 7, the premix batch run experiment showed higher k_1 and k_2 values when the same mass of material (0.1 g) and initial concentration of EE2 (2000 ng/L) was used. Comparing Figure 64 and Figure ***45*** a higher transformation of EE2 was observed in the liquid phase when exposed to premix, which can be attributed to the presence of a higher concentration of the catalytic agents mediating the transformation of EE2 in the premix. Comparing the liquid phase of the premix with that of the RF, it appears that premix reached self-inhibition within a shorter time interval.

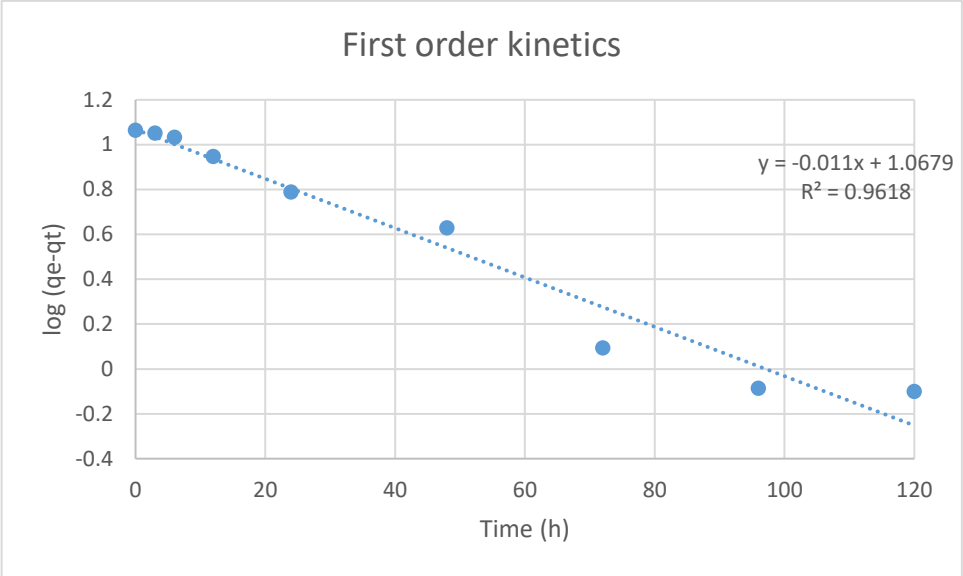


Figure 66 Pseudo-first order kinetics of EE2 with "premix"

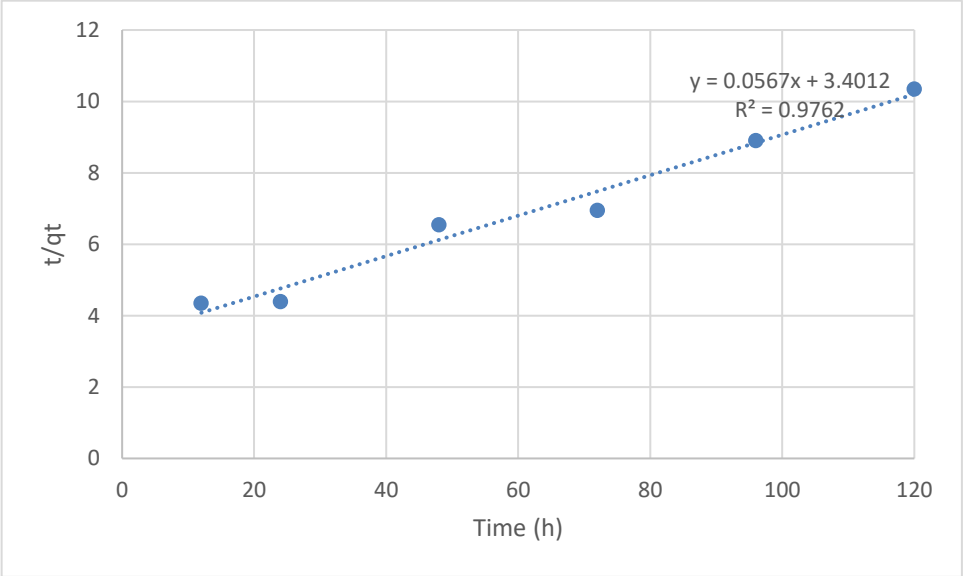


Figure 67 Pseudo-second order kinetics of EE2 with "premix"

D. Conclusion

Mass of EE2 that was adsorbed q_e and transformed m_t for different materials were determined in this chapter. Carrot leaves, corn, and radish leaves had comparable m_t values to those of RF, radish leaves exhibited the highest m_t of all materials tested.

Transformation of EE2 upon exposure to micronutrients improved significantly in the presence of a solid matrix, which indicates that transformation reactions occur in the adsorbed state.

Vegetables used in this study are considered as inexpensive materials, which were found to promote the removal of EE2 via enhanced adsorption and/or catalytic reaction. Radish and radish leaves showed promising results and should be further studied and characterized. This may provide valuable insight into underlining the mechanism of abiotic transformation of EE2 and other phenolic compounds upon exposure to vegetable wastes. Vitamins and minerals also had an impact on the transformation of EE2, with vitamins B7 and D3 showing improved m_t values over those of the premix. The premix batch experiment results proved to be comparable to those of RF. Transformation of EE2 took place in both liquid and solid phases. A pseudo-second-order model described the experimental data well. The resulting k_2 and q_e were 0.00055 g/ug.min, and 17.6 ug/g, respectively.

CHAPTER VII

CONCLUSIONS AND RECOMMENDATIONS

Studies conducted during the course of this work indicated that EE2 went through catalytic transformation. Based on earlier work, the mechanism governing this transformation is most likely an oxidative coupling mechanism. The ultimate mechanism for this process is still unclear.

Extractions done using ASE and ultra-sonication techniques proved to be the most efficient for recovering EE2 from various substrates. These techniques proved to be accurate and less time-consuming, when compared to conventional extraction procedures and Soxhlet extraction. Furthermore, both ASE and sonication produced reproducible results and eliminated the need for freeze-drying (lyophilizing).

Results obtained from two batch experiments provided evidence abiotic transformations in both liquid and solid phases represent the major driver for the removal of EE2 in the presence of RF. Results reported by Marfil Vega et al. (2011) on E2 exposure to RF were similar to those observed for EE2. Column experiments demonstrated the effectiveness of RF as a sorbent for the removal of EE2 from aqueous and solid phases. The Behavior of breakthrough curves and RF adsorption capacity was found to be influenced by flow rate. Thomas, Bohart-Adams, and the Yoon-Nelson model were used to model the behavior of RF in the column runs. Both Thomas and Yoon-Nelson models agreed with the experimental data. The Bohart-Adams model deviated from the experimental data with time. Error analysis showed the Thomas model to be the most descriptive of the column runs. Absolute error analysis indicated that Thomas model

produced the most accurate predictive results. We assumed that irreversible adsorption onto RF did not take place, since the surrogate d4 EE2 added immediately after samples were taken showed recoveries within acceptable ranges. Additionally, Marfil-vega et al. (2010) showed that compounds of very similar structures such as, testosterone, androstenedione, and progesterone did not exhibit any polymerization and yielded complete recoveries upon extraction from RF.

Vegetable matter provides inexpensive material for promoting the removal of estrogens via enhanced adsorption and/or catalytic reaction. Different vegetables contain groups typical of cellulose, hemicellulose, lignin, and proteins, with an amorphous, fibrous, and porous surface. Our theory is, in addition to the abiotic transformation of EE2 in the liquid phase, sorption onto solid surface occurs followed by transformation. The behavior of EE2 upon exposure to leaves (carrot leaves and radish leaves) had a higher impact on transformation of EE2 than that observed for the edible parts of the root vegetable.

During the premix and RF batch runs, it was observed that EE2 was transformed catalytically into unknown byproducts. A pseudo-second-order model described the experimental data of the premix batch experiment rather well.

In summary, future work should focus primarily on the identification of the byproducts generated by the catalytic reaction. Additionally, other pollutants with similar structures should be assessed including: bisphenols, alkylphenolic surfactants, and other phenolics compounds. Research should also focus on the design of technologies that maximize the rate of abiotic transformation of EE2 and other estrogens.

Palletization of vegetable wastes, most importantly leaves and employing the use of porous pellets in column runs might raise interesting possibilities for the future of

wastewater treatment technologies. A look into more abundant agricultural wastes such as soy bean leaves and corn husks is needed. Abundance of such wastes might make up for lower removal capacities of EE2. The abundant presence of rooted plants in wetlands also raise an opportunity to assess the removal of EE2 and other estrogens.

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