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Occurrence and Transformation of Pharmaceutical and Antibacterial Compounds in the Environment

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OCCURRENCE AND TRANSFORMATION OF PHARMACEUTICAL AND ANTIBACTERIAL COMPOUNDS IN THE ENVIRONMENT

By

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The presence of pharmaceuticals and personal care products (PPCPs) in the environment has become a matter of concern during the last decade. Increased production of PPCPs along with their increased use has led to release of these compounds in the environment via various routes. PPCPs includes large group of compounds including veterinary and human antibiotics, analgesics and anti-inflammatory drugs, psychiatric drugs, β-blockers, X-ray contrasts, and steroid hormones, etc. Many of the compounds used in PPCPs have been shown to possess adverse effects to living organisms and act as endocrine disrupting agents (ECDs). This dissertation includes the investigation of the occurrence of antibiotic compounds added to personal care product and the transformation of hormones used in pharmaceuticals such as contraceptives. The results obtained in this study can provide information on the fate and transformation of the studied compounds once released in the environment.

An analytical method employing sonication extraction and HPLC-ESI-MS detection was developed. The developed method was used to detect antibiotic compounds triclosan (TCS) and triclocarban (TCC) in biosolids-applied soil and biosolids. Both TCS

and TCC were detected at high concentrations in biosolids and at lower concentrations in biosolids-applied soil. TCS and TCC concentrations decreased in biosolids composts and in biosolids-applied soil collected at deeper depths. The developed method was able to provide efficient detection limits and reliable quantification of target compounds.

A molecularly imprinted polymer (MIP) was synthesized to achieve efficient clean-up of TCS and TCC from biosolids-applied soil and biosolids samples using 4,4'-DBP-4-vp-EGDMA. The motivation behind this project was to be able to eliminate the use of expensive instruments such as LC-MS and employ easily available instruments such as LC-UV. The synthesized MIP was able to achieve efficient clean-up and allowed quantification and identification of TCS and TCC in a complex matrix.

Transformation of hormones such as 17β-estradiol, estriol, ethynlestradiol, estrone and testosterone was studied by employing Fe (III)-saturated montmorillonite catalysts. The use of Fe (III) – saturated montmorillonite as a catalyst proved be to very efficient in transformation of the studied hormones. Complete removal of hormones was observed in aqueous environment. LC-UV was used for detection and quantification of hormones.

Key words: triclosan, triclocarban, estrogens, molecularly imprinted polymers, oxidative transformation, Fe (III) – saturated montmorillonite, montmorillonite

DEDICATION

I would like to dedicate this work to my father Santosh Kumar Verma, mother Sukanya Devi and husband Binay Kumar Chaudhary.

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LIST OF ABBREVIATIONS

PPCPs	Pharmaceuticals and personal care products
EDCs	Endocrine disrupting compounds
DES	Diethylstilbestrol
WWTPs	Wastewater treatment plants
AOPs	Advanced oxidation processes
ESI	Electrospray ionization
MS	Mass spectrometry
HPLC	High performance liquid chromatography
GC	Gas chromatography
USEPA	United states environmental protection agency
TCS	Triclosan
TCC	Triclocarban
CAN	Acetonitrile
m/z	Mass to charge ratio
E2	17β-Estradiol
E1	Estrone
E3	Estriol
EE2	Ethynlestradiol
SPE	Solids-phase extraction
LLE	Liquid-liquid extraction

ASE	Accelerated solvent extraction
MIP	Molecularly imprinted polymers
NIP	Non-imprinted polymers
MISPE	Molecularly imprinted solid phase extraction
NISPE	Non-imprinted solids phase extraction
Fe (III)-SM	Fe (III)-saturated montmorillonite
NM	Non-modified montmorillonite

CHAPTER I

INTRODUCTION

1.1 Pharmaceuticals and personal care products (PPCPs) in the environment

Regulatory bodies in every country make a list of Priority Pollutants and develop water quality criteria. The US Environmental Protection Agency (US EPA) has a list of 129 priority pollutants that does not appropriately represents the vast number of chemicals present in wastewater and other water resources.[1] In countries like the UK, a few compounds such as nonylphenol, flame retardants and chlorinated paraffins are strictly regulated from a list of 92 identified chemicals of concern. More attention is paid towards conventional Priority Pollutants listed under "persistent organic pollutants"; PPCPs are not included in this list. There is a much higher need to study the occurrence and fate of "emerging" pollutants used daily in domestic and industrial application.[1]

The presence of pharmaceuticals and personal care products (PPCPs) in the environment has raised concerns during the last decade. The reason for increased interest is due to the fact that these PPCPs can have adverse impact on the environment and living organisms. Despite the possible known effects of these compounds, PPCPs are not included in priority list either in the US or in Europe. There are no set criteria or regulations for PPCPs treatments in water or wastewater.[2] The PCPPs has been detected in various environmental matrices including drinking water, waste water, surface water, biosolids and agricultural soils.[2-6]

Table 1.1 Pharmaceutical and personal care products uses and associated compound of concern [1]

Compound classification	**PPCPs**
Pharmaceuticals	
Veterinary & human antibiotics	Trimethoprim, erythromycin, licomycin
Analgesics & anti-inflammatory drugs	Ibuprofen, diclofenac,fenoprofen, paracetamol, acetominophen
Psychiatric drugs	Diazepam, carbamazepine, primidone, salbutamol
Lipid regulators	Clofibric acid, bezafibrate, fenofibric acid
β-Blockers	Metoprolol, propranolol, timolol, sotalol, atenolol
X-ray contrasts	Iopromide, iopamidol, diatrizoate
Steroid hormones	Estradiol, estrone, estriol, diethylstilbestrol (DES), testosterone
Personal Care Products	
Fragrances	Nitro, polycyclic and macrocyclic musks, phthalates
Sun-screen agents	Benzophenone, methylbenzylidene camphor
Insect repellents	N,N-diethyltoluamine
Antibiotics	Triclosan, Triclocarban, chlorophene

The urbanization along with increased concern about personal and health care has lead to wide spread production and use of PPCPs. Koplin et al., measured concentration of 95 PPCPs in water samples from a network of 139 streams in the US, and detected several PPCPs in 80 % of the streams.[7] PPCPs include a diverse class of compounds; a list of several PPCPs along with their applications is shown in Table 1.1.

These compounds are specifically designed to produce biological a effects at low concentrations and hence when they enter into water resources they could cause significant impacts on aquatic and terrestrial organisms.[8]

1.2 Sources of PPCPs in the environment

Pharmaceutical compounds are administered in body where they get absorbed and are subjected to further metabolic reactions before being removed from the body. This can happens in two ways, they can undergo hydrolysis, oxidation, reduction, alkylation and dealkylation or the parent compound undergo conjugation to increase the solubility in urine and feces, which then are excreted out of the body. However some portion of the parent compound does not undergo any transformation in the body and is excreted as it is. [9] Another source of pharmaceuticals entering in sewer system is the disposal of unused or expired medication by people or hospitals in a drainage system. Once excreted out of the body or being dumped down the drain, the PPCPs enter the sewer system and the WWTPs. Direct release of PPCPs in surface water and the ocean is possible. Domestic communities which do not have a sewage systems, dispose as raw sewage directly into the streams by straight-piping.[10]

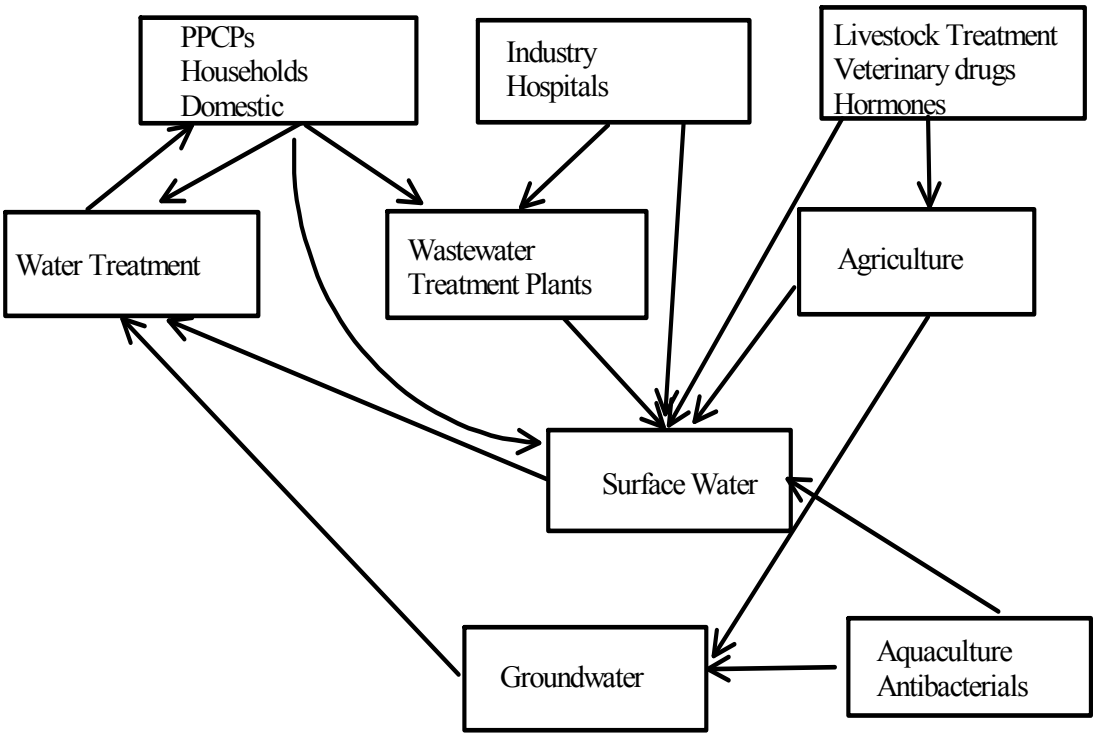


Figure 1.1 Pathways for PPCPs introduction in environment.[1, 11]

The WWTPs play an important role in introduction of PPCPs to the environment. These drugs, based on their physiochemical properties, can either get absorbed onto solids, or can be transformed in the WWTP treatment process. If PPCPs get sorbed to biosolids, a part of it can be transformed and the remaining can still be in the biosolids. Application of this biosolids as fertilizers for land applications can further distribute PPCPs in the soil environment.[12] Figure 1.1 shows the pathways for PPCPs introduction into the environment. Studies have shown that removal of PPCPs from wastewater by WWTPs is often incomplete and the effluent contains residual amount of organic pollutants. The effluents are usually allowed to flow into the surface waters, hence large number of PPCPs have been detected in surface water. Hirsch et al., detected five pharmaceutical compounds, dehydrated erythromycin, roxithromycin, clarithromycin,

sulfamethoxazle and trimethoprim in river water samples at levels between 0.06-1.70 µg/L.[13]

Stolker et al., also measured the concentration of several pharmaceuticals in surface and ground water. They found that 80% of the groundwater samples contained at least three pharmaceutical drugs. While surface water showed presence of upto eight pharmaceuticals, the concentration of these compounds ranged from 10-100 ng/L.[14] Boyd et al., developed a method for analysis of nine PPCPs in surface water, STP effluent and water from drinking water treatment plants during various stages of treatment in Louisiana, USA and Ontario, Canada. Naproxen and triclosan were detected in effluents from STPs in Louisiana; the concentration detected was between 10-106 ng/L. Only naproxen was detected in drinking water effluents in a drinking water treatment plant in Louisiana and its concentration ranged within 63-68 ng/L. While clofibric acid and naproxen were detected at a concentration of 103 and 63 ng/L respectively, in drinking water treatment plants in Ontario, Canada.[15] Kosmo et al., studied occurrence and removal of PPCPs in municipal and hospital WWTPs. They found that salicylic acid, ibuprofen, paracetamol, caffeine and gemfibrozil were present in both municipal and hospital WWTPs influents and effluents. The concentration of these PPCPs in the municipal WWTPs influent ranged from 0.7-164 µg/L, while in effluents it ranged from 0.5-13.9 µg/L. In the hospital WWTPs influent these PPCPs were present in concentration ranging between 1.1-70.1 µg/L and it ranged from 0.5-14.6 µg/L in effluents.[2] Schlusener et al., developed a method for detection of antibiotic compounds in soil samples. They detected tiamulin at a concentration of 0.7 µg/kg in soil.[16]

Table 1.2 Occurrence of various PPCPs in the environment.

Class/Compound	Matrix	Detected concentration	Ref.
Veterinary & human antibiotics	River water	0.06-1.70 µg/L	13
Several Pharmaceuticals	Surface water	10-100 ng/L	14
Nine PPCPs	WWTPs effluent Drinking water effluent (US) Drinking water effluent (Canada)	10-106 ng/L 63-68 ng/L 63-103 ng/L	15
PPCPs	Municipal WWTPs Influent Effluent Hospital WWTPs Influent Effluents	 0.7-164 µg/L 0.5-13.9 µg/L 1.1-70.1 µg/L 0.5-14.6 µg/L	2
Antibiotic	Soil	0.7 µg/kg	16
Anti-inflammatory drugs	WWTPs Influents Effluents	 2.39-2.74 µg/L 0.21-0.55 µg/L	17
veterinary antibiotics Sulfonamides	Pig manure	20 mg/kg	18
Veterinary drugs Tetracyclines	Soil	198.7 µg/kg	19

Rodriguez et al., detected two anti-inflammatory drugs ibuprofen and naproxen in WWTPs influent and effluent. Ibuprofen was detected at a concentration of 2.74 µg/L and 0.55 µg/L in influents and effluents respectively. While naproxen was detected at a

concentration of 2.39 µg/L and 0.21 µg/L in influents and effluents respectively.[17] Table 1.2. lists occurrence of various PPCPs in the environment.

Alternatively PPCPs can also enter the environment through direct release. Personal care products such as sunscreens, lotions, shampoos and several others can be directly released in surface and ground waters. Even though the contribution by direct release is not significant.[1] PPCPs can also be directly introduced in surface water by runoff from agricultural areas where animals are treated with veterinary drugs.[7, 20] Veterinary medicines are used to treat animals to keep them healthy. Some compounds are added as feed additives to help animals grow faster. The drug supplements that farmers feed to animals include antimicrobials, antiprotozoals, ecto- and endo-parasiticides and hormones.[21] As shown in Figure 1.1 the residues from drugs used for veterinary practice can enter the environment directly by application of manure to the soil or application after storage as sludge. Once applied to the soil these residual drugs can accumulate or can go into surface water during runoff events or can leach into ground water resources.[22] Haller et al., measured the concentration of veterinary antibiotics in pig manure, they detected sulfonamides in the manure pits on farms where medical feed had been used. Sulfonamides were detected at concentration upto 20 mg/kg in liquid manure.[18] Hamscher et al., studied the persistence of veterinary drugs tetracycline and chlortetracycline in soil after application of manure. The highest detected concentration for tetracycline was up to 198.7 µg/kg in top 30 cm of the soil, while 4.6-7.3 µg/kg of chlortetracycline was present in top 30 cm of the soil layers. They concluded that tetracyclines might show potential for bio-accumulation in soil.[19]

1.3 Process of PPCPs removal in conventional wastewater treatment plants

The presence of PPCPs in effluents from wastewater treatment plants suggests that conventional wastewater treatment plants are not efficient in removal of these compounds from wastewater influent.[9, 23] The technology that the WWTPs use currently are not suitable for removal of PPCPs, these WWTPs have been designed for the removal of carbon, nitrogen and phosphorous.[24, 25]

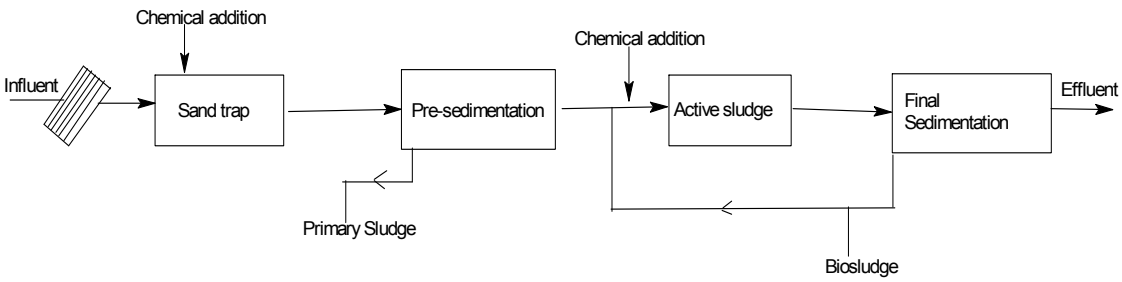


Figure 1.2 Diagram of a Sewage treatment plant process.[26]

Figure 1.2 shows the design of a conventional WWTP. A conventional WWTP goes through preliminary and primary treatments, secondary biological treatment, and a tertiary treatment for disinfection of the treated water and finally the sludge disposal for land application.

1.3.1 Preliminary treatment

Preliminary treatment is usually employed before the wastewater enters in the primary and secondary treatment chambers. This treatment usually employs a screen to remove large floating objects.[27]

1.3.2 Primary treatment

The primary treatment in WWTPs uses a coarse grid to remove large objects such as plastic bottles and other debris.[28] During this step there is little or no removal of organic compounds.[29] Sand trap, primary sedimentation and chemical addition treatments are carried during primary treatment.

1.3.2.1 Sand trap

The sand trap is a chamber where the wastewater is allowed to settle. In this process high density materials like sand , gravel, grit would settle down and the effluents pass to the primary sedimentation tank.[27]

1.3.2.2 Primary sedimentation

Once the heavy density material such as grit is removed in the sand trap, wastewater enters the sedimentation tank. At this stage wastewater still contains dissolved organic and inorganic components along with solid waste. During this step suspended solids can be removed by sedimentation or gravity settling, chemical coagulation or flocculation and filtration. The suspended solids generated during primary sedimentation are called primary sludge.[27]

1.3.2.3 Chemical addition

The use of chemical additives in WWTPs can change the sorption properties of several organic compounds with high sorption coefficients and can improve the removal efficiency of these compounds. Chemicals additives that can cause coagulation, flocculation and tensoactives are usually added.[30] Carballa et al., showed 50 - 70 % removal of PPCPs with high sorption properties using ferric and aluminum salts as coagulating agent. Compounds with lower sorption coefficient were removed with lower

efficiency of 10-25 %. Higher fat content in the wastewater improved the efficiency of chemical addition treatment.[30]

1.3.3 Secondary treatment

After the pre-sedimentation step there is still a large amount of organic matter in the primary effluent. This organic matter which is a rich source of carbon can be used by micro-organisms. Many PPCPs are thought to undergo biodegradation and transformation, since certain microorganisms present in WWTPs might be able to use them as carbon source.[31] A quick and complete degradation of pollutants present in the sludge is brought under aerobic conditions through catabolic pathways.[32] Many wastewater treatment plants use biodegradation processes such as activated sludge treatment, biobeds and trickling filters.

1.3.3.1 Activated sludge treatment

Activated sludge treatment happens to be one of the frequently used methods for removal of organic matter from wastewater influents. In the active sludge treatment chamber, the incoming wastewater influents are mixed with microorganisms with continuous aeration and stirring action. Once the influents are filtered, the effluent either goes through a disinfection step or is released into the environment. A portion of sludge solids are returned to the tank for next batch to come in and the remaining sludge is treated and disposed accordingly.[32]

Even though the activated sludge process is efficient in removal by adsorption or bio-degradation of many organic compounds, the complete removal of PPCPs does not takes place. Johnson et al. reported in their study that the average removal of 17β-estradiol (E2) and estrone (E1) was about 88% and 74%, respectively from WWTPs

employing activated sludge treatment.[33] Baronti et al., reported the removal efficiencies of six WWTPs using activated sludge treatment in Rome to be around 85% for 17α-ethinylestradiol (EE2), 61% for E1, 87% for E2 and 95% for estriol (E3).[34]

A low removal efficiency was found by Ternes et al., for E1 and EE2 < 10%, while two-thirds of E2 was eliminated in the WWTPs by activated sludge treatment.[29] In the studies conducted by Svenson et al., three different treatment methods were studied: trickling filter, biorotors and activated sludge. They found that biorotors and trickling filter were the least efficient of all the treatments; they were able to remove only 28% and 33.3% of the total estrogenic compounds. While activated sludge removed 81% of the estrogenic compounds.[26] Komori et al., observed removal efficiency of 47% for E1, while 100% of E2 was removed and 98% removal of E3 was obtained.[35]

Biobeds or trickling filters are also used for biological treatment alone or in combination with activated sludge. Some WWTPs use nitrifying and denitrifying chambers.[26] Hence different types of removal pathways are possible for organic compounds in WWTPs including adsorption, volatilization, chemical and biological degradation.

1.3.4 Tertiary treatment

WWTPs employ tertiary treatments after the primary and secondary treatment. Tertiary treatment is usually disinfection process of the wastewater effluents. Disinfection is required because wastewater contains micro-organisms and pathogens that can cause diseases.[27] Commonly employed methods for disinfection include chlorination, ozonation and UV radiation.

1.3.4.1 Chlorination

Chlorination is widely used all around the world for disinfection of water and wastewater due to its strong oxidative properties, convenience and cost.[36] The chlorination process is usually fast but is accompanied by formation of by-products which can exhibit carcinogenic or mutagenic properties.[37]Chlorination of acetaminophen, a common pain reliever used in paracetamol and 4-acetamidophenol was investigated using NaOCl. Eleven chlorination products were produced, out of which two products were identified to be 1,4-benzoquinone and N-acetyl-p-benzoquinone imine. These are toxic products causing lethal effect associated with overdose of acetaminophen.[38]

1.3.4.2 Ozonation

Ozone is very effective is elimination of viruses and bacteria. But the cost associated with production of ozone makes it an economically unfavorable disinfection method.[27]

1.3.4.3 UV radiation

UV radiation kills micro-organisms by penetrating the cell walls of the micro-organisms and retards their ability to survive by damaging their genetic material. UV radiation can also bring about destruction of trace amount of organic compounds remaining in effluents. But micro-organisms can survive low dose of UV radiation, hence appropriate UV dose needs to be applied for the disinfection process.[27]

Table 1.3 PPCPs removal efficiency of various treatment process used in conventional WWTPs

Class/Compound	Treatment used	Removal efficiency	Ref.
Pharmaceuticals	Activated sludge (AS)	90 %	39
31 Pharmaceuticals	Membrane bioreactor (MBR)	> 80%	40
Hormones	AS	74-88%	33
Hormones	Chemical treatment (Fe, Al) Activated sludge Trickling filter Biorotors	18 % 81% 28% 33.3%	26
Various PPCPs	Chemical treatment (Fe, AL)	10-60 %	30
13 PPCPs	Activated sludge	20-60%	41
Various PPCPs	Activated sludge	20-95%	42
Various PPCPs	Sand filtration Sand filtrations, ozonation, AS	0% 80%	43
55 PPCPs	Trickling filter Activated sludge	70% 85%	44
Musk Fragrance	Activated sludge Trickling filter	88-99.9% 71.3-98.6%	45

1.3.5 Advanced treatments for physical removal of PPCPs

Advanced treatments for physical removal of organic compounds are usually employed as an extension of secondary treatment.[27] Physical process such as coagulation, sedimentation, chemical precipitation, filtration and membrane separation are commonly employed processes in WWTPs.[24, 46]

1.3.5.1 Sand filtration

A sand filtration chamber contains a layer of raw water over a layer of fine sand supported on a thin layer of gravel; this chamber is connected to several drains and inlets. A schmutzdecke layer is formed on the top of sand over which the raw sewage water flows, the water penetrates the bed of sand and gravel due to the gravity.[47] During this step, both physical and biological processes can eliminate many organic compounds. This process, however, does not have much impact on the removal of free and conjugated estrogens from the raw sewage.[48, 49]

1.3.5.2 Activated carbon

Another way of physical removal of PPCPs is by using activated carbon system (AC). Activated carbon is produced either from coal, wood or coconut shells.[46] It can be either in powdered or granular form. The activated carbon is highly porous and the pore size varies over a broad range, the adsorption takes place due to intermolecular attractions in the pores. The adsorption results into organic molecule or gases getting trapped inside the pores of the activated carbon.[46] However, the disadvantage of using AC is that they need regular regeneration, studies showed that AC filters that were not regenerated showed no removal of target compound.[50]Once the granular or activated carbon loses its

activity, it has to be either regenerated, landfilled or incinerated, which can certainly add up to the cost of the process.[31, 51]

1.3.5.3 Membrane filtration

To overcome the limitations of AC, use of membrane filtration is becoming a wide spread practice for removal of organic contaminants. [52] Removal of trace micropollutants depends on their size of the compound, the physicochemical properties of the feed solution and membrane material. Studies conducted to test the efficiency of various filtration processes showed that the nanofiltration (NF), tight and small pore size membranes (reverse osmosis (RO)) can effectively retain estrogen hormones. Predicted value of retention by RO was 95 % according to Huang et al.[53] Nghiem et al., showed that the porous membranes lost their adsorption capacity with increase in pressure resulting in breakthrough of the target compound from the membrane surface. They also showed that presence of organic matter is important for retention of estrogenic hormones.[54] Yoon et al., studied retention of 52 EDCs and PPCPs on NF and ultrafiltration (UF) membranes, they concluded that NF was able to retain more target compounds based on size exclusion and hydrophobic adsorption, while UF membrane retained fewer compounds of hydrophobic nature based on a hydrophobic mechanism.[55]

Membrane filtration seems to be an efficient method for removal of organic pollutants, but these membranes are expensive. Fouling of membranes is another problem, regular cleaning is required to maintain the efficiency of the membrane.[31]Not every WWTP would consider this option as economical, which will limit the use of this process.[56]

1.3.6 Advanced oxidation processes (AOPs) for removal of PPCPs

Advanced oxidation processes are normally used for disinfection /oxidation of toxic or hazardous compounds.[57]AOPs are widely studied for removal of various organic pollutants which have high chemical stability and/or low biodegradability in WWTPs.[58] The mechanism of AOPs in elimination of target compound is by using strong oxidizers through oxidation-reduction reactions. Transformation occur by complete mineralization into CO_2, water and inorganic compounds, or transformation into less toxic compounds compared to the parent compound.[59]

There are several combination of reagent systems used in AOPs, but all of them produce $OH^{\bullet}$ radicals which are non-selective towards the target compounds, these radicals are very reactive species and have a redox potential of 2.80 eV.[52]

1.3.6.1 UV based AOPs

1.3.6.1.1 UV AOPs

Direct exposure of a target compound in water with the UV light can cause the molecule fragmentation by mechanism shown in equation 1.1 & 1.2.[60]

$$\text{Target compound} \xrightarrow{h\nu} \text{Intermediates} \quad (1.1)$$

$$\text{Intermediates} \xrightarrow{h\nu} CO_2 + H_2O + Cl^- \quad (1.2)$$

Kim et al., studied photolysis of 30 PPCPs, the PPCPs selected in study included 10 analgesics, three antiarrhythmia agents, nine antibiotics and two bronchodilators, anti-itch drug, anticonvulsant, antineoplastic agent, insect repellent, carbadox (antiparasitic agent) intermediate and NMDA (N-methyl d-aspartate) receptor antagonist. They calculated the dose of UV light required to cause 90 % degradation of all the selected PPCPs, the required UV dose ranged from 38 mJ cm^{-2} to 5644 mJ cm^{-2}. From the

required dose time they calculated, that the UV irradiation by itself would not be able to remove selected PPCPs from WWTPs, because the UV dose used in WWTPs is usually less than the calculated value.[61]

1.3.6.1.2 UV/H_2O_2 process

In this process $OH^{\bullet}$ radicals are produced by cleavage of HO-OH bond (equation 1.3). The propagation and termination during this process occurs as shown in equations 1.4 through 1.8.

Initiation Reaction:

$$H_2O_2 \xrightarrow{h\nu} 2\,HO^{\bullet} \quad (1.3)$$

Propagation Reaction:

$$H_2O_2 + HO^{\bullet} \longrightarrow O^{\bullet}\!-\!OH + H_2O \quad (1.4)$$

$$H_2O_2 + O^{\bullet}\!-\!OH \longrightarrow HO^{\bullet} + O_2 + H_2O \quad (1.5)$$

Termination Reaction:

$$HO^{\bullet} + HO^{\bullet} \longrightarrow H_2O_2 \quad (1.6)$$

$$O^{\bullet}\!-\!OH + HO^{\bullet} \longrightarrow H_2O + O_2 \quad (1.7)$$

$$O^{\bullet}\!-\!OH + O^{\bullet}\!-\!OH \longrightarrow H_2O_2 + O_2 \quad (1.8)$$

This reaction is not pH dependent, and increases under alkaline conditions.[58]

Kim et al., studied the photolysis of 30 PPCPs, they found that use of H_2O_2 along with UV irradiation lead to 90% removal of all the PPCPs except for 7 PPCPs in 30 min. The UV dose calculated was 691 mJ cm^{-2}. They concluded that UV/H_2O_2 was an efficient method for removal of selected PPCPs from WWTPs.[61] Li et al., studied degradation of clofibric acid (CA) in WWTPs effluent using UV/H_2O_2, they found that 99 % of CA was removed within 15 min at 30°C.[62]

1.3.6.1.3 UV/O_3 process

The photolysis of O_3 at 253.7 nm UV radiation produces $OH^{\bullet}$ radicals by photodecomposition of ozone (equation 1.9). These hydroxyl radicals than combine to produce hydrogen peroxide, which in turn would cause further decomposition of residual ozone (equation 1.10). The simplified reaction mechanism is given below.[63]

$$O_3 + H_2O \xrightarrow{h\nu} O_2 + H_2O_2 \quad (1.9)$$

$$2\,O_3 + H_2O_2 \xrightarrow{h\nu} 2HO^{\bullet} + 3O_2 \quad (1.10)$$

This system is capable of producing OH radicals ($OH^{\bullet}$) by three mechanisms namely, UV radiation, ozone and hydrogen peroxide.

The transformation of ECDs 17β-estradiol (E2) and bisphenol A (BPA) was investigated using UV/O_3. They found that when UV was coupled with ozone, there was a reduction in the consumption of ozone required for transformation of E2. Also the time required for transformation of E2 was decreased. However, no difference in the amount of O_3 required for transformation of same amount of BPA was observed with O_3 and UV/O_3.[64]

1.3.6.1.4 UV/O_3/H_2O_2 process

In this advanced oxidation process, hydrogen peroxide is added to the UV/O_3 process. Addition of hydrogen peroxide accelerates the production of $OH^{\bullet}$ radicals as shown in equation 1.11, but the cost of this process is very high, since two reagents are used when compared to other processes mentioned earlier in which only one reagent is used. The resulting reaction is shown in equation 1.11.

$$2\,O_3 + H_2O_2 \xrightarrow{h\nu} 2HO^{\bullet} + 3O_2 \quad (1.11)$$

1.3.6.1.5 Fe^{+3}/UV process

In Fe^{+3}/UV AOPs, the $OH^{\bullet}$ radicals are produced due to photo-excitation of Fe (III). The process is of interest due to its potential application in wastewater treatment plants. The solar energy can be used to excite Fe(III), which is ubiquitous in natural water.[65] The dominant form of Fe (III) in acidic solution between pH values 3.0 to 4.5 is $Fe(OH)^{+2}$. This form undergoes a photolysis to yield $OH^{\bullet}$ radicals with the highest quantum yield at 300 nm. Equation 1.12 shows the oxidation process involved with Fe (III)/UV AOPs.

$$Fe(OH)^{+2} \xrightarrow{h\nu} Fe^{+2} + HO^{\bullet} \qquad (1.12)$$

The initial intermediate products produced during degradation of organic compounds include oxygenated Fe(III) complex which can further undergo mineralization without using $OH^{\bullet}$ radicals.[66] This reaction was successfully studied by Du et al., and Rodriguez et al., for degradation of atrazine and nitrobenzene.[65, 67] However, when concentration of Fe (III) decreases in the reaction mixture, production of $OH^{\bullet}$ radicals and degradation process also decreases. Hydrogen peroxide can be used to oxidize Fe (II) to Fe (III) but use of hydrogen peroxide as a reagent increases the cost of the process, use of oxygen seems to be a cheaper alternative. Du et al., compared the concentration of Fe(III) in the reaction mixture with and without oxygen, they found that 25% more Fe(III) was present in the reaction mixture with oxygen.[65] Fe (III)/UV/O_2 can serve as a cheaper and efficient AOPs for removal of organic pollutants from water or wastewater treatment plants. The only problem associated with implementing this technology is strict control of pH of wastewater, which can be an additional cost for the WWTPs.

1.3.6.1.6 Photocatalysis

This process uses semiconductor metal oxide as catalyst and oxygen as oxidizing agent. This type of photocatalysis is also called heterogeneous photocatalysis because there are two active phases, solid and liquid.

The mechanism of photocatalytic degradation of organic pollutants over TiO_2 involves absorption of photon of energy equal to or higher than the band gap energy width, 3.2 eV (Figure 1.3). This can be achieved by photo-irradiation of TiO_2 at wavelengths greater than 400 nm. On receiving the required energy a electron is promoted from valence band to the conductance band leaving an electron vacancy or "hole" in the valance band.[68] Several chalcogenides (oxides and sulfides) have been tested as photocatalysts: TiO_2, ZnO, CeO_2, ZrO_2, SnO_2, MnO_2, $SbrO_4$, CdS, ZnS, MgO, Fe_2O_3 and their various combinations have been used for the degradation of organic pollutants.

The anatase form of TiO_2 is the most commonly used form, since it the most active form of TiO_2. Other forms of TiO_2 are also available such as natural forms rutile and brookite, and artificial forms TiO_2-B, TiO_2-H.[68, 69]

$$TiO_2 \xrightarrow{h\nu} e^- + h^+ \quad (1.13)$$

$$TiO_2\,(h^+) + H_2O_{ad} \longrightarrow TiO_2 + H^+ + HO^{\bullet}_{ad} \quad (1.14)$$

$$TiO_2\,(h^+) + HO_{ad} \longrightarrow TiO_2 + HO^{\bullet}_{ad} \quad (1.15)$$

$$e^- + h^+ \longrightarrow \text{heat} \quad (1.16)$$

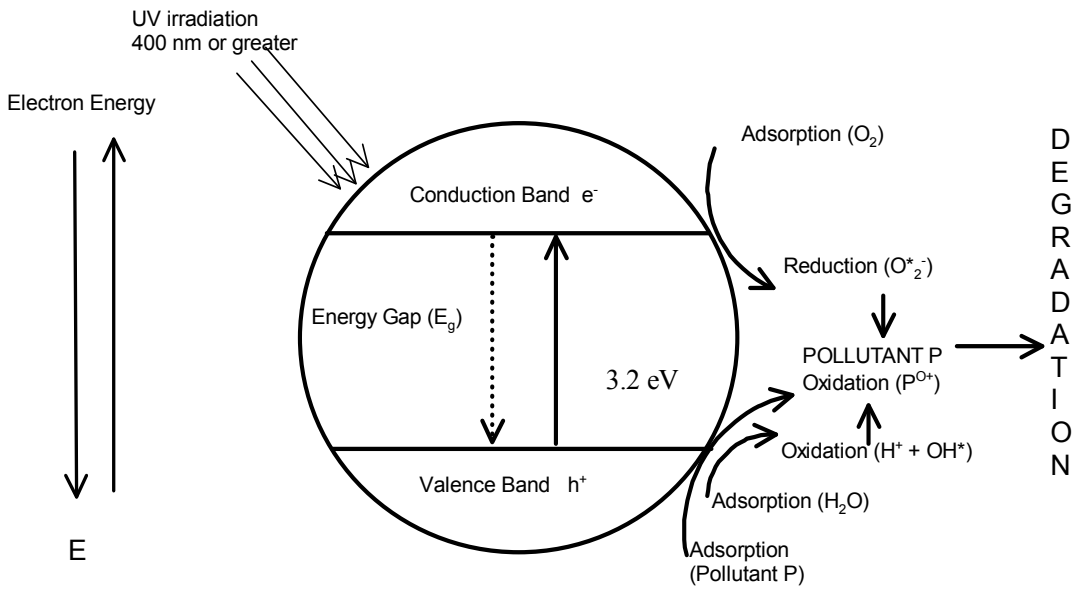


Figure 1.3 Energy band diagram of photocatalysts TiO_2.[69]

The electrons formed during the process of initiation are able to reduce metals and dissolved oxygen to produce superoxide radical ion $O_2^{\bullet -}$. The holes formed during the process oxidize adsorbed H_2O or OH^- to form reactive $HO^\bullet$ radicals (equation 1.13 & 1.15). As shown in Figure 1.3, certain pollutants get adsorbed on the surface of TiO_2 can directly undergo oxidization by electron transfer reaction.[59] But a significant part of electron-hole pairs are lost due to combining of electrons and holes to produce heat (equation 1.16). An ideal photocatalysts should have following properties:[68]

1) Photoactivity,
2) It should be biologically and chemically inert,
3) Photostable,
4) Should be able to utilize visible and/or near-UV light,
5) Inexpensive, and
6) Low toxicity.

TiO_2 is almost an ideal catalyst, but it does not absorb energy in the visible range of the spectrum. To increase the performance of TiO_2 and increase its applications, TiO_2 is doped with other metals such as Pt, Rh or Ni. Doping is expected to increase the absorption spectrum of TiO_2, which can facilitate the use of TiO_2 as a photocatalysts when irradiated with the solar irradiation.[59] Until this limitation of TiO_2 is overcome, its application on an industrial scale seems to be far away from reality.

Cao et al., used TiO_2 photocatalysis to study degradation of chlorfenapyr. Chlorfenapyr is an insecticide used to control various insect, mite pests on cotton and vegetables. They compared photolysis of chlorfenapyr with photocatalysis and found that the photocatalysis reaction was 2.5 and 3 times faster than photolysis at 300 and 350 nm, respectively. Half-lives were measured at different pH values, a faster half-life value of 8.7 min was observed at pH 11. The degradation rate was also dependent on TiO_2 dosage.[70] Degradation of another organophosphorus pesticide terbufos was studied by Wu et al. They found that 99 % of pesticide was degraded after 90 min of UV irradiation in TiO_2 suspension.[71]

1.3.6.1.7 Fenton and photo-fenton process

H.J.Fenton described in 1894, production of hydroxyl radical when certain organic compounds were oxidized with H_2O_2 in the presence of Fe(II) catalyst.[72] Later it was discovered that oxidation was carried due to the presence of $OH^{\bullet}$ radicals.[58] Addition of UV/Vis radiation to the Fenton reaction was found to produce more $OH^{\bullet}$ radicals and efficient recycling of Fe(II) catalyst.[73]

In the Fenton reaction, Fe(II) reacts with hydrogen peroxide to produce OH^- and $OH^{\bullet}$ as shown in equation 1.17.[74]

$$Fe\ (II) + H_2O_2 \longrightarrow Fe\ (III) + OH^- + HO^{\bullet} \quad (1.17)$$

Fe (III) can then be reduced by excess H_2O_2 to form Fe (II) ions and more radicals (Equation 1.18-1.20).

$$Fe\ (III) + H_2O_2 \longleftrightarrow FeOOH^{+2} + H^+ \quad (1.18)$$

$$FeOOH^{+2} \longrightarrow O^{\bullet}{-}OH + Fe\ (II) \quad (1.19)$$

$$Fe\ (III) + O^{\bullet}{-}OH \longrightarrow Fe\ (II) + O_2 + H^+ \quad (1.20)$$

In the Photo-Fenton process, the regeneration of Fe(II) is carried out by photo-reduction of Fe(III) ion, $OH^{\bullet}$ radicals are also produced in this process (equation 1.21-1.22).[74]

$$Fe\ (III) + H_2O \longrightarrow Fe(OH)^{+2} + H^+ \quad (1.21)$$

$$Fe(OH)^{+2} \xrightarrow{h\nu} Fe\ (II) + HO^{\bullet} \quad (1.22)$$

A lot of research has been devoted to the Fenton process. But this process has limitations, such as strict control of the pH and the iron sludge waste which is produced as by-product of the Fenton process. Which requires further disposal, adding to the cost of the AOPs.[59] Use of UV along with the Fenton process reduces the production of sludge.[58]

Xu et al., investigated degradation of melatonin, which is a hormone. Melatonin can suppress production of testosterone and can effect semen quality. Fenton and Photo-Fenton reactions were used to study the degradation of melatonin in aqueous solution. They found that both the Fenton and Photo-Fenton reactions were able to completely remove melatonin, but with Fe^{+2}/H_2O_2, 100% removal of melatonin took place in 60 min, while it took only 30 min for 100% removal using $Fe^{+2}/H_2O_2/UV$.[75]

The advanced oxidation process represents very a powerful means for removal of toxic organic compounds from water and wastewater when compared with the

conventional treatment methods. AOPs can provide a cheaper way to eliminate organic pollutants which are not removed by conventional treatments. Advanced technology for the treatment of wastewater can have an impact on the economy and the environment due to increased energy consumption and CO_2 emissions. The cost associated with the treatment facilities would eventually be passed down to the consumers.[76] Efforts to increase the efficiency and use of the solar radiation could be a more economical solution for water purification.[59]

1.4 PPCPs used in the study

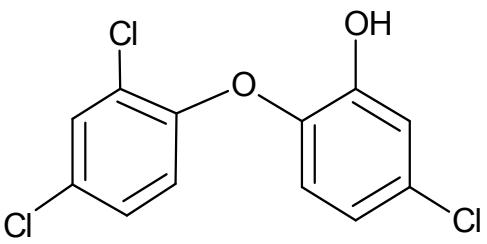
Synthetic organic antibacterial compounds are a class of compounds which are frequently detected in municipal wastewaters due to their domestic, commercial and industrial use.[77] The primary route of these compounds getting in the environment is through discharge of effluent from WWTPs and the on going practice of using sewage sludge as fertilizer.[78] Antibacterial compounds triclosan (2, 4, 4'-trichloro-2'-hydroxydiphenyl ether) and triclocarban (3, 4, 4'-trichlorocarbanilide) happen to be of particular interest because of their acute and chronic toxicity to aquatic organisms.

Many organic compounds such as PPCPs when released in the environment, produced as result of human activities, can act as endocrine disrupting chemicals (EDCs). EDCs are those substances which interrupt with normal endocrine system function by mimicking endogenous hormones.[79] Steroid hormones are a class of EDCs that are a potential threat to the aquatic and other living organisms including humans. The steroid hormones can be natural or synthetic hormones. Natural steroid hormones are excreted daily by humans and animals in environment by urine and feces. 17β-estradiol, estrone, estriol and testosterone are examples of naturally occurring steroid hormones and

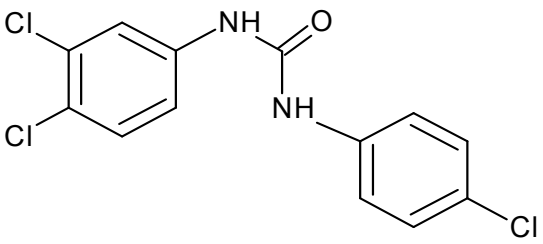
synthetic steroid hormone includes 17α-ethinylestradiol.[80] Both 17β-estradiol and estrone are of major concern because they exert ECDs properties to certain aquatic organisms at low concentration of 1 ng/L.[79]

1.5 Triclosan and triclocarban

Triclosan (TCS) and triclocarban (TCC), widely used antibacterial agents, are added at a level of 0.1- 0.3 % (w/w) and 0.5 – 5% (w/w), respectively, in hand lotions, soaps, toothpaste, and many other consumer products.[81-84] Both compounds in consumer products are usually washed down the sewage systems and end up in wastewater treatment plants. These are major point sources of pharmaceutical and personal care products (PCPPs) in the environment. It is estimated that more than 300 t $year^{-1}$ TCS is disposed into wastewater , it is also ranked in the top ten among 96 organic wastewater contaminants, with an appearance frequency of 58% in US water streams.[77] The use of antibacterial compounds is increasing steadily around the world. TCS and TCC are included in the "Emerging Organic Pollutants" group which mostly includes endocrine-disrupting chemicals (EDCs).[85] Some studies suggest that TCS has EDC properties, when Long Evans rats where given oral dose of TCS; they found that TCS interrupted with thyroid hormone hemostasis.[86, 87] TCC being structurally very similar (Figure 1.4) to TCS can pose same risk. It is possible that TCC may have higher risk potential , since it appears to be more persistent and in higher concentration than TCS in WWTPs.[88]



TCS



TCC

Figure 1.4 Structure of triclosan and triclocarban

Table 1.4 Physiochemical properties of TCS and TCC

Property	TCS	TCC
CAS registry no.	3380-34-05	101-20-2
Formula	$C_{12}H_9Cl_3O_2$	$C_{13}H_9Cl_3N_2O$
Molecular weight (g/mol)	289.55	315.59
Water solubility (mg/L at 25°C)	1.97-4.6	0.65-1.55
Kow (at 25°C, pH 7)	4.8	4.9
Vapor pressure (Pa at 25°C)	-	7×10^{-4}
pKa	7.9	12.7

Taken from reference[89, 90]

1.5.2 Toxicology of TCS and TCC

Studies of the toxicological properties of TCS and TCC are limited. Studies suggest that triclosan blocks the lipid biosynthesis by specifically inhibiting the enzyme enoyl-acyl carrier protein reductase and may induce bacterial resistance towards the compound itself.[91] Physical properties listed in Table 1.4, shows that TCS and TCC both have low water solubility and octanol-water partition coefficient, which allows it to accumulate in fat. As a result several aquatic species were found to have bioaccumulated TCC or TCS and its metabolite in their tissue.[92, 93] One study found that TCS has high

toxicity towards the early life stages of medaka, and it metabolites would probably act as weak estrogenic compounds. Like estrogenic compounds these metabolites are capable of inducing vitellogenin of male medaka.[94] In another study TCS showed acute toxicity towards zebra fish embryo with (96 h LC_{50} = 0.42 mg/L). The survived embryo showed several malformations.[95] Triclosan can also undergo photolysis to form dioxin, which is known as the most carcinogenic chemical in the world.[96] Little information is available regarding the toxicity of TCC and whatever information is available points towards negative impact of TCC. It has been found to cause impaired reproduction in laboratory rats and the metabolites produced by degradation of TCC are carcinogenic.[97] With so much existing toxicity evidence, it is important to understand the fate of these compounds when they enter into our environment.

1.5.3 Occurrence and fate of TCS and TCC

Triclosan is one of the most frequently detected organic pollutant, TCC and methyl-TCS are co-contaminants with TCS in waste water and surface water.[97] Understanding the occurrence and environmental fate of potential pollutant is important to determine its environmental exposure and the risk associated with the exposure of the pollutants.

1.5.3.1 Occurrence and fate of TCS and TCC in wastewater and surface water

There are many reports of detecting TCS in wastewater, surface water, groundwater, even in fish and human milk.[84, 98-100] Whereas TCS is being studied extensively, the occurrence and environmental fate of TCC is less investigated.[89, 101] Because of their similar properties (Table 1.1, Figure 1.1), usage, and disposal, co-occurrence of TCC and TCS in the U.S. water resources have been observed. Halden's

group measured 42 environmental samples from the greater Baltimore region and observed presence of both TCS and TCC in all the samples.[89] In another study conducted by Bester et al, in Germany, concentration of TCS was measured in surface water and effluent from sewage treatment plant (STP). The TCS concentration detected in surface water ranged from 3 - 10 ng/L, while in STP effluents TCS concentration was much higher and ranged from 10 – 600 ng/L.[99]

Table 1.5 Occurrence of TCS and TCC in water or wastewater samples from various locations

Location	Matrix	TCS (ng/L)	TCC (ng/L)	Ref.
USA	WWTPs Influent effluent	6100 ± 100 35 ± 20	6700 ± 100 110 ± 10	89
Germany	Surface water WWTPs effluent	3-10 10-600	-	99
USA	WWTPs influent effluent	3800 - 16600 200-2700	-	81
Switzerland	River water	74	-	102
China	Surface water	478	338	103
China	WWTPs influent WWTPs effluent	533-774 80 – 250	-	104

McAvoy et al., studied the fate and removal of TCS in wastewater treatment systems and they found that the concentration of TCS in influent ranged from 3.8 – 16.6 µg/L and 0.2 - 2.7 µg/L in the final effluent.[81] In Switzerland, Lindstrom et al., detected TCS at concentration upto 74 ng/L in lakes and river water.[102] In the Pearl River system

in China, J.-L.Zhao et al., measured the concentrations of TCS and TCC as high as 478 ng/L and 338 ng/L in surface water, they also measured the concentrations of TCS and TCC in the river sediment and found the concentrations as high as 1329 ng/g and 2633 ng/g for TCS and TCC respectively.[103] The concentration of TCC usually appears to be few orders higher than that of TCS. Concentration of TCS in raw wastewater samples collected from WWTPs in the Shanghai ranged from 553 – 774 ng/L and ranged from 80.14 – 249.72 ng/L in the effluent samples.[104] High levels of TCS and TCC were found to be removed from wastewater during the wastewater treatment processes by sorption onto wastewater particular matter, resulting in their high concentrations in biosolids.[82, 84, 101] Table 1.5 summarizes the concentration of TCS and TCC detected in wastewater around the world.

1.5.3.2 Occurrence and fate of TCS and TCC in biosolids and biosolids applied soil

Studies have shown that TCS and TCC can sorb to sewage sludge during WWTP treatment process, resulting in their concentration in biosolids. Studies have shown that activated sludge treatment was able to remove 96% of TCS from wastewater while trickling-filter treatment only removed TCS between 58 – 86%.[81] Concentrations up to 16.79 mg/kg of TCS has been detected in biosolids collected from WWTPs in Australia.[84] While TCC was also detected in biosolids up to concentrations of 7 mg/kg.[85]

Since application of biosolids to the agricultural fields as fertilizer or soil conditioner is becoming a common practice, plants and soil organisms in the biosolids applied field can potentially be exposed to these chemicals which are present at high levels. In addition, there is a potential for these chemicals to get into aquatic systems via surface runoff and leaching from fields applied with biosolids. Limited information is

available on the fate of these two compounds in soils receiving TCC and TCS containing biosolids.[78] Studies estimate that in the USA approximately 7 million dry tons of biosolids was produced in 2004, out of which 50% was used as fertilizer and 45% was disposed in landfills.[4] Lozano et al., measured the concentration of TCS in farm soils that were fertilized with biosolids, the concentration of TCS after a sampling period of 16 months was in the range of 4.1 – 4.5 ng/g dry wt. When sampling was done after a period of 12 months from the day of biosolid application the TCS concentration ranged from 23.6 – 66.6 ng/g dry wt, with an estimated half-life of 107.4 days.[86] Chu et al., measured concentration of TCS and TCC in biosolids collected from WWTPs in Ontario, Canada. They found that TCS and TCC co-occur in municipal sludge and treated biosolids at cocentrations ranging from 0.62-11.55 µg/g and 2.17 – 5.97 µg/g dry weight for TCS and TCC respectively.[88] The concentration of TCS ranged from 0.09 – 16.79 mg/kg dry weight in biosolids collected from WWTPs in the Australia.[84] The concentration of TCS and TCC in biosolids collected from the Michigan WWTPs ranged from 90 – 7060 ng/g and 4890 – 9280 ng/g, respectively. In the same study concentration of TCS and TCC was also determined in biosolid applied soil samples which were collected over the period of two years, the concentration of TCS ranged within 0.16 – 1.02 ng/g and 1.20 – 65.10 ng/g for TCC.[85] Table 1.6 summarizes the occurrence of TCS and TCC studied by different research groups in biosolids and biosolids-applied soil.

Table 1.6 Occurrence of TCS and TCC in biosolids-applied soil and biosolids from various locations

Location	Matrix	TCS (ng/g)	TCC (ng/g)	Ref.
USA	Biosolids-applied soil	4.1-4.5	-	86
USA	Biosolids Biosolids-applied soil	90-7060 1.24-7.01	4890-9280 1.20-65.10	85
Canada	Biosolids	620-11550	2170-5970	88
Australia	Biosolids	90-16790	-	84
China	River sediment	1329	2633	103

1.5.4 Transformation of TCS and TCC in the aqueous environment

1.5.4.1 Methylation

Methyl triclosan is thought to be produced as a result of bio-methylation reaction of triclosan, which is more lipophilic than TCS and hence has a higher potential of bioaccumulation.[102] The concentration of methylated derivative of TCS was much lower than TCS when determined in wastewater from a sewage plant.[105] The concentration of TCS and methyl-TCS was measured in the lakes and river water in Switzerland. They found the concentration of TCS up to 74 ng/L and methyl-TCS concentration up to 2 ng/L.[102]

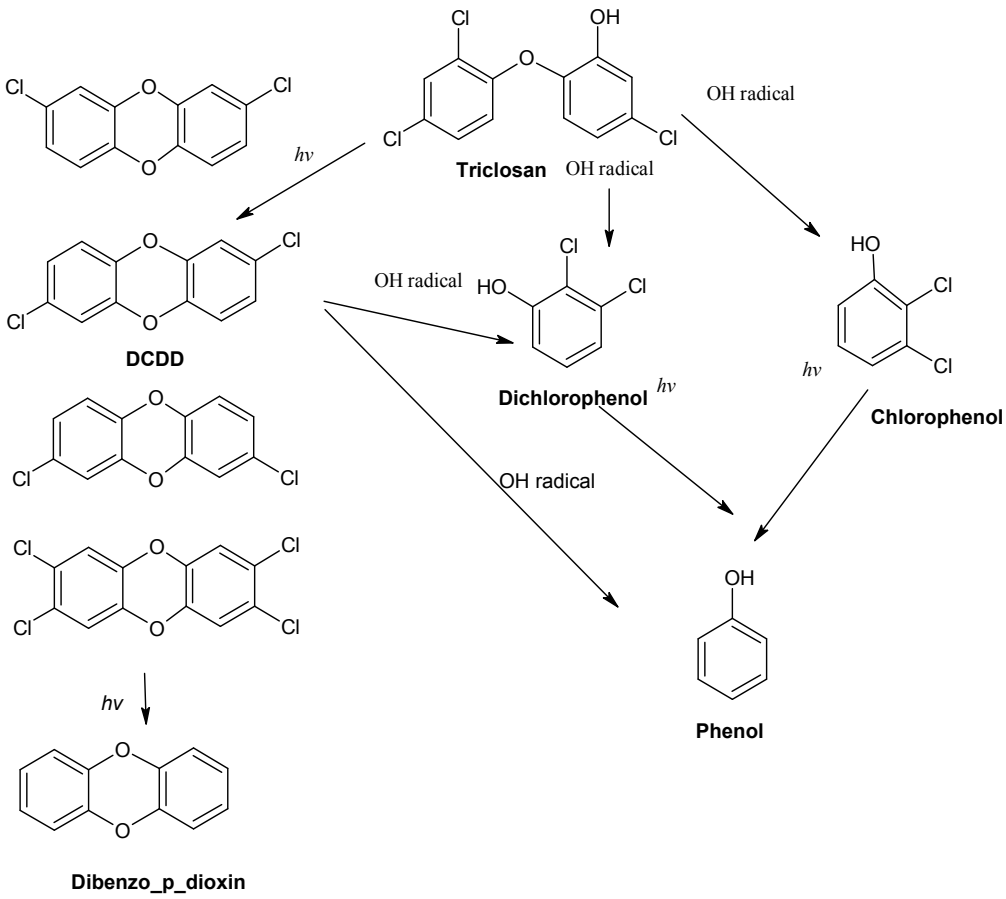


Figure 1.5 Photolysis and photo catalysis degradation kinetics of TCS.[91, 96, 106]

1.5.4.2 Photolysis

Triclosan itself undergoes photolysis reaction to produce 2,8-dichlorodibenzo-p-dioxin (DCDD) (Figure 1.5).[91] TCS when in it phenolate form is photodegradable, while is stable photolytically in its phenolic form. When TiO_2 was used as a catalyst in the photocatalysis reaction, dioxins were not produced instead phenol-type intermediates were formed.[96] A suggested transformation pathways for the photolysis and photocatalysis at 364 nm is shown in the Figure 1.5. Dioxin and phenol-type intermediates are highly toxic and show potential for bioaccumulation, a need for a degradation method producing environmentally less toxic intermediates is important. Photolysis at low intensity (365 nm) produces low numbers of $OH^{\bullet}$ radicals, which leads

to the production of a larger quantity of dioxin. While photocatalysis produces large number of $OH^{\bullet}$ radicals and as a result phenol- type intermediates are produced.

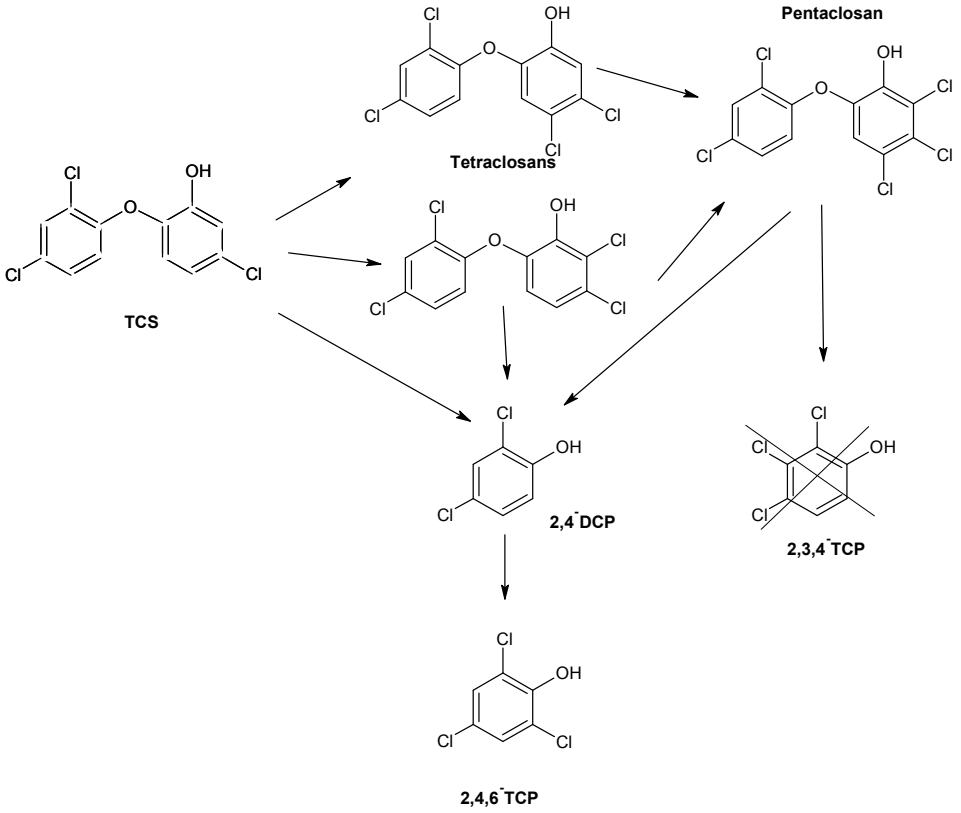


Figure 1.6 Degradation pathway of TCS when reacted with free chlorine.[37]

1.5.4.3 Chlorination

A study was done to address the presence of TCS in tap water which is usually chlorinated to bring water upto drinking standards. Reaction of chlorine with TCS lead to formation of five major chlorinated TCS products.[37] The reaction pathway, as described by the Canosa et al., is shown in the Figure 1.6. The group concluded that chlorination of the phenolic ring and breakage of the ether bond were main degradation pathways (Figure 1.6). The higher chlorinated TCS derivatives pentaclosan and tetraclosan are unstable products. Production of endocrine disrupters 2,4-DCP and 2,4,6-TCP was also

observed via photolysis.[37] The chlorinated derivatives produced during chlorination of TCS can undergo furthur tranformation to produce highly toxic chlorinated dioxins when exposed to heat or UV irradiation.[107]

1.5.5 Transformation of TCS and TCC in soil environment

The information regarding transformation of TCS and TCC in a soil environment is very limited.[78] Wu et al., studied transformation of TCS and TCC in soil and biosolids-amended soil. TCS and TCC were found to be biodegradable under aerobic conditions in soils and soil amended with biosolids. Half-life of TCS was calculated to be 20-58 days and for TCC the half-life was calculated to be 87-231 days. TCC showed less transformation in all the experimental set-up when compared to TCS. No transformation was observed under anaerobic conditions for either compounds.[108] Ying et al., studied the biological transformation of TCS and TCC in soil under aerobic and anaerobic conditions. They found that TCS and TCC transformed slowly under aerobic conditions, but were persistent during 70 days in anaerobic conditions. They also found that TCS and TCC did not show any negative effect on the microbial community of the soil. But when the soil was sterilized to kill all the microbial population in soil, no transformation was observed, which suggest the importance of biotic transformation of TCS and TCC in soil. Half-live of 18 and 108 days was calculated for TCS and TCC, respectively.[78] Xu et al., studied transformation of TCS in agricultural soils under a controlled laboratory environment. They found that within 16 days 50% of TCS has degraded in soil. They calculated the half-life of TCS to be 15.68 days in soil.[12] In another study soil bacteria *Pseudomonas putida* and *Alcaligenes xylosoxidans* subsp. *Denitrificans* were used to study transformation of TCS. Decrease in TCS concentration was observed with 6 and 24

h with both the bacterial cultures, complete reduction in TCS concentration happened within 96 and 228 hr of inoculation.[109]

1.5.6 Analytical methods for detection of TCS and TCC in environmental samples

To carry out studies related to fate and transformation of TCS and TCC, developing analytical methods capable of detecting TCS, TCC and other transformation products is necessary. The possible harm related with the exposure of these compounds to aquatic organisms has led to considerable research on detection and analysis of TCS; however, very few methods exist for analysis of TCC. This section gives an overview of the analytical method developed to detect TCS and/or TCC and their transformation products in environmental samples. Instruments usually employed for analysis of TCS, TCC or methyl-TCS in wastewater, biosolids samples are GC/MS, GC/MS/MS, LC/MS and LC/MS/MS.[97] Table 1.7 shows brief details about some methods that have been developed for analysis of TCS or TCC or both.

Cha et al., developed a method for determination of TCS and TCC in biosolids amended soil samples. The method employed pressurized liquid extraction (PLE) as their extraction method, ultra performance LC system coupled with MS detector using electrospray ionization for TCC and atmospheric pressure chemical ionization (APCI) for TCS. Recovery rates above 95 % were obtained and method detection limit (MDL) obtained for biosolids was 3.08 ng/g (TCC), 0.1 ng/g (TCS) and in soil samples was 0.58 ng/g (TCC), 0.05 ng/g (TCS).[85] The problem with this method is that TCS and TCC cannot be analyzed together because different ionization methods are used for TCC and TCS, which increases the cost of analysis of samples. In the method developed by Bester et al., sludge samples were first lyophilized and than extracted for 6h with soxhlet

extraction. The extract solvent was exchanged with toluene and the resulting extract was cleaned with silica SPE cartridges. The cleaned extract than was injected in gel perfusion chromatography (GPC)-column and the fraction containing TCS was collected. The samples solvent was again exchanged with toluene and finally analyzed using GC-MS. Recovery rates of 94% were obtained and the limit of quantification (LOQ) was determined to be 4 ng/g.[105] The developed method is very time consuming and involves many steps. The same group developed a method for analysis of triclosan and methyl-triclosan in surface water. Target compounds were extracted using liquid-liquid extraction (LLE) of 1L water samples with 10mL toluene. The organic phase was removed for water and residual water was removed by freezing overnight. The extract was evaporated and analyzed with GC-MS. The limit of quantification for TCS was 3 ng/L and for methyl-TCS 0.3 mg/L. The recovery rate of the method was between 88% - 100%.[99] The method developed can be used for analysis of TCS and methyl-TCS from surface water, this method would not work well with complicated matrixes such as soil or biosolids. Also the extraction requires the extract to be freezed overnight in order to remove water.

Zhao et al., developed a method for analysis of TCS and TCC in surface water and sediment of the Pearl River in China. The method called for extraction of water samples using SPE and sediment samples were extracted using ultrasonic extraction. Clean-up of the surface water and sediment extract was carried out using silica gel column. The final extract was evaporated and redissolved in methanol and analyzed using rapid resolution liquid chromatography-tandem mass spectrometry (RRLC-MS/MS) with ionization source electrospray ionization (ESI). Recovery rates of TCS were 106 and 110 % in surface water and sediment respectively, while for TCC it was 94 and 106 %

respectively. Limits of detection for TCS was 1.2 ng/L and for TCC was 1.2 ng/L in surface water and 0.6ng/g and 0.6 ng/g for TCS and TCC in sediment.[103] The disadvantage of this method is that it requires a good clean-up method to achieve sensitivity using instruments such MS/MS. Chu et al., developed a method for simultaneous determination of TCS and TCC in biosolids. ASE was used as the extraction method and the extract was concentrated, the obtained extract was clean with Oasis HLB cartridge. The clean extract was evaporated to dryness, reconstituted in methanol, and analyzed using LC-MS/MS using negative mode ESI. The method recovery rates were 97.7 and 98.3% for TCS and TCC respectively. The LOQ was determined to be 5.0 and 0.5 ng/g for TCS and TCC respectively.[88] The samples are extracted using ASE extraction hence a good clean-up method is required before the extract can be injected in LC-MS/MS.

Table 1.7 List of various analytical methods employed for analysis of TCS and TCC in the environmental samples

Sample	Extraction Method	Instrumental Method	Detection limit (ng/g)		Concentration detected		Ref.
			TCS	TCC	TCS (ng/g)	TCC (ng/g)	
Biosolids	Accelerated solvent extraction (ASE)	UPLC-ESI-APCI Triple quadrupole	Biosolids MDL:0.1	3.08	90 - 7060	4890 - 9280	85
Soil			Soil 0.05	0.58	<0.05 – 1.02	1.20 – 65.10	
Sewage sludge	Soxhlet extraction - SPE	GC - MS	LOQ : 4		1000-8000		105
Surface water	Liquid-liquid extraction (LLE)	GC-MS	LOQ: 3 ng/L		< 3 – 10 ng/L	7500-25,900	99
Sludge	ASE	LC-ESI-MS-MS Triple quadrupole			320		110
Biosolids	Ultrasonic extraction -SPE	LC-ESI-MS-MS Triple quadrupole	LOQ : 57				5
Marine sediment	ASE-solid phase extraction	GC-ESI-MS, LC-ESI-MS-MS	LOD: 3.50		0.27- 130.7		106
Digested sludge	Supercritical fluid extraction	GC-MS	LOQ : 70		500 – 15,600		81

Table 1.7 List of various analytical methods employed for analysis of TCS and TCC in the environmental samples. (Continued)

Sample	Extraction Method	Instrumental Method	Detection limit (ng/g)		Concentration detected		Ref.
			TCS	TCC	TCS (ng/g)	TCC (ng/g)	
Biosolids	LLE-SPE	GC-MS	LOQ : 5		90 – 16,780		111
Wastewater	SPE	HPLC-UV	MDL : 3.91 ng/L		533-774 ng/L		104
Surface water Sediment	SPE Ultrasonication-silica gel	Rapid Resolution LC-MS/MS Triple quadruple	LOD: 1.2 ng/L		478 ng/L 1329 ng/L	338 2633	103
Agricultural soil	ASE	LC-MS Triple quadruple	MDL :1.0		4.1 - 4.5		86
Biosolids	ASE – SPE	LC-ESI-MS-MS Triple quadruple	LOQ : 5.0	0.5	680 –11,550	3050 - 5970	88

MDL: Method detection limit, LOQ: Limit of quantification, LOD: Limit of detection.

1.6 Estrogenic hormones

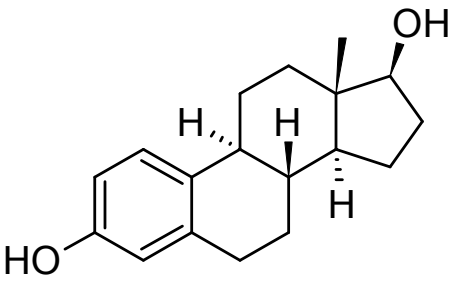
A study suggests that estrogenic hormones are detected in more than 20% of water streams in the United States.[7] The estrogens such as 17 β-estradiol (estradiol, E2), 16 α-hydroxy-17β-estradiol (estriol, E3), estrone (E1), testosterone and progesterone are naturally occurring estrogens and more biodegradable than synthetic estrogens.[112] Synthetic estrogens diethylstilbestrol (DES) and 17 α-ethinylestradiol are considered to be very potent EDCs and are more stable in water than natural estrogens. All steroid hormones have the same tetracyclic structure as their parent compound, which consist of a phenol, two cyclohexane and a cyclopentane rings (Figure 1.7). The difference in the structure arises at position C16 and C17 as can be seen with 17β-estradiol which has an upward facing –OH group to the molecular plane, while estriol has two –OH groups at C16 and C17 positions and estrone has a carbonyl group at C17 position.[113] Synthetic estrogens such as ethinylestradiol (EE2) and mestranols (MeEE2) are synthesized by addition of ethinyl and methyl groups to estradiol. These synthetic estrogens are the main ingredients in formulations for contraceptive pills.[28] Estrogenic compounds can be classified in two categories as maintained earlier, but their source of origin is not necessarily animals and humans.

Natural estrogens include:

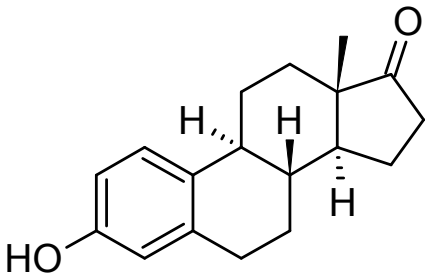
- Steriod hormones such as estrone, 17β-estradiol, estriol, testosterone and progesterone are naturally occurring hormones produced by humans and animals.[28]
- Phytoestrogens, are hormone like chemicals produced by plants.[114]

Synthetic estrogens include:

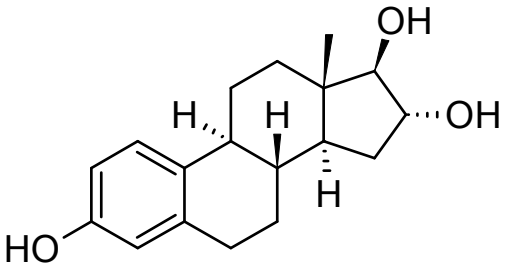
- Artificially produced hormones that are used in contraceptive pills, additives in animal feed and hormonal therapy includes; EE2, diethylbestrol and mestranol.
- Xenoestrogens are synthetic compounds that exhibit estrogenic activity, these compounds include pesticides (dieldrin, DDT) , plasticizers (bisphenol A), paints and detergents and several others compounds produced by breakdown of compounds such as nonylphenol.[28]



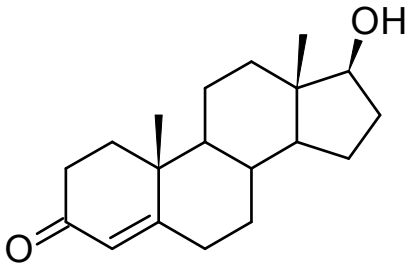
a) 17β-estradiol (E2)



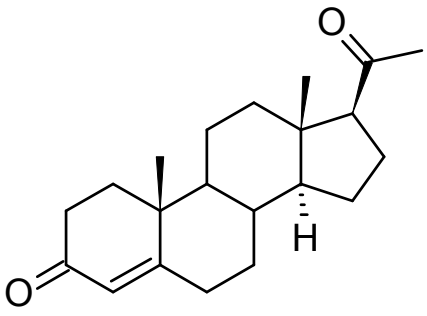
b) Estrone (E1)



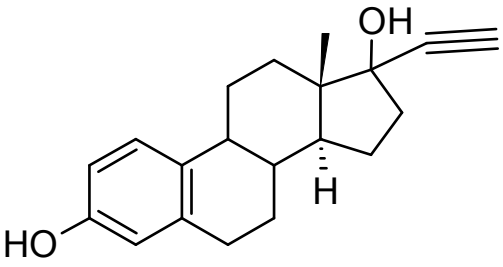
c) Estriol (E3)



d) Testosterone



e) Progesterone



f) 17α-ethynylestradiol (EE2)

Figure 1.7 Molecular structure of natural and synthetic steroid hormones.

1.6.2 Toxicity of estrogenic hormones

Estrogen steroid hormones are a class of endocrine-disrupting chemicals (EDCs) that can bind to estrogen receptors in cells, which can lead to abnormal hormone secretion and diseases such as abnormal development, reproductive disorders, reduced male sperm counts, as well as increased chances of testicular, prostate and breast cancer. [115-117] Studies show that parts per trillion (ppt) levels can have a significant effect on male fish reproductive system, and they can start producing female-specific egg protein [118]. When amphibian embryos were exposed to 25 μmol/L E2, 100% embryos died within 48 hrs of exposure.[119] Studies have shown that when rats are exposed to hormones during prenatal or early postnatal life, permanent modifications takes place in endocrine and reproductive organs. Male and female organs were affected equally.[120] There has been several reports of male fish producing female specific egg protein called vitellogenin, when the male fish were exposed to WWTPs effluents, which suggest the presence of estrogenic compounds in the effluents.[121] Studies have also shown alterations in the endocrine reproductive system of birds, reptiles and mammals.[25] There is no study to prove that there is a direct relationship between estrogen hormones and human health, but minimal evidence are pointing towards a possible connection.[121]

1.6.3 Occurrence and fate of estrogenic hormones

Humans, wildlife and animals naturally synthesize and excrete endogenous estrogens in urine [118]. Free estrogens undergo catabolism in liver where they are conjugated by substitution at C3 and/or C17 position with sulfate, glucuronide, to produce conjugated estrogens.[28] The steroid hormones are usually excreted in free or conjugated form, the conjugated form can undergo biotransformation in the environment to form free estrogens.[122] A cycling women can produce somewhere between 10 – 100 μg

of E2, EE2, E1 and E3 and pregnant women can release up to 30 mg of estrogen mostly E3 daily.[34] Total daily estrogen concentration of a calve producing estrogens can be up to 45 µg/day, while a cycling cow can produce about 299 µg estrogen per day and bulls can produce 540 µg estrogen per day.[123] About 90% of the endogenous hormones in US are excreted by pregnant and cycling cows. The estrogen production in the European Union and the USA as calculated by Lange et al., is 49 tons and 33 tons per year respectively.[123] Studies suggest that the two main sources leading to hormone contamination in the environment are WWTPs and concentrated animal feeding operations (CAFOs).[124] Application of animal waste generated as a result of CAFOs to agricultural land can lead to loading of estrogen and its metabolites in agricultural fields.[116] In municipal waste water treatment plants (WWTP's) , 90% or greater amounts of the natural estrogens are removed from effluents during various treatment processes.[118] The effluent from the WWTPs is usually allowed to flow in the surface water sources such as streams, rivers and estuaries, since WWTPs are only approximately 90% effective in removing estrogens from treated water, our water resources are bound to have trace amounts of estrogen hormones. Onces in the surface water the concentration of these estrogens should go down even further, due to various processes such as dilution, degradation and sorption.[125] The fate of these compounds in aquatic environments is highly dependent on the physicochemical properties of these hormones. The physical and chemical properties of several estrogenic hormones are listed in Table 1.8. As shown in Table 1.8 the vapor pressure of the compounds suggest that these compounds are not volatile hence they would stay in water. Kow is the measure of partition of the compound in the water and organic phase. Higher Kow suggest that the compounds are hydrophobic and would partition in organic matter such as sediment or natural organic matter.[126]

Table 1.8 Physical and chemical properties of the natural and synthetic estrogen.[28, 125]

Compound	Molecular weight (g/mol)	Water Solubility (mg/L at 20^0C)	Vapor pressure (mmHg)	Log K_{ow}	Log K_d
Estrone (E1)	270.4	13	2.3×10^{-10}	3.43	2.44-2.72
17β-Estradiol (E2)	272.4	13	2.3×10^{-10}	3.94	2.68-2.83
17α-Estradiol (E2α)	272.4	3.9	2.25×10^{-9a}	3.57	NA
Estriol (E3)	288.4	13	6.7×10^{-15}	2.81	NA
17α-Ethinylestradiol (EE2)	296.4	4.8	4.5×10^{-11}	4.15	2.65-2.86
Testosterone[b]	288.4	18-25	NA	2.9	3.2-4.8
Mestranol (MeEE2)	310.4	0.3	7.5×10^{-10}	4.67	NA

NA: data not available, a: taken from reference [113], b: taken from reference [80]

Summary of a few studies reporting occurrence of estrogens in the environment is listed in Table 1.9.

Zheng et al., detected three estrogenic hormones E2, E2α and E1 in dairy wastewater and lagoon water, the concentrations of these compounds in solid waste ranged from parts-per-billion to parts-per-million, in lagoon wastewater the concentration ranged in parts-per-trillion.[124] Concentrations of hormones were determined in sewage collected from German municipal sewage treatment plants (STP), two hormones E2 and E1 were detected at a concentration of 0.015 μg/L and 0.027 μg/L respectively. The removal efficiency of STP for E2 and 16α-hydroxyestrone was higher compared to EE2 and E1. In the same study in the Canada and Germany E1, E2, EE2 and 16α-hydroxyestrone were detected frequently in the STP discharge. The concentrations of these compounds ranged within the low ng/L level and highest concentration detected was for estrone which was 70 ng/L.[29] Xu et al., determined the concentration E2 in WWTPs effluent and influent using YES assay.[127] Zhang et al., measured the concentration of estrogenic hormones in effluents from WWTPs, E1, E2, EE2, and 16α-OH-E1 were detected at, upstream , downstream and sewage outfall.[128] Kuch et al., developed a method for analysis of estrogens in wastewater, surface and drinking water. They detected E1, E2, E2α and EE2 in WWTPs effluents at concentration range of 4.5 – 18 ng/L. In river water the concentration of the hormones ranged at 2.0-5.1 ng/L and in drinking water it was 0.30-2.1 ng/L.[129] Xiao et al ., analyzed the concentration of estrogens in environmental water and treated effluents in UK. They found that estrone was the most persistent among all other hormones with concentrations ranging 6.4-29 ng/L in effluents and 0.2-17 ng/L in the River Thames water.[130] A method was developed by Fine et al., for positive identification and quantification of estrogen in swine lagoon

samples , E1, E2 , E3 were detected in swine lagoon, their concentration ranged from 19 – 25,700 ng/L.[112]

Table 1.9 Occurrence of estrogenic hormones in the environment

Location	Matrix	Hormones detected	Concentration	Ref.
USA	WWTPs-effluent Influent	E2	4.2 3.7 ng/L	127
UK	WWTPs effluent	E1, E2, EE2, 16α-OH-E1	0.3-41 ng.L	128
USA	Dairy Solid waste	E1, E2 ,E3, E2α, progesterone	76 – 2103[a] µg/kg	124
Germany	WWTPs effluents	E1, E2	15, 27 µg/L	29
Canada	WWTPs effluents	E2, EE2, 16α-OH-E1	42-64 µg/L	
Germany	River water	E1	1.0-1.6 µg/L	
Germany	WWTPs effluent River water Drinking water	E1, E2, E2α, EE2	4.5 – 18 ng/L 2.0-5.1 ng/L 0.30-2.1 ng/L	129
UK	River water	E1, E2, E3, EE2	0.2 – 17 ng/L	130
	WWTPs effluent	E1, E2, E3, EE2, 4-OH-E1, 2-OH-E1, 2-OH-E2	6.4– 29 ng/L	
USA	Swine lagoon	E1, E2, E3	19 – 25,700 ng/L	112

a- Given concentration is sum of all hormones

1.6.4 Methods for analysis of estrogenic hormones

The concentration of estrogenic hormones detected in the environment is in ng/L, which makes analysis and detection of these compounds difficult. Commonly employed methods for detection of estrogens in biological samples include bioanalytical assays such as (YES, ELISA and E-screen). Quantitative and qualitative analysis in the environmental samples usually employ chromatographic techniques such as LC-MS/MS, GC-MS, LC-florescence and so on.[131]

Analytical method development for analysis of estrogens in wastewater and other matrixes is challenging because of the complexity of the matrix and because of the required low detection limits (~ 1 ng/L). The concentration of these compounds found is in ng/L, but it is high enough to cause adverse effect on the endocrine system of living organisms. Hence the methods usually developed for analysis of estrogens are time consuming, labor intensive, costly and complicated.[132] The following section discusses a few of the many analytical methods developed for analysis of estrogens so far.

Benijts et al developed a method for simultaneous determination of 35 EDCs compounds, including hormones such as E2, E1 and EE2 in the environmental water. The sample extraction and clean-up was achieved with wide spectrum SPE (Oasis HLB) cartridges, and the sample analysis was done with positive and negative ESI with LC/MS/MS. Stable isotopes-labeled standards were added as internal standards to compensate for the lose of analyte during extraction and matrix effect. Recovery rates of 80% to 110% were observed , and quantitation limits ranged between 0.1 to 20 ng/L.[133]

Komori et al., described an analytical method for extraction and analysis of several free and conjugated estrogens from influent and effluent of secondary settling tanks in WWTPs. The extraction and clean-up method used SPE with Oasis HLB,

supersonic liquid extraction for suspended matter followed by two clean-up steps and the cleaned up extract is than analyzed by LC/MS/MS. The detection limit for this method ranged between 0.1 – 1.4 ng/L.[35]

Liu et al., developed a method for extraction of endocrine disrupting chemicals (EDCs) including 17β-estradiol, estrone, 17α-ethynylestradiol, 16α-hydroxyestrone, 4-nonylphenol, 4-*tert*-octylphenol and bisphenol A in river sediments. They used microwave-assisted extraction (MAE) as their extraction method followed by silica gel clean-up and GC-MS analysis. The target compounds were derivatized with BSTFA prior to GC-MS analysis. The limit of detection for the method ranged between 0.2 – 1.0 ng/g.[134] Hibberd et al., also developed similar methods for the simultaneous analysis of phenolic and steroidal estrogens in water and sediment using MAE for sediment samples and SPE for aqueous samples. The BSTFA derivatized compounds were analyzed with GC-MS/MS when the limit of detection ranged from 0.01 to 0.49 ng/L in water and from 0.05 to 0.14 ng/g in sediment.[135]

Liu et al., developed a method for simultaneous analysis of free and conjugated estrogen in wastewater. Hydrophilic-lipophilic balance solid phase extraction (Oasis HLB SPE) method was used for separation of free estrogens and their conjugates. And the conjugates were hydrolyzed using enzyme into their free forms. The free estrogens were derivatized with N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) prior to GC-MS analysis. The method recovery rate for most of the analytes ranged from 73.3 -114.9%. They used the method to detect free estrogens and their conjugates in influent and effluent, they found that concentration of E1 was highest at 44 ng/L in the effluent.[136]

Penalver et al., developed a method for analysis of estrogenic compounds in water samples using solid-phase microextraction (SPME)-HPLC-UV-electrochemical detection (ED). For SPME extraction of water samples polyacrylate fibers (85 μm) were used. Limit of detection for E2 was found to be between 0.3 -1.1 μg/L with UV, while the LOD was between 0.06-0.08 μg/L with ED. The concentration of E2 was determined using this method in wastewater samples which was found to be in between 1.9 – 2.2 μg/L.[137] Carpinteiro et al., developed a method for five estrogens in water samples based on SPME extraction, but they also included on-fiber derivatization of estrogens with MSTFA for GC-MS analysis. The detection limits obtained with this method ranged from 0.2-3 ng/L. The developed method was successfully used for analysis of estrogens in sewage water.[138]

Biosensors are also used for detection of EDCs in environment, Rodriguez-Moza et al., developed an optical immunosensor prototype called RIANA, for simultaneous determination of atrazine, isoproturon and estrone in river waters. The immunosensor is based on rapid fluoroimmunoassay on an optical transducer chip. The transducer chip is chemically modified with derivatives of the target compounds in discrete location. The achieved detection limits with the immunoassay were 0.155, 0.046, and 0.084 μg/l, for atrazine, isoproturon, and estrone, respectively.[139] Schneider et al., developed an immunoassay method for detection of EE2 in water and wastewater samples using an alternative approach. In this approach a long chain biotinylated EE2 derivative was used in a direct competitive ELISA. The limit of detection was found to be 14 ng/L, and cross reactivity issues were observed among metabolites of EE2, the presence of humic acid causes an overestimation of EE2 concentration.[140]

Leusch et al., compared five in vitro bioassays namely the yeast estrogen screen (YES), ER-CALUX, MELN, T47D-KBluc, and E-SCREEN assays for measuring estrogenic activity in environmental waters. The YES assay is based on activity of β-galactosidase of yeast strain which is expressed after being in contact with estrogens. A yellow chromogenic substrate CPRG (chlorophenol red β-galactopyranoside) is added to the assay and is transformed to red product because of activity of β-galactosidase which is than measured with a spectrophotometer at 540 nm. The ER-CALUX, MELN, T47D-KBluc assay are based on the expression of the estrogen receptor gene and detection of luciferase activity by luminescence. In E-SCREEN assay MCF-7BOS breast cancer cells are seeded and incubated with a compound of interest, and after 5 days number of live cells are determined with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In the study YES assay had the highest method quantification limit of 3.5 and 5 ng/L, the other bioassays had lower method quantification limits of 0.27, 0.2, 0.2, and 0.1 ng/L for MELN, KBluc, E-SCREEN and ER-CALUX respectively. Out of all the assays ER-CALUX was able to detect estrogenic activity in 97% samples.[141]

A summary of various analytical methods developed for analysis of estrogens in environment is listed in Table 1.10.

Table 1.10 List of analytical methods developed for analysis of estrogen in environmental sample

Sample	Extraction & Clean up Method	Instrumental Method	Detection limit	Hormones Detected	Concentration	Ref.
Water	SPE	LC-Ion Trap-MS/MS	LOQ : 0.1 – 20 ng/L	Estrone	21.7 ng/L	133
Wastewater	SPE Supersonic liquid extraction	LC/MS/MS ESI	MDL: 0.1 – 1.4 ng/L	E1, E2, EE2, E3, E1-S, E2-S, E3-S, E1-G, E2-G, E2-G	0 – 100 ng/L	35
Sediment	MAE, Sonication Silica Gel	GC-MS Ion trap	LOQ : 0.5 – 1.4 ng/L	E1, E2, EE2, 16α-OH-E1	0-12 ng/g	134
Wastewater	SPE	GC-MS	LOQ : 5.4 -6.2 ng/L	E1, E2, EE2, 16α-OH-E1	3.2- 44 ng/L	136
Wastewater	Solid-phase microextraction (SPME)	HPLC-UV-Electrochemical detection (ED)	LOD :0.3-1.1 µg/L 0.06-0.08 µg/L	E2	1.9 – 2.2 µg/L	137
Water	SPE	Immunoassay	LOD : 0.084 µg/L	E1	-	139
Surface water Drinking water	Online SPE	LC/MS/MS Triple quadrupole	LOQ: 0.02 -1.02 ng/L	E2, E1, E3,E2-17G, E1-3S, EE2, DES	0.22 – 0.68 ng/L	142

1.6.5 Transformation of estrogens in the environment

1.6.5.1 Chlorination

Deborde et al., studied the chlorination of E1, E2, E3 and EE2 and concluded that chlorination products at the beginning 10 mins of chlorination do not show reduced estrogenicity. Further chlorination causes the ring opening of the estrogen hormones leading to formation of products that show less estrogenic activity than parent compounds.[143] All the studies suggest that further understanding of the risk associated with by-products produced by chlorination of estrogenic hormones should be investigated. [36, 144] A pathway proposed by Hu et al., for chlorination of E2 with HOCl is shown in Figure 1.8.

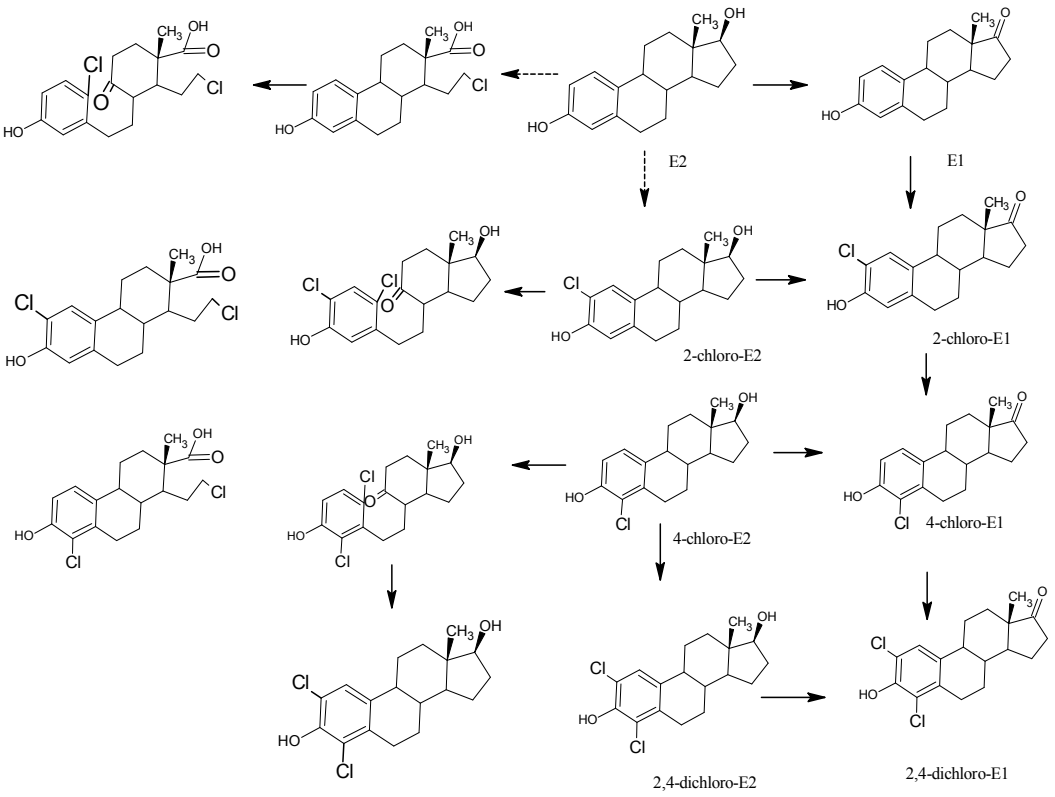


Figure 1.8 Proposed pathway for chlorination of E2 using HOCl.[144]

1.6.5.2 Photolysis

Direct photolysis of E2 and E1 was carried out by Liu et al., they studied that E2 undergoes photolysis reaction at 254 nm wavelength while E1 can be degraded photolytically at 365 nm. The rate of photolysis decreases with increasing initial concentration of hormones, pH values also play a significant role in photolysis; the lowest degradation rate was observed between pH 4-5.[145] Rosenfeldt et al., studied the degradation of E2 and EE2 using UV advanced oxidation process. They used UV light by itself and in combination with H_2O_2. No intermediate products were identified in the study, but they tested the estrogenic activity of the oxidation product solution. They found that both UV and UV/H_2O_2 degrades E2 and EE2, with UV/H_2O_2 having higher removal efficiency greater than 90%. They concluded that if there is slight estrogenic

activity associated with oxidation products it is still significantly less than the parent estrogens.[146] Leech et al., studied the effect of natural organic matter and sunlight on the degradation of E2. The simulated sunlight (290-700nm) was able to degrade 26% of E2 after 6h of exposure, the presence of natural organic matter increased the degradation rate to 50%, when 15mg/L of humic acid was added. The degradation rate decreased to 20% when 2-propanol, a free radical scavenger, was added.[147] The difference in the transformation rates observed in above studies can be due to initial concentration used, Rosenfelt et al., used 3 μg/L while Leech et al., used 0.27 mg/L.

In the study by Zang et al., degradation of EE2 was studied in aqueous solution by UV and UV/H_2O_2. They found that the degradation rate was dependent on the concentration of EE2, H_2O_2 and UV intensity. The UV radiation partially removed EE2, but H_2O_2 itself was not able to oxidize EE2.[148]

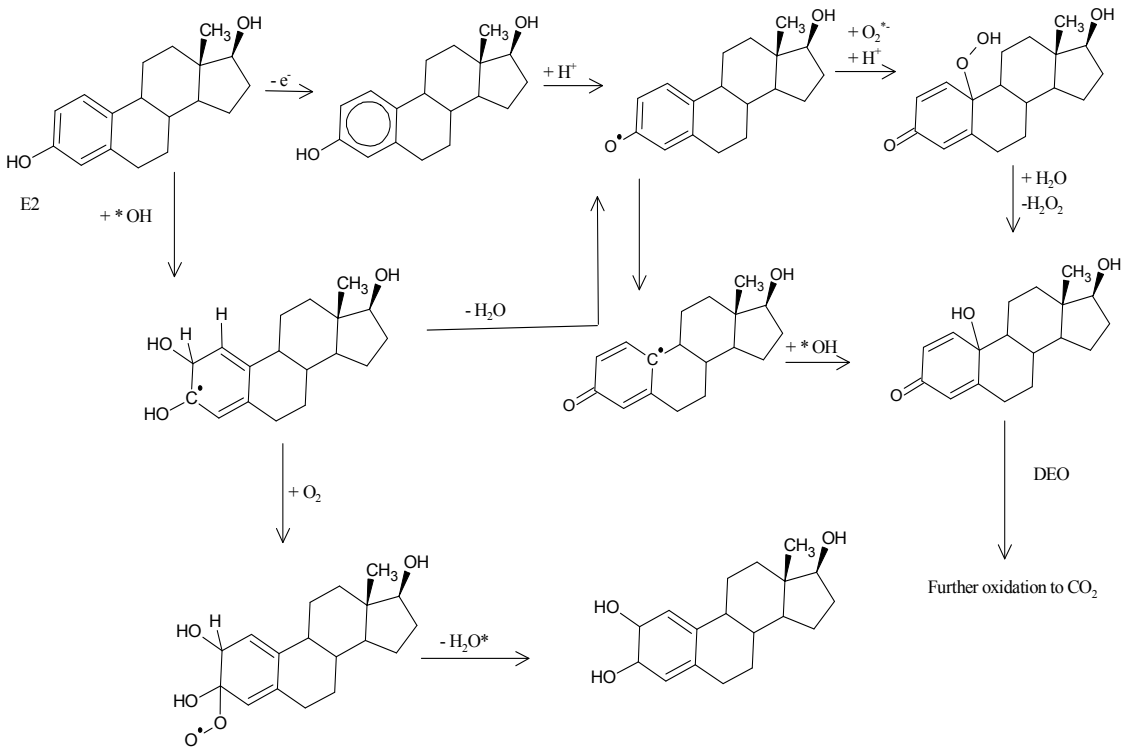


Figure 1.9 Predicted pathway for degradation of E2 by TiO_2.[149]

Ohka et al., studied the degradation of E2 by TiO_2 photocatalysis. Their results predicted complete mineralization of 10^{-6} M E2 into CO_2 with 1.0g/L TiO_2 suspension under UV irradiation for 3 h. A mechanism was also proposed for mineralization (Figure 1.9). According to the study they concluded that the phenol group on the benzene ring should be the site for initiation of photocatalytic oxidation. This phenol group is responsible for binding with estrogen receptor, hence its activity should be lost upon initiation of the photocatalytic degradation.[149] In another study by Mitamura et al., degradation of 3-hydroxy conjugated estrogens was compared with estrogens that where not conjugated at 3-hydroxy position, TiO_2 was used as photocatalysts. They found that 3-hydorxy conjugated estrogen degraded at slower rate than estrogens which were not conjugated at 3-hydroxy position. This study supports the hypothesis that hydroxyl

radicals generated during irradiation of TiO_2 with UV light oxidizes the 3-hyroxy group of estrogen , which is the initiation of photodegradation reaction.[150]

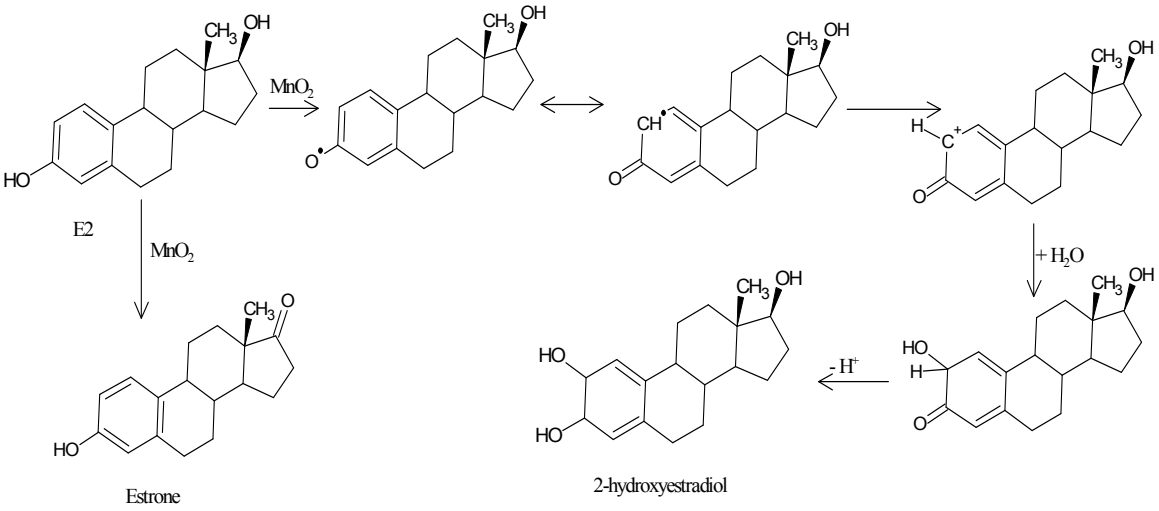


Figure 1.10 Possible degradation pathway of E2 by MnO_2 as proposed by Jiang et al.[151]

Jiang et al, studied the degradation of E2 with MnO_2, they found that 90% of E2 can be oxidized by MnO_2 within 8h. The proposed E2 transformation pathway using MnO_2 is shown in Figure 1.10. The reaction followed first-order kinetics for the first 2h, after which the reaction progresses slowly. They also identified two intermediate products estrone (E1) and 6-hydroxyestradiol. On the basis of previous studies based on TiO_2 and obtained products, a degradation mechanism was proposed as shown in Figure 1.10.[151] In another study by Xu et al., oxidative degradation of E1, E2, E3 and EE2 was performed with MnO_2, they found 20% of all the estrogens were removed within first 20 min of the treatment, and complete degradation took place in 220 min. No mechanism or oxidation products were identified.[116]

1.6.5.3 Fenton and photo-Fenton

Feng et al., studied photo-Fenton like degradation of E2 in water. They found that, when only H_2O_2 was used with UV irradiation, only 29.6% of E2 had degraded after 160 min, while when only Fe (III) was used with UV irradiation 15.9% E2 was degraded after 160 min, but when Fe (III)/H_2O_2/UV-vis was combined together, after 160 min the degradation rate went up to 61.7% which was more than combined sum of the binary systems. They found that the pH also played important role in the degradation process. At pH 3, Fe (III) existed in the $Fe(OH)^{+2}$, which is the most potent form of Fe (III). When the pH changed to 3.7, Fe (III) was present as $Fe(OH)_3$, which decreases the concentration of available Fe (III) for the AOPs. The degradation rate increased with concentration of H_2O_2, but mineralization efficiency stayed low at 21.6%, from this they concluded that mineralization only occurred at the aromatic ring and the alicyclic rings were not destroyed.[152] The same group also studied the degradation of E1, EE2, E2 and DES, 14.2 % E1 degraded during 160 min with UV/H_2O_2, 38.6 % degraded with UV/Fe(III) and 75.4 % degraded when Fe(III)/H_2O_2/UV-vis was used as a degradation method. They found that DES degraded fastest of all, followed by E2, EE2 and E1 respectively.[153] Yaping et al., studied degradation of E2 with heterogeneous photo-Fenton catalyst, they prepared β-FeOOH loaded resin which was used along with H_2O_2 and weak UV irradiation. They found that the estrogenic activity of E2 went from 1511 units to almost zero after 22 h of UV irradiation over β-FeOOH/resin. The reduction in activity of E2 was directly proportional to the concentration of E2 degraded, which went down from 272 µg/L to 0.23 µg/L after 22 h of irradiation.[154]

1.7 Research objectives

The research was focused around three main objectives:

1.7.1 Occurrence and detection of triclosan and triclocarban in biosolids applied soil and biosolid samples using HPLC/ESI/MS

Very few methods exist for simultaneous measurement of both TCS and TCC in biosolids and biosolids-applied soils. Compared to water samples, the matrixes for biosolids and soil samples is more complex due to higher contents of organic matter and soil minerals. Both organic matter and soil minerals in the matrixes can result in significant interferences during extraction, clean-up, and analysis of target analytes. Therefore, the methods developed for water samples may not work well for biosolids and soil samples. The objective of this study (Chapter II) was to develop a sensitive LC/MS method for simultaneous extraction and determination of TCS and TCC in biosolids and biosolids-applied soil and study the occurrence of TCS and TCC in the environment.

1.7.2 Molecularly imprinted solid phase extraction for TCS and TCC analysis

Tailor made sorbents for selective extraction/clean-up of analytical compounds are gaining popularity. Complex matrices such as biosolids are not easy to clean, while sophisticated instruments such as LC/MS are sensitive, but at the same time, they are also sensitive to interferents. A good clean-up method is always in the order. So an attempt to develop molecularly imprinted polymer for analysis of TCS and TCC was made (Chapter III). The prepared MIP was used for clean-up of real biosolids - applied soil and biosolids samples and compared with the C-18 SPE clean-up method.

1.7.3 Oxidative transformation of estrogenic hormones

The main objective of this study was to determine the oxidative transformation of several estrogens in aquatic systems (Chapter IV). The oxidative transformation of 17β-estradiol, estrone, EE2, estriol and testosterone was studied. A house made catalysts Fe (III)-saturated montmorillonite was used and compared with other minerals such as Fe_2O_3 and MnO_2. Furthermore, the influence of organic matter on transformation of E2 was evaluated by using lake water matrix. The effect of Fe (III) as catalysts with different anions was compared with Fe (III)-saturated montmorillonite and Na-saturated montmorillonite. The influence of $NaNO_2$ on the oxidative transformation of E2, EE2 and E3 was studied. Oxidative transformation of E2 was also studied at different pH values. UV light was used for some of the compounds to compare the influence of UV light on the transformation of the selected hormones. Attempts were made to identify the transformation products using LC/MS whenever possible.

CHAPTER II

DETECTION OF TRICLOSAN AND TRICLOCARBAN IN BIOSOLIDS APPLIED SOIL AND BIOSOLID SAMPLES USING HPLC/ESI/MS

2.1 Introduction

Understanding the occurrence and fate of TCS and TCC helps better predict the potential exposure of living organisms. Research is required to confirm that the concentration of TCS and TCC in biosolids and biosolids-applied soil samples detected by other groups are true representatives of biosolids and biosolids-applied soil all over the country.[155] The composition and application of biosolids as fertilizers is controlled by regulations, such as suitable weather and soil conditions during application, appropriate application methods and rates, and biosolids composition. But still it needs to be determined if the current regulations are good enough to protect surface and ground water resources which can get contaminated by runoff water during rains from land amended with contaminated biosolids.[156] A study by Lapen et al showed that triclosan can be transported in runoff from an agricultural field amended with biosolids and triclosan can persist in runoff for up to 266 days after application of biosolids.[156] Therefore it is important to measure the concentrations of TCS and TCC in biosolids and biosolids applied soil.[88]

2.2 Analytical method development

Analytical method development consists of several collective steps. The flow chart in Figure 2.1, shows the major steps involved in method development.[157] The first

step is sample collection, which is usually done outside the laboratory, and involves collecting water samples from rivers, streams, lakes, collecting animal or plant samples, soil samples etc. As shown in Figure 2.1, the next step in method development involves sample pretreatment, extraction and removal of interference. During this step, aqueous samples are usually extracted using LLE or SPE. In LLE, aqueous samples are extracted using immiscible solvents and the organic layer is collected. Extraction with organic solvent is repeated several times until complete removal of the target compound is achieved. In SPE of aqueous solvents, a large volume of aqueous solution is passed through a bed of sorbent (C18 in case of RP) and extracted using solvents with reduced polarity. Solid samples need to be extracted first before using SPE or LLE. Extraction techniques such as ASE, soxhlet, sonication, and ultrasonication are widely employed for extraction of solid samples.[158] After extraction, the extract can be cleaned using SPE and LLE as used for aqueous samples. Finally the collected extract after cleanup is evaporated and redissolved in the solvent appropriate for instrumental analysis. Commonly employed instruments in analytical method development are LC-MS, GC-MS and LC-UV.[88, 159]

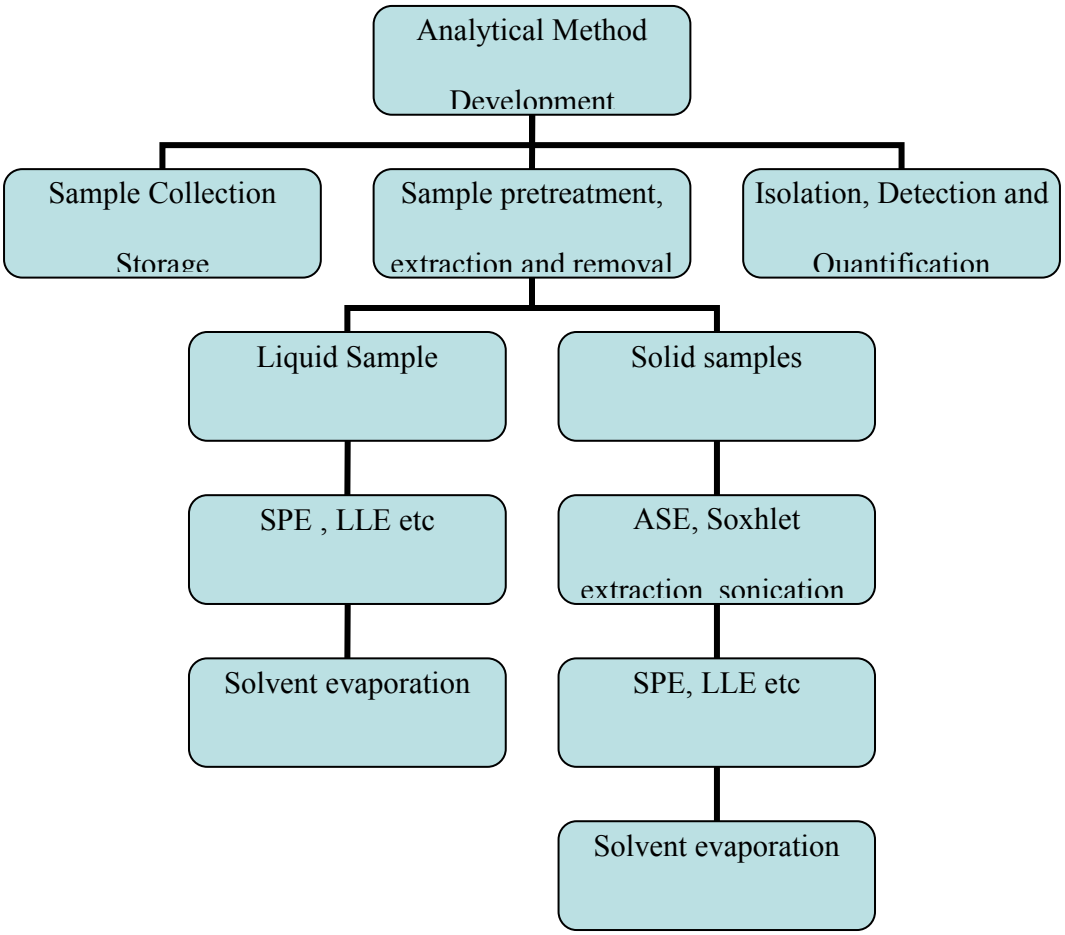


Figure 2.1 Organization chart showing different steps in the analytical method development process [157]

2.3 Analysis of triclosan and triclocarban

The analysis of TCS and TCC from various environmental matrices is a challenging process, because of the complexity of the matrix such as wastewater, soil, biosolids, tissues etc., thus complicated, time- and labor-consuming analytical procedures are usually required for accurate determination of these compounds from their respective matrixes.

Analytical methods have been developed for determining concentration of TCS in foodstuff using reversed phase liquid chromatography coupled with ultraviolet detection [98, 160], and in cosmetics and pharmaceutical products using liquid chromatography method

with a refractive index detector.[83] Several methods have been developed for determining TCS in water, sludge, and sediment samples using liquid chromatography mass spectrometry (LC/MS) or gas chromatography/mass spectrometry (GC/MS). Most of the GC/MS methods using electron impact ionization require derivatization of TCS to its trimethysilylethers derivatives before analysis, generating various recovery rates from as low as 36% [81] to as high as around 100 % [84]. Compared to GC/MS method, the LC/MS is easier because it does not require the extra step of derivatization.

The LC-MS/MS method developed by Chu and Metcalfe provides mean recovery of 98±6% for both TCS and TCC-spiked biosolids and method detection limits of 1.5 and 0.2 µg/kg for TCS and TCC, respectively.[88] For the method used by Halden and Paull et al., recovery rate of 95±9% was achieved with method detection limits ranging from 3 to 50 ng L^{-1} depending on the matrix.[81, 82, 88] Recovery rates of 79±21% and 84±21% were achieved for TCS-spiked wastewater and sewage sludge, respectively, with limits of quantitation at 100 ng/L for the wastewater and 70 µg/kg for the sewage sludge for method developed by McAvoy et al.[81]

Few methods have been found for simultaneous measurement of both TCS and TCC in biosolids and biosolids-applied soils. Cha et al., developed a method for determination of TCS and TCC in biosolids amended soil samples. The method employed pressurized liquid extraction (PLE) as their extraction method, Ultra performance LC system coupled with MS detector using electrospray ionization for TCC and atmospheric pressure chemical ionization (APCI) for TCS. Recovery rates above 95 % were obtained and method detection limit (MDL) obtained for biosolids was 3.08 ng/g (TCC), 0.1 ng/g (TCS) and in soil samples was 0.58 ng/g (TCC), 0.05 ng/g (TCS).[85] The problem with this method is that TCS and TCC cannot be analyzed together because

different ionization methods are used for TCC and TCS, which increases the cost of analysis of samples. Chu et al., developed method for simultaneous determination of TCS and TCC in biosolids. ASE was used as the extraction method and the extract was concentrated, the obtained extract was clean with Oasis HLB cartridge. The clean extract was evaporated to dryness, reconstituted in methanol, and analyzed using LC-MS/MS using negative mode ESI. The method recovery rates were 97.7 and 98.3% for TCS and TCC respectively. The LOQ was determined to be 5.0 and 0.5 ng/g for TCS and TCC respectively.[88] The developed method uses expensive instruments such as ASE and LC-MS/MS which increases the cost of sample analysis. Moreover such instruments are not available for routine use in every environmental laboratory. There is scarcity of information regarding concentrations of TCS and TCC in soil receiving continuous and long-term application of biosolids.[161]

Current methods employed for analysis of TCS and TCC uses expensive instruments such as LC-MS and GC-MS. Extraction methods such as ASE and SPE employed for TCS and TCC extraction from soil and biosolids samples are expensive and time consuming. To study environmental occurrence and fate of TCS and TCC, a sensitive analytical method is needed to detect their concentrations in complicated environmental matrixes at trace levels.

2.4 Methods

2.4.1 Chemical and Reagents

- Acetonitrile, formic acid and acetone- LC grade (Fisher Scientific, Fair Lawn, NJ, USA).
- LC purity water. – Milli-Q purification unit (Millipore Bedford, MA)

- Reference standards. – Triclosan (CAS no.3380-34-5) and Triclocarban (CAS no. 101-20-2) were purchased from Fluka Biochemika and Aldrich respectively. TCS had a purity of 97%, while TCC was 99% pure.
- LC mobile phase. – Acetonitrile and water containing 0.1 % formic acid was used.

2.4.2 Apparatus

- LC system. - The Waters Alliance 2695 separations module (Waters, Milford, MA, USA) is equipped with quaternary, low pressure mixing pump and inline vacuum degasser and a photodiode array detector with a wavelength range of 190-800 nm.
- UV detector. – Model 2990 (Waters, Milford, MA, USA)
- MS detector. – Waters Quattro-micro mass spectrometer (Micromass, Manchester, UK) is a triple quadrupole mass spectrometer equipped with electrospray ionization source (ESI).
- Analytical Column. – Reversed Phase Xterra, 5um, 4.6 × 150 mm id (Waters).

2.4.3 Standard solutions

Primary stock solution containing both TCS and TCC was prepared in acetonitrile (ACN) at a concentration of 1000 mg/L. The stock solution was diluted with ACN: H_2O (70:30, v/v) to obtained intermediate standard solutions of TCS and TCC at a concentration range of 0.004 to 5 mg/L. Solutions were stored in a refrigerator (4 – 10^{o} C) between use. A new stock solution was prepared every six months.

2.4.4 Optimization of the LC conditions

Acetonitrile (ACN) was chosen as organic modifier in the experiments, due to its stronger elution properties, and miscibility with aqueous phases. Several combinations of mobile phase and mobile phase pH levels were tested. Several C18 HPLC analytical columns such Nova-pak, C18, 3.9×150 mm (Waters) , Synergi, 4µm, Hydro-RP, 250 × 4.6 mm (Phenomenex), Kingsorb, 3µm , C18, 4.6 × 150 mm (Phenomenex), µBondapak C18, 10µm, 3.9 × 300 mm (Waters) and X-terra RP C18, 5 µm, 4.6 × 150 mm (Waters) were tested to achieve the best separation of TCS and TCC. The initial method development was done on HPLC-UV at 280 nm for TCS and 260 nm for TCC. An isocratic elution profile was chosen during the beginning of the method development and the run time was 20 min. The method was changed to the gradient method described in Table 2.2.

Table 2.1 Chosen properties of C_{18} columns used in the study.

Column name	Particle substrate	Particle shape	pH stability	Ref
Nova-pak, C18	Silica	Spherical	2.0-8.0	162
Synergi, 4 µm, Hydro-RP	Silica	Spherical	1.5 – 7.5	163
Kingsorb, 3 µm , C18	Silica	Spherical	2.0-9.0	164
µBondapak,10 µm,C18	Silica	Irregular	2.0-8.0	162
X-terra RP, 5 µm C18	Hybrid	Spherical	2.0-12.0	162

After selection of a C_{18} analytical column and a mobile phase for HPLC/UV analysis, the method was transferred onto the LC-MS. A gradient system equipped with

a reversed phase separation column Xterra RP 5 μm (4.6 × 150 mm id) was used for analysis of TCS and TCC in the sample. Formic acid was added to the mobile phase to facilitate ionization of target compounds. Detection was performed using electrospray ionization- mass spectrometry (LC-ESI-MS) in negative ionization mode using single ion monitoring (SIM) on a micromass quattro micro triple- quadrupole mass spectrometer (Micromass, Manchester, U.K.). The operating conditions are listed in Table 2.2.

Table 2.2 Operation parameters for LC/MS.

LC conditions	
Parameter	**Setting**
Column	Reversed Phase Xterra , 5um, 4.6 × 150 mm id (Waters)
Mobile phase	A: acetonitrile containing 0.1 % formic acid B: water containing 0.1 % formic acid
Solvent gradient	0 - 2 min 10% A; 2 - 25 min 10%A to 90%A; 25.1 min 10%A for 10 min
Flow rate	1 mL/min
column temperature	Ambient
injection volume	50 μL
MS conditions	
ionization mode polarity	electrospray ionization, negative ion
capillary voltage	3.50 kV
cone voltage	22 kV
source temperature	80 ^{o}C
Desolvation temperature	300 ^{o}C
cone gas flow	35 L/h
desolvation gas flow	410 L/h
SIM ($[M-H]^-$)	287 m/z for TCS and 313 m/z for TCC

2.4.5 LC/MS linearity and range

The MS data acquisition and analysis was performed using MassLynx version 4.0 software. Linearity was determined by constructing two calibration curves. For the construction of each calibration curve, four standard concentrations of TCC and TCS in the range of 0.05 – 0.5 μg/mL were prepared in ACN. Three replicates of 50 μL injections were made for the standard solution to verify the repeatability of the detector response at each concentration. The peak areas of the chromatograms were plotted against the concentrations of TCC and TCS to obtain the calibration curve. The four

concentrations of the standard solution were subjected to regression analysis by the least-squares method to calculate the calibration equation and correlation coefficient.

2.4.6 Optimization of the extraction technique

Two extraction techniques namely ASE and sonication were tested for extraction of TCS and TCC from biosolids-applied soil and biosolids.

2.4.6.1 ASE extraction

For the ASE extraction, 3 g ± 0.01 g of each blank soil and biosolids sample was spiked with 300 µL TCS and TCC standard solution (10 mg/kg) before mixing thoroughly with mixed with 5 g of hydromatrix. The spiked sample-hydromatrix mixture was placed in a 22 mL ASE stainless steel cell. A glass fiber filter was placed inside the bottom end of the ASE cell to filter the extract before it was collected in ASE vials. The sample and hydromatrix mixture was packed in the cell and a glass fiber filter was also placed on the top layer after which the cells were sealed tightly and loaded onto the ASE extractor (ASE 200, Dionex). The following extraction conditions were employed, two 5 min cycle at 100^{o} C, 1500 psi, static time of 2 min, flush volume 100 % and purge value of 60 sec. Hexane, dichloromethane, and acetone were tested for extraction.

After an ASE extraction was completed, the extract volume (~ 40 mL), was evaporated in a turbo evaporator and redissolved in 1.5 mL of ACN for soil samples and control samples and redissolved in 50 mL ACN for biosolids sample. Finally, the solution was filtered with Millex Millipore filter (0.45 µm, PTFE, 13 mm) and transferred to an LC vial before analyzing the target compounds on LC/MS. To make sure no adsorption of TCS and TCC occurred onto the filter; standard solutions were passed through the filter and tested for any decrease in concentration after filtration process.

2.4.6.2 Sonication extraction

For sonication extraction, 3 ± 0.01 g of air-dried blank biosolids or soil were spiked with 300 µL solution (10 mg/kg) of the TCS and TCC dissolved in acetonitrile, to obtain a final concentration of 1 mg/kg in the soil sample. The spiked samples were thoroughly mixed and stored in a dark refrigerator for 1 day before the extraction and analysis of the target analytes. Weighed and spiked air dried soil was transfered in a 25 mL beaker. The target compounds were extracted in a sonicator (Bransonic 52) by three separate 15-min sonications with acetone (3× 7 mL) for the soil sample, and four 15-min sonications with acetone (4× 10 mL) for the biosolids sample.

After each sonication, the sample was centrifuged at 3500 g for 10 min and the supernatant was pulled into the same vial. The final supernatant was evaporated to complete dryness using turbo evaporator at 40^{o}C and then redissolved in 1.5 mL and 50 mL ACN for the soil and biosolids, respectively. Finally, the solution was filtered with Millex Millipore filter (0.45 µm, PTFE, 13 mm) and transferred to an LC vial before analysis for the target compounds on LC/MS. With sonication only acetone was tested as extraction solvent.

2.4.7 Matrix effect

A mechanism called ion suppression was introduced by Kebarle and Tang [165], they showed that electrospray ionization response towards the target organic bases decreased in the presence of other organic bases. Components from the matrix can induce ion suppression and result in decrease detector response towards the target compound [165]. To measure the matrix effect in this study the following method was used; 3 ± 0.01 g of

blank soil or biosolids was weighed in a beaker, extracted using sonication method described in the previous section. After the extract was collected, acetone was evaporated and reconstituted in 2 mL ACN. To make the matrix-matched standards, a 500 µL of extract was mixed with 500 µL of 1 mg/L or 3 mg/L TCS & TCC standards solution in ACN to achieve final concentrations of 0.5 and 1.5 mg/L. The two matrix-matched standards were injected in LC-ESI-MS and peak areas of TCS and TCC were measured .TCS and TCC standard with a concentration of 1 mg/L in ACN was also injected in LC-ESI-MS. All injections were made in triplicates and a volume of 50 µL was injected. The following formula was used to calculate matrix effect (ME).

$$ME\ (\%) = 100 \times \frac{A - B}{S} \quad . \qquad (2.1)$$

A = Peak area of matrix matched standard 1.5 mg/L

B = Peak area of matrix matched standard 0.5 mg/L

S = Peak area of standard 1 mg/L

2.4.8 Biosolids and soil samples

Soil samples were collected from different depths of three fields receiving 33-consecutive year application of biosolids at four different rates (Table 2.3). The field plots, from which soil samples were collected, were established by Metropolitan Water Reclamation District of Greater Chicago (MWRDGC) at a land reclamation site located in Fulton County, Illinois. Dewatered biosolids samples were collected from sixteen different wastewater treatment plants (WWTP) in the United States. Detailed description about the characteristics of the WWTPs from where biosolids were collected is given in Table 2.4. Both biosolids and soil samples were kept frozen at -20^{o} C until analysis.

Table 2.3 Information of the biosolids-applied soil used in this study

Soil Treated with Biosolids	**Sample name Depth 0-6 inch**	**Sample name Depth 6-12 inch**	**Sample name Depth 24-48**
Treatment 1 (control)	03-1 16-1	03-2 16-2	03-4 16-4
Treatment 2 (7.5 ton biosolids/ha)	01-1 14-1	01-2 14-2	01-4 14-4
Treatment 3 (15 ton biosolids/ha)	02-1 13-1	02-2 13-2	02-4 13-4
Treatment 4 (30 ton biosolids/ha)	04-1 15-1	04-2 15-2	04-4 15-4

Table 2.4 Characteristics of WWTPs from where biosolid samples were collected.

WWTP #	Location (State)	Population	Treatment	Sample type
1	GA	25,000	Aerobic	Biosolids
2	SC	125,000	Anaerobic	Biosolids
3	SC	30,000	Aerobic	Biosolids
4	SC	30,000	Aerobic	compost from WWTPs 3
5	SC	60,000	Aerobic	Biosolids
6	SC	60,000	Aerobic	compost from WWTPs 5
7	SC	50,000	Aerobic	Biosolids
8	SC	50,000	Aerobic	compost from WWTPs 7
9	SC	25,000	Aerobic	Biosolids
10	SC	25,000	Aerobic	compost from WWTPs 9
11	GA	40,000	Anaerobic	Biosolids
12	GA	40,000	Anaerobic	compost from WWTP 11 (3 months)
13	GA	40,000	Anaerobic	compost from WWTP 11 (12 months)
14	GA	70,000	Aerobic	Biosolids
15	GA	70,000	Aerobic	compost from WWTP 14
16	GA	30,000	Anaerobic	Biosolids
17	GA	30,000	Anaerobic	compost from WWTP 16
18	GA	29,000	Aerobic	Biosolids
19	GA	29,000	Aerobic	Compost from WWTP 18
20	GA	600,000	Anaerobic	Biosolids
21	GA	175,000	Anaerobic	Biosolids

2.4.9 Sample preparation

Frozen biosolids and soil samples were thawed, air dried, and ground to 1 mm average size using a Wiley mill before extraction. Three grams of air dried sample was

weighed in a 25 mL beaker. The target compounds were extracted using sonication. Sonication was chosen as an extraction method because our test data suggest sonication is better than ASE extraction method. Further information on the results obtained for extraction methods is given in section 2.5.3. The target compounds were extracted by three separate 15-min sonications with acetone (3× 7 mL) for the soil sample, and four 15-min sonications with acetone (4× 10 mL) for the biosolids sample in a sonicator (Bransonic 52). After each sonication, the sample was centrifuged at 3500 g for 10 min and the supernatant was pulled into the same vial. The final supernatant was evaporated to complete dryness using a turbo evaporator at 40^{o}C and then redissolved in 1.5 mL and 50 mL ACN for the soil sample and biosolids, respectively. Finally, the solution was filtered with a Millex Millipore filter (0.45 µm, PTFE, 13 mm) and transferred to an LC vial before analysis for the target compounds on LC/MS.

2.4.10 Analytes identification and quantification

The identification of TCS and TCC in the samples was achieved by comparison with the chromatogram retention time and the mass spectrum of the standard compounds. Quantification of TCS and TCC was carried out with a calibration curve obtained from external standards for soil samples. Matrix matched calibration curves were prepared for biosolids. The obtained results suggest only significant matrix effect for biosolids. Detailed results showing the matrix effect observed in biosolids is given in section 2.5.4. The calibration curve was a plot of peak area of TCS and TCC against their concentrations. All the calibration curves used for quantification had r^2 values of 0.970 or better.

2.5 Results and discussion

2.5.1 Optimization of the LC conditions

Due to the structural similarities between TCS and TCC, their separation on a LC column was difficult to achieve. The initial column selection was carried out on LC-UV. Several reverse phase columns, namely, Nova-Pak C18 (Waters), Synergy Hydro-RP (Phenomenex), Kingsorb C18 (Phenomenex), µ-Bondapak C18 (Waters) were tested, but none of the columns were able to completely separate the two target analytes. Performance of all the columns evaluated is listed in Table 2.5. Briefly, Nova-Pak C18 column did not retain TCS or TCC on the column, even using high aqueous phase concentrations in the mobile phase did not help with the retention of these compounds on the column and both compounds were eluted in the void volume. With Synergi Hydro-RP column one peak was observed for both the compounds, when percent of water was increased in the mobile phase peak broadening took place but no separation was achieved. With Kingsorb C18 column TCC was retained on the column but TCS eluted in the void volume. Changing the polarity of the mobile phase did not changed retention time of TCS on the column. While µ-Bondapak C18 showed some retention of TCS and TCC at higher pH range above 8, but the suggested pH range for this column is between 2-8, hence this column was not used for further tests for separation of TCS and TCC. Finally, Xterra (Waters) was used, as satisfactory separation was achieved with a

Table 2.5 Tested combinations of mobile phase and C18 column for separation of TCS and TCC

Column name	**Mobile Phase**	**TCS retention time**	**TCC retention time**
Nova-pak, C18	ACN: H_2O (55:45, v/v)	Eluted in void volume	Eluted in void volume
Synergi, 4µm, Hydro-RP	ACN: H_2O (80:20, v/v) 10 mM Acetic Acid	No separation Peak overlap	No separation peak overlap
Kingsorb, 3µm , C18	ACN:H_2O (70:30, v/v) pH. 9	Eluted in void volume	5.47 min
µBondapak,10µm,C18	0.1 M Sodium Borate buffer: ACN (30:70, v/v), pH. 8	Eluted in void volume	5.00 min
	0.025 M KH_2PO_4: ACN (30:70, v/v), pH. 7.5 & 6	Eluted in void volume	Eluted in void volume
X-terra RP, 5µm C18	ACN: H_2O (70:30, v/v), pH. 3	Eluted in void volume	Eluted in void volume
	0.025 M Sodium Bicarbonate buffer: ACN (30:70, v/v), pH. 10	1.93 min	9.1 min
	0.25 M KH_2PO_4 buffer : ACN (30:70, v/v), pH 7.5	5.46 min	7.03 min
	ACN:H_2O (70:30, v/v)	5.49 min	7.45 min

retention time of 5.49 min for TCS and 7.25 min for TCC (Figure 2.4). The UV spectrum of TCS and TCC are shown in Figure 2.5. The UV spectrum was used to identify TCS and TCC during the initial method development stage with analysis using LC-UV.

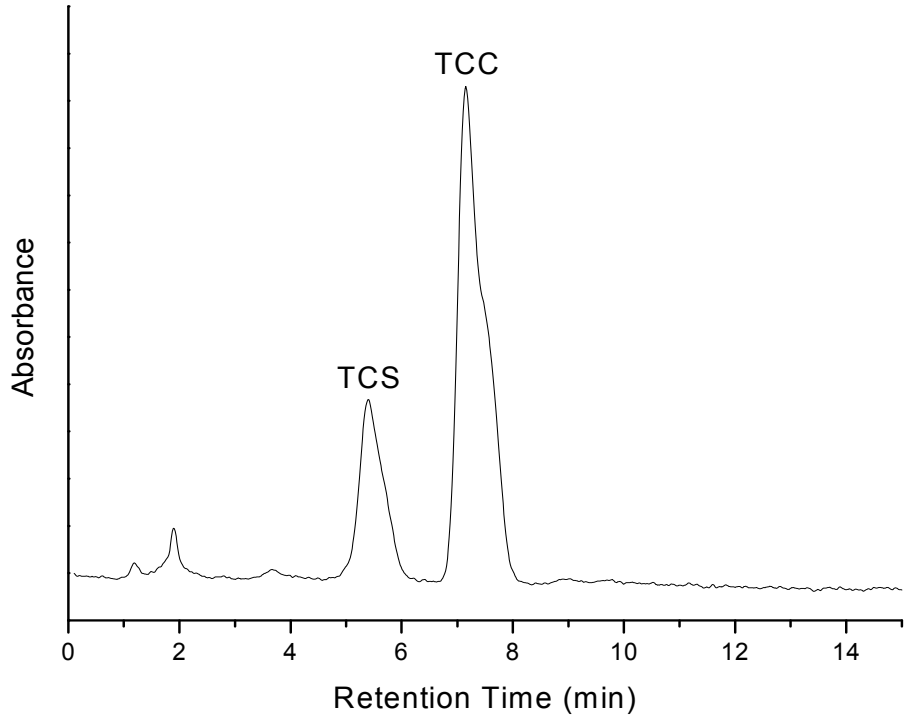


Figure 2.2 HPLC-UV chromatogram of standard TCS and TCC (1 mg/L).

When the method was transferred to LC-ESI-MS a gradient mobile phase method was used. The gradient method is used to achieve better separation of target compounds in a complex matrix (Figure 2.6). Figure 2.6, shows completely resolved peaks of TCS and TCC using gradient method. With the chosen gradient method a retention time of 17.60 ± 0.03 min for TCS and 18.80 ± 0.03 min for TCC was obtained, see Figure 2.7.

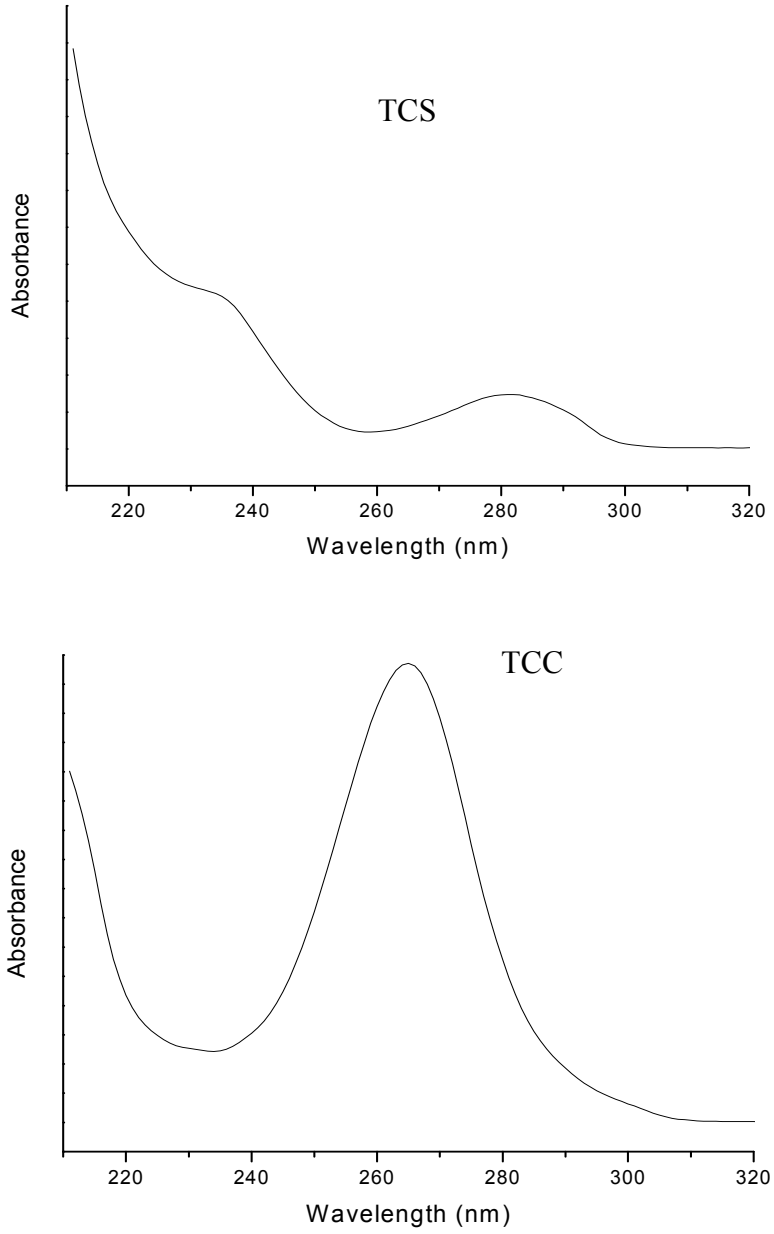


Figure 2.3 UV spectrum of TCS and TCC

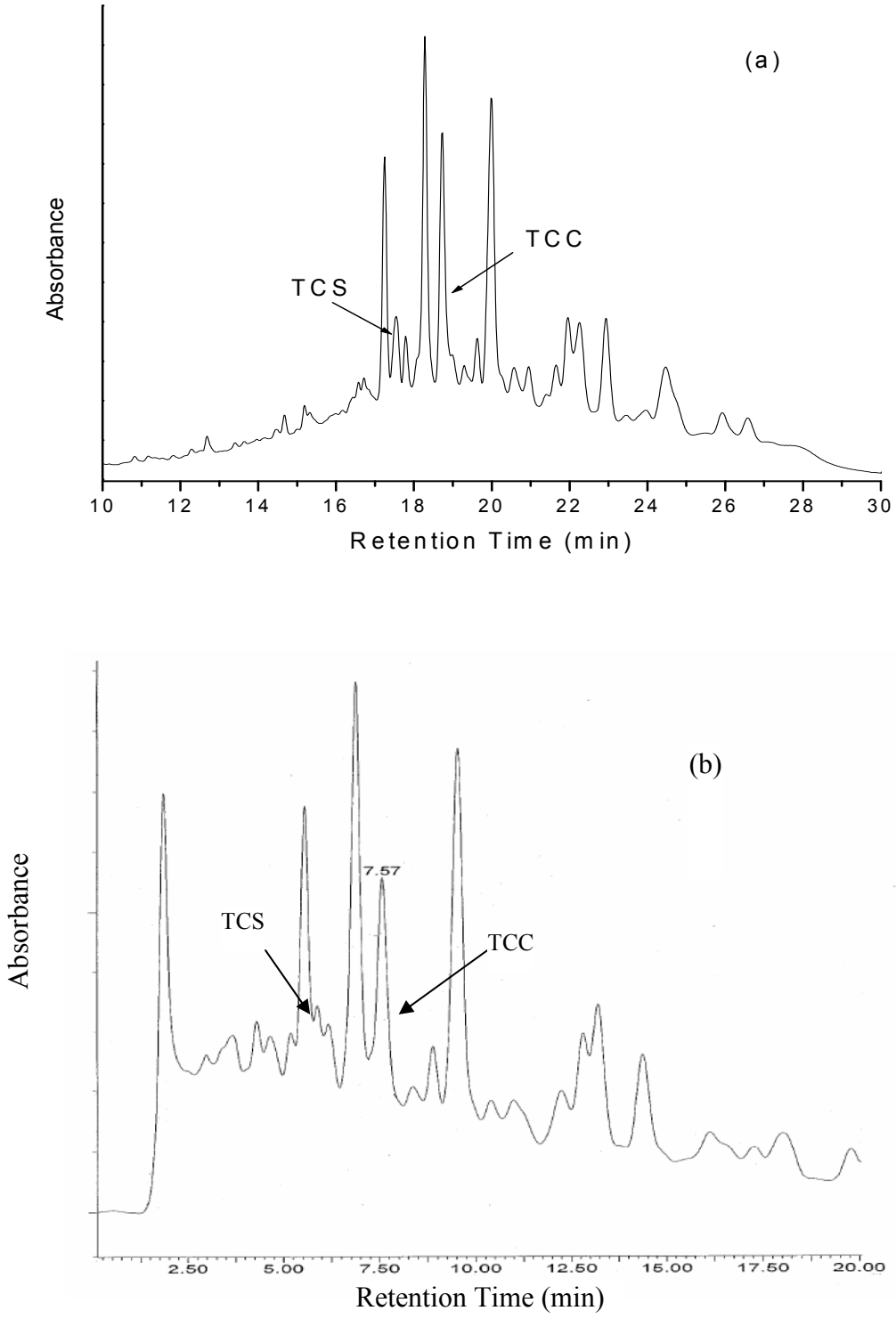


Figure 2.4 LC-UV chromatogram of a biosolids extract at 280 nm (a) using gradient method, and (b) using isocratic method

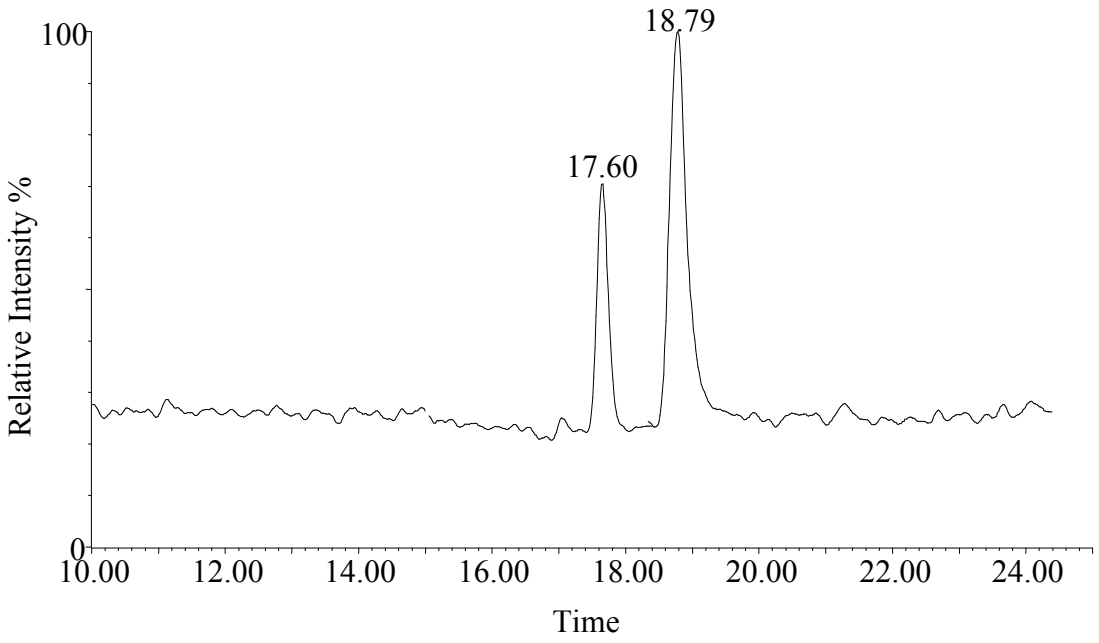


Figure 2.5 Total ion chromatogram of 2 mg/L TCS and TCC standard solution using the developed LC-ESI-MS method.

2.5.2 LC/MS linearity and range

The standard calibration curves were prepared at concentrations of 0.005, 0.01, 0.05, 0.1, 0.5 and 1 mg/L for both TCS and TCC. A linear relation was observed for TCS at all concentrations shown in Figure 2.6 (A). A second order polynomial relation was observed between TCC concentration and the obtained peak area Figure 2.6 (B). The relation was linear for TCC at a concentration range of 0.005 to 0.1 mg/L (Figure 2.6 (C)). However deviation from linearity was observed at higher concentrations of TCC. To eliminate the issue with non-linear calibration curve, biosolids extract was diluted with 50 mL ACN so that TCS and TCC concentration in the extract of samples was within the linear range.

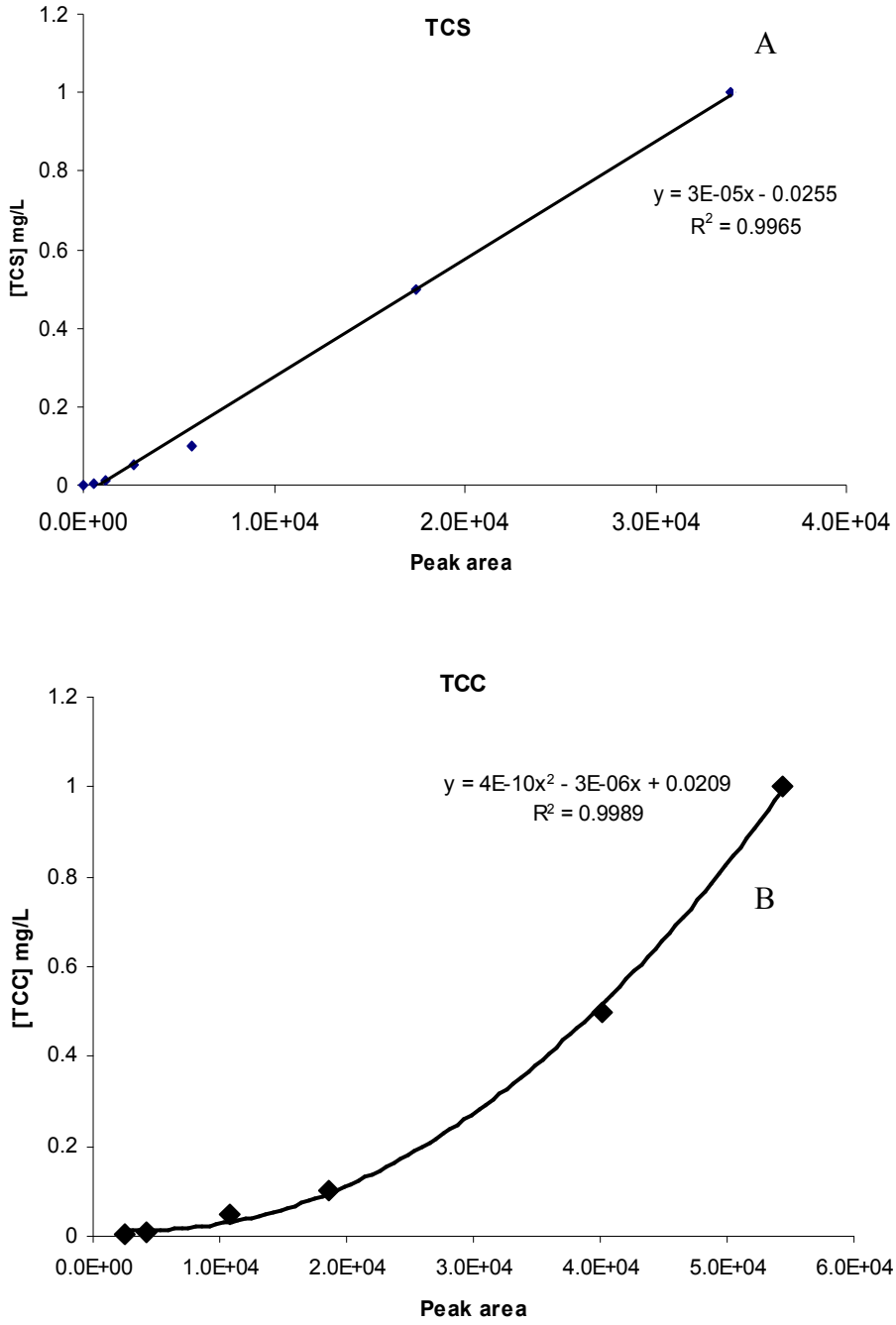


Figure 2.6 Calibration curve showing a linear relationship between the TCS concentration and detector response (A). Plot showing second order polynomial relation between the TCC concentration and the detector response (B). Plot showing linear relationship for TCC between 0.005 – 0.1 mg/L (C).

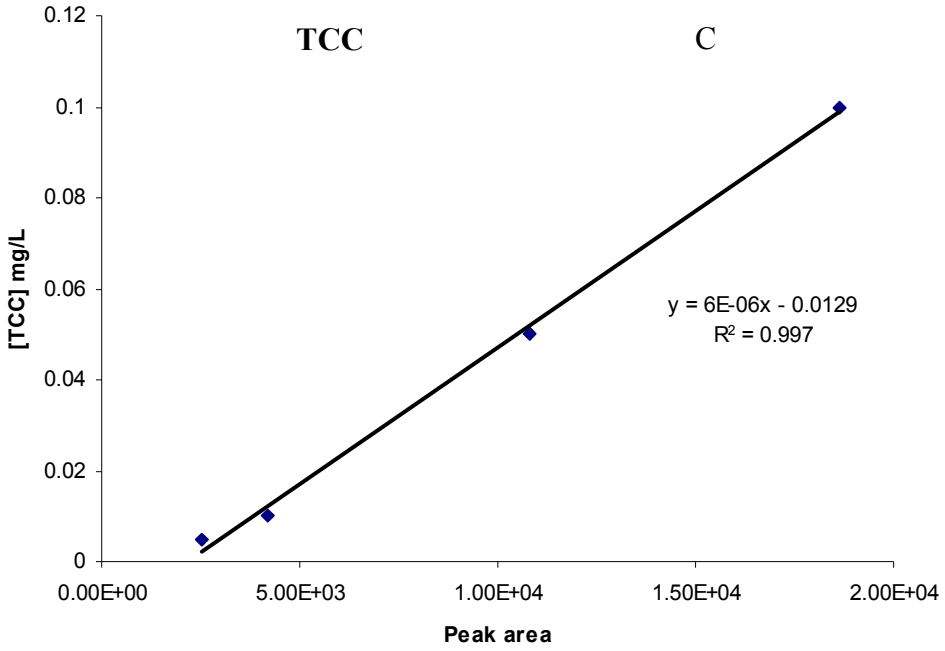


Figure 2.6 (continued)

2.5.3 Optimization of the extraction technique

Accelerated Solvent Extraction (ASE) and sonication were tested for sample extraction (Table 2.6). The ASE extraction using hexane yielded 0% recovery of TCS and TCC from biosolids-applied soil and biosolids samples. The ASE extraction of biosolids-applied soil samples with dichloromethane following method by Singer et al., also yielded low recovery rates for both compounds (less than 20 %). The ASE extraction of biosolids-applied soil samples with acetone yielded 42.9 % and 87.1 % recovery rates for TCS and TCC, respectively. Extraction recovery of all the tested solvent is given in Table 2.6.

After ASE extraction it was determined that non-polar solvents were not suitable for extraction of TCS and TCC from soil and biosolids samples and polar solvents like acetone gave better extraction recoveries. Hence acetone was the only solvent that was

chosen for extraction using sonication. Sonication of soil samples with acetone yielded recovery rates at 110.5% and 92.2% for TCS and TCC, respectively. The extraction recoveries for biosolids-applied soil and biosolids quality control samples are shown in Table 2.6. ASE showed lower recoveries compared to sonication method for both biosolids-applied soil and biosolids samples. Since ASE extraction is carried at high temperature and pressure a lot of the matrix is also extracted along with the target compound. As a result the extracts obtained with ASE required additional clean-up step. ASE was therefore not chosen as an extraction method for simultaneous extraction of TCS and TCC in our investigation.

Table 2.6 Extraction recoveries of TCS and TCC in biosolids-applied soil and biosolids samples using ASE and Sonication (n = 3).

	TCS		**TCC**	
ASE	**Soil (recovery % ± SD)**	**Biosolids (recovery % ± SD)**	**Soil (recovery % ± SD)**	**Biosolids (recovery % ± SD)**
Hexane	0	0	0	0
Dichloromethane	9.0 ± 1.9	4.08 ± 2.8	10.1± 2.4	19.6 ± 3.1
Acetone	42.9 ± 2.8	80.7 ± 8.5	87.1 ± 3.6	97.9 ± 4.0
SONICATION				
Acetone	110.5 ± 2.1	126.6 ± 6.6	92.2 ± 1.6	91.7 ± 2.3

2.5.4 Matrix effect

No matrix effect was observed for biosolids-applied soil. While 37% suppression of signal was observed for TCS and 34% suppression was observed for TCC in biosolids

matrix. For the further analysis and quantification of biosolids, matrix matched standards were used. A calibration curve was prepared with standards added to blank biosolids extracts. This procedure is important when ion suppression is observed because ion suppression can lead to inaccurate quantitation of analytes in the desired samples. Since no ion suppression was observed in biosolids applied soil samples normal calibration curve was used for quantitation of TCS and TCC in soil samples.

2.5.5 Limits of detection and quantification

The limit of detection (LOD) is usually defined as 3×signal-to-noise (S/N) ratio. MassLynx 4.0 software was used for calculation of S/N. The instrument LOD was determined to be 2 µg/kg for both compounds at an S/N ratio of 4. The LOD for biosolids-applied soil samples were considered to be same as that of instrumental LOD, due to lower levels of interference. Limit of quantification (LOQ) is usually defined as 10 × S/N ratio. The LOQ values for TCS and TCC in soil samples were determined to be 5 µg/kg. For biosolids, method LOD and LOQ for TCS was 8 µg/kg and 12.5 µg/kg, respectively. While for TCC, the LOD was 4 µg/kg and LOQ was 8 µg/kg. The LOD and LOQ in biosolids sample are higher due to the complexity of the matrix and possible interference.

2.5.6 Recovery and precision

The control soil samples were spiked with standards for determining the recoveries of TCS and TCC at a spiking concentration of 1mg/kg and 2 mg/kg. Triplicate analysis at each concentration was performed. Blank biosolids were spiked at a 1mg/kg level. The average recoveries along with the coefficient of variation (CVs) are listed in

Table 2.7. The CVs were calculated using standard deviation to mean ratios. Each sample was analyzed 3 times to measure the precision of the method.

Table 2.7 Recoveries (%) of triclosan and triclocarban from soil and biosolids samples using sonication extraction with acetone (n=3).

extraction method	Sample	TCS		TCC	
		recovery (%)	coefficient of variances	recovery (%)	coefficient of variances
Sonication	Soil	110.5 ± 2.1	1.91	92.2 ± 1.6	1.79
	Biosolids	126.6 ± 6.6	2.25	91.7 ± 2.3	2.53

2.5.7 Analytes identification and quantification

The identification of target compounds TCS and TCC was carried out by comparison of the retention time and mass spectrum obtained with standards. The elution profile of TCC in biosolids-applied soil sample is shown in Fig. 2.7 (D) and the elution profile of the two compounds in standard solution at a concentration level of 2 mg/L is shown in Fig. 2.7 C, 2.7 F. Positive identification of TCS and TCC was carried out by LC-ESI-MS in negative mode and compared with the authentic standards. Triclosan (molecular weight 289.55) in the negative ion mode ESI mass spectrum was detected at m/z 287 $[M-H]^-$. Some minor peaks due to naturally occurring ^{37}Cl atoms at m/z 289, 291 and 293 for TCS were also detected (Figure 2.8(A)). Triclocarban (molecular weight 315.59) was identified by its molecular ion peak at m/z 313 $[M-H]^-$ and its formic acid adduct at m/z 359, other minor peaks due to ^{37}Cl atoms were detected at m/z 315, 317, 319, 361, 363 and 365 (Figure 2.8 (B)). Mass spectrum of TCS and TCC detected in representative biosolids and biosolids-applied soil samples is shown in Fig. 2.8 (C, D, E, and F). The quantification was done by comparing the peak area of TCS and TCC of tested samples with the standard curves of both analytes. The final concentration of both analytes in the tested samples was calculated based on the dry weight of the tested samples.

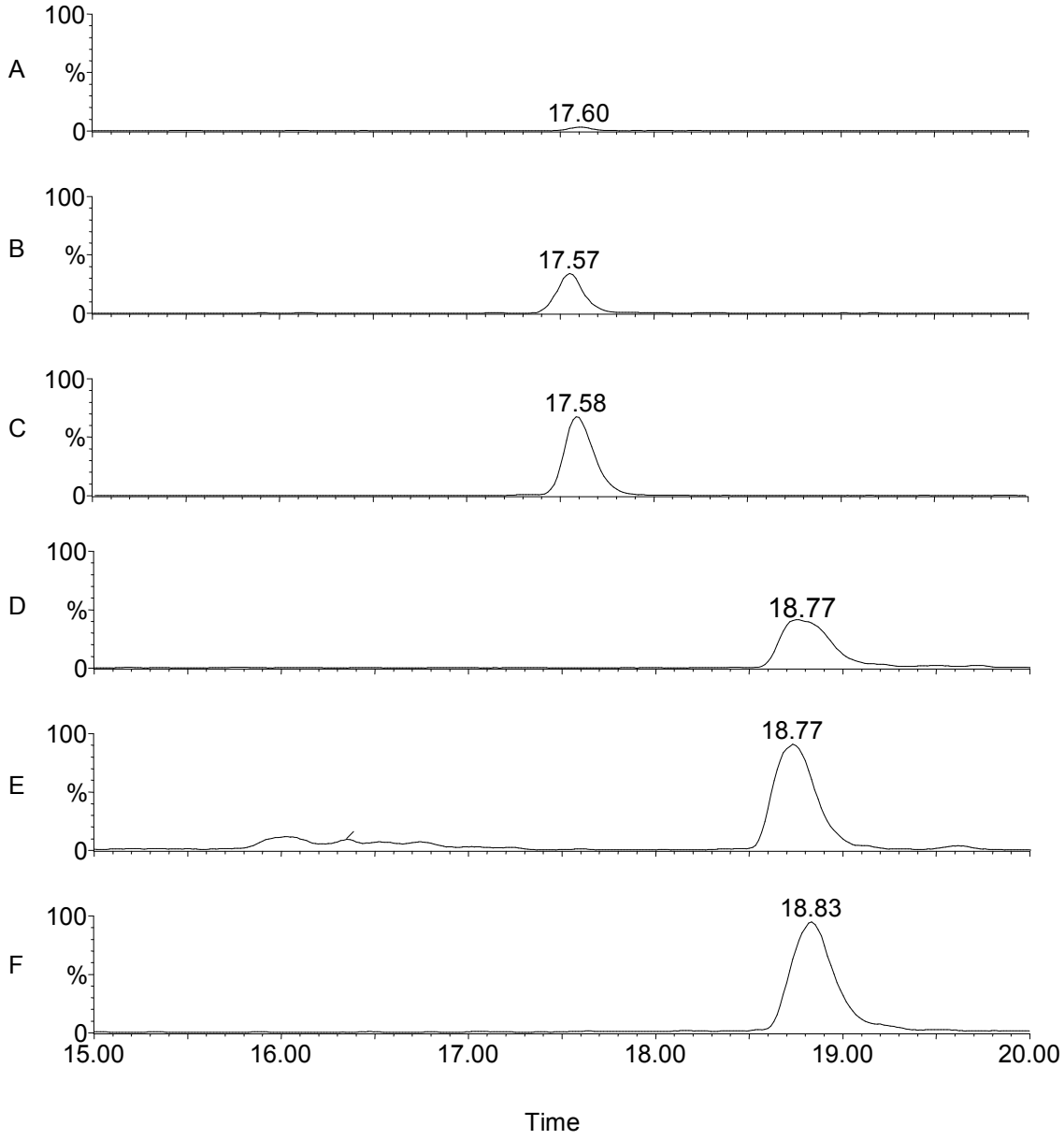


Figure 2.7 Representative chromatograms of TCS and TCC obtained by negative ion LC-ESI-MS at single ion monitoring (SIM) mode. Triclosan detected in biosolids-applied soil (A), biosolids (B) and standard at 2 mg/kg level (C). Triclocarban in biosolids-applied soil (D), biosolids (E) and standard at 2 mg/kg level (F).

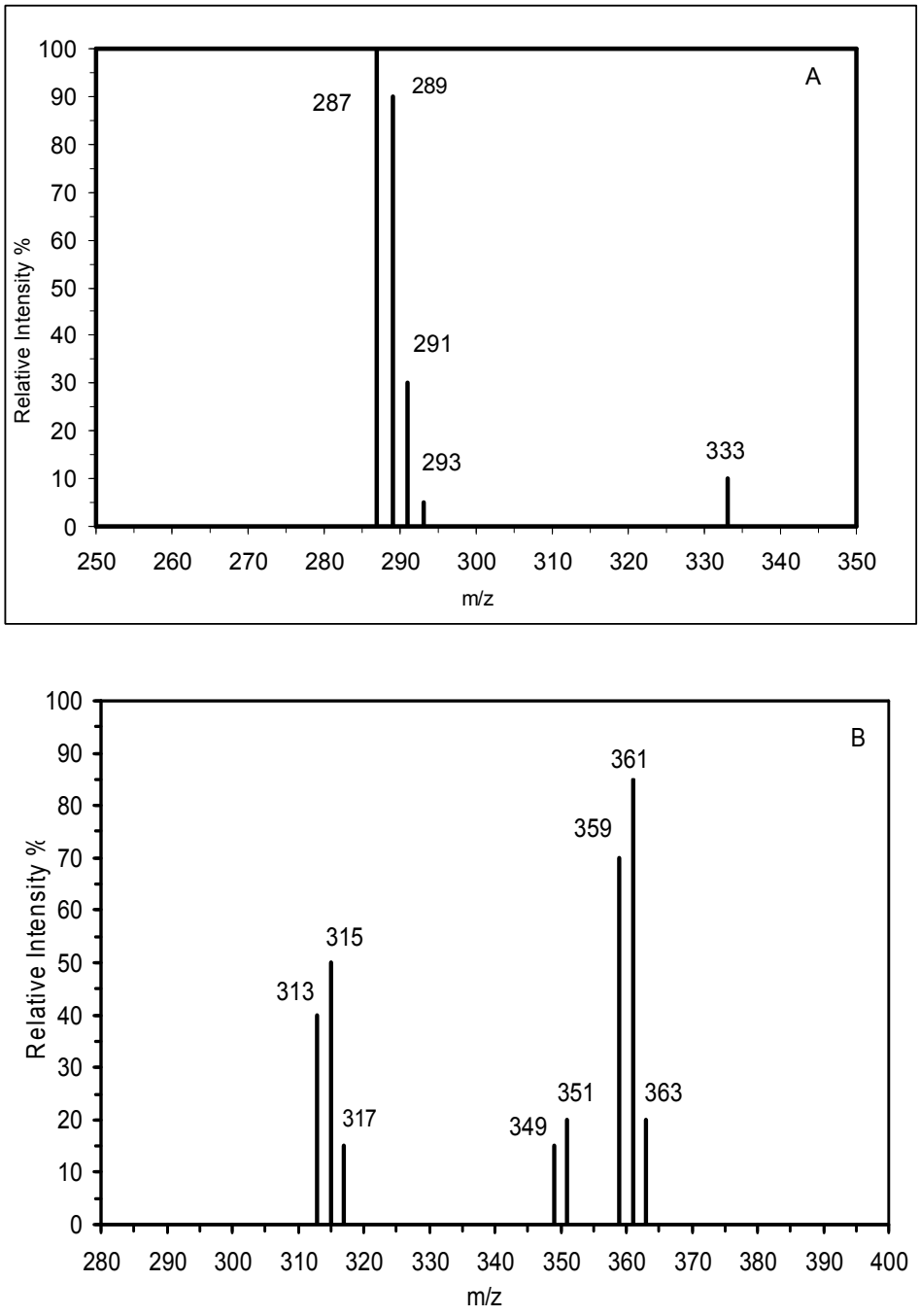
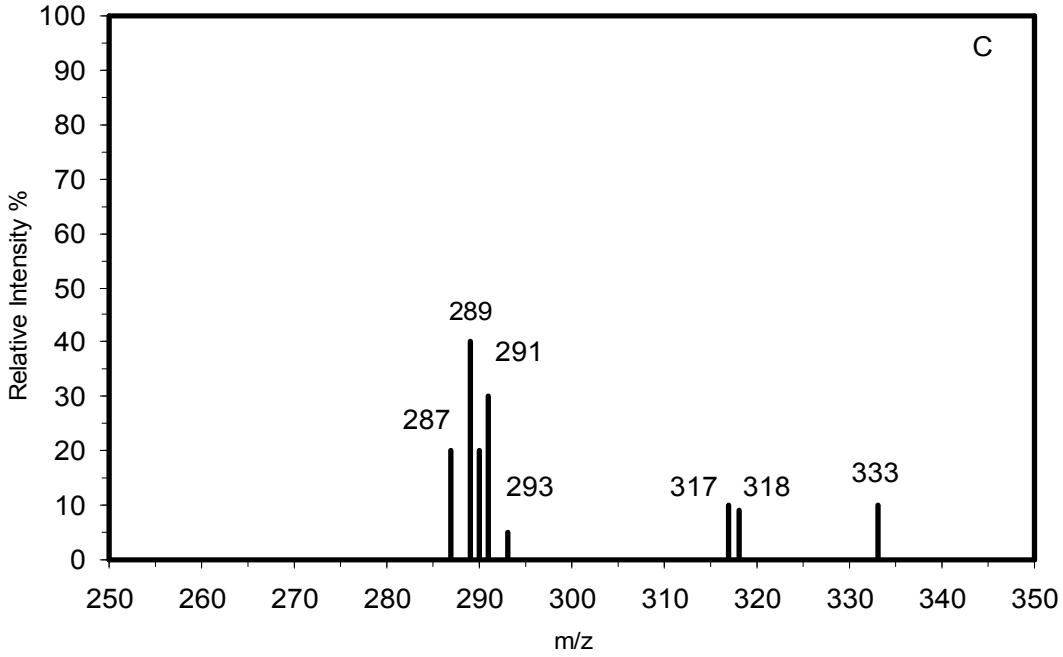


Figure 2.8 Full scan mass spectra at negative ESI mode for triclosan at 2 mg/kg level (A). Full scan mass spectra of triclocarban at 2 mg/kg level (B). Mass spectra of triclosan (C) and triclocarban (D) detected in biosolids-applied



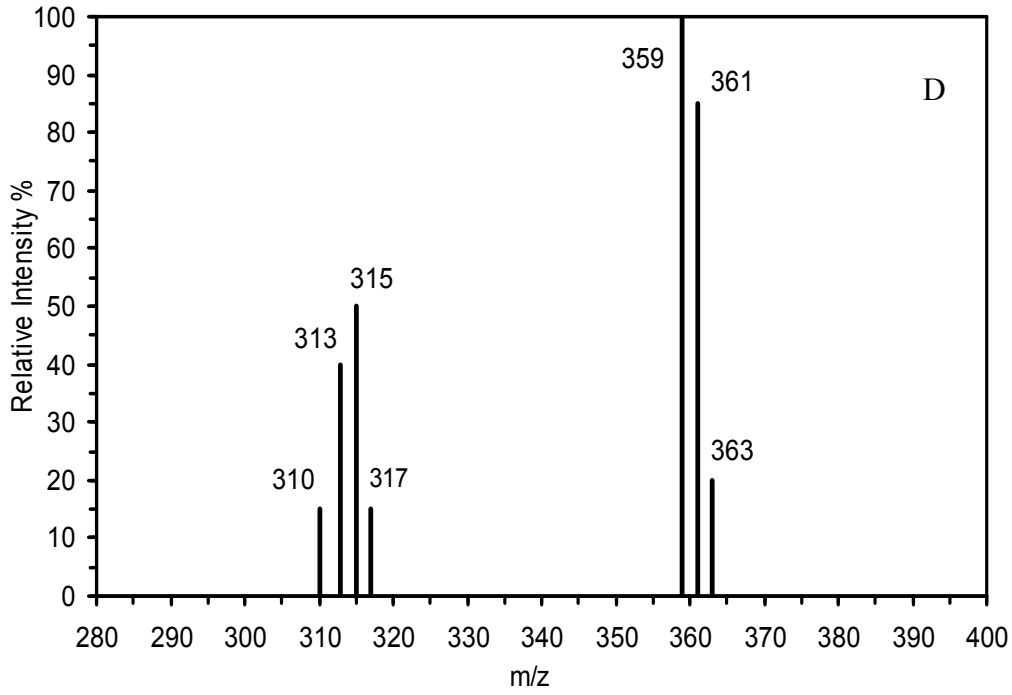


Figure 2.8 (continued)

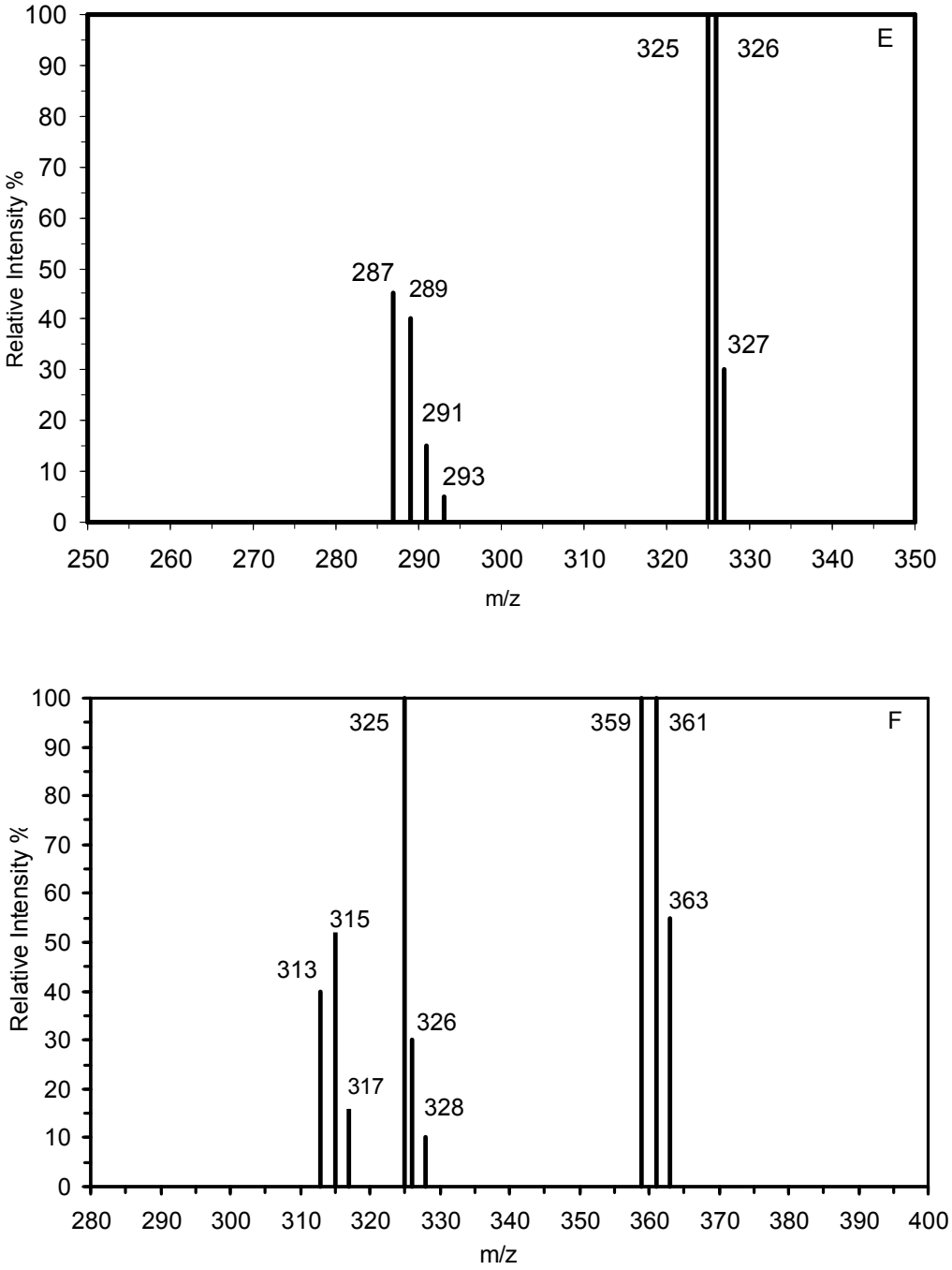


Figure 2.8 (continued)

2.5.8 Analysis of TCS and TCC in biosolids - applied soil samples

The applicability of the proposed method was demonstrated using the method for analysis of TCS and TCC in large number of biosolids-applied soil and biosolids samples. Triclosan and TCC were detected in most of the biosolids-applied soil samples analyzed in the study. The highest concentration of TCS and TCC observed in biosolids-applied soil was 81 µg/kg and 1244 µg/kg respectively. The lowest observed concentration for TCS and TCC in biosolids- applied soil samples was 1.5 µg/kg and 14 µg/kg, respectively. The concentration of TCC was always several magnitudes higher than TCS.

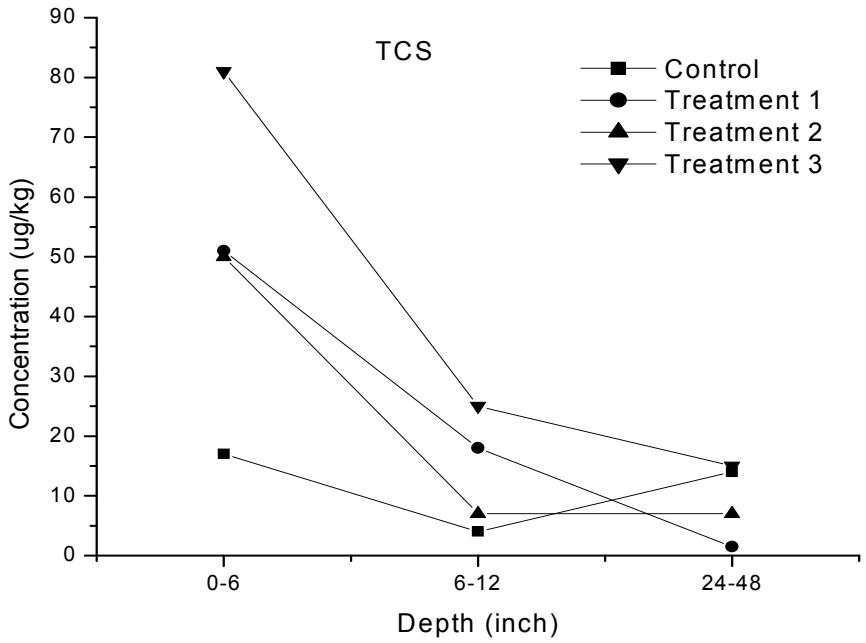


Figure 2.9 Concentration of TCS and TCC in biosolids-applied soil collected at various depths.

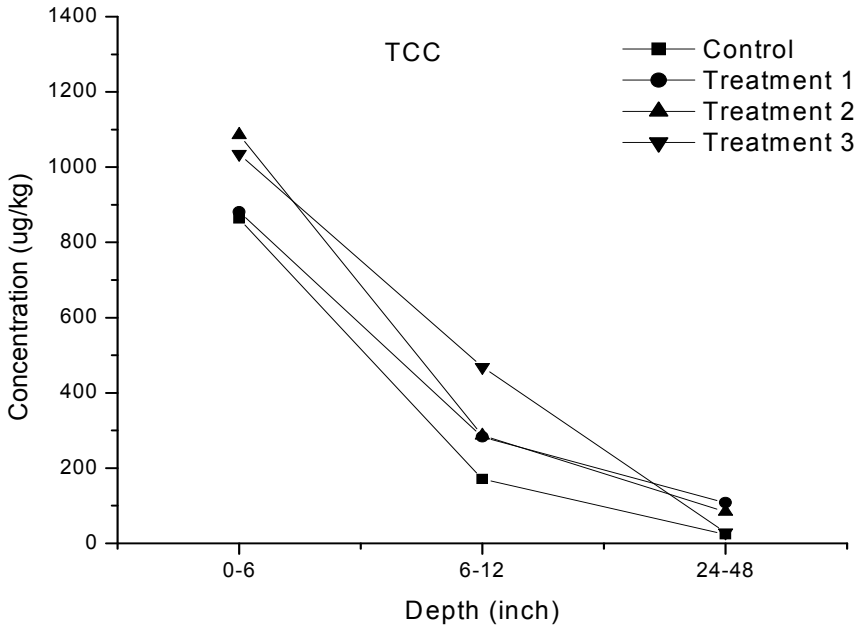


Figure 2.9 (continued)

The determined concentration of TCS and TCC in all the soil samples analyzed using LC-ESI-MS and LC-UV are presented in Table 2.8. The concentration of TCS and TCC in biosolids-applied soil decreased with increasing soil depth (Figure 2.9). The highest concentration was found in the top 0-6 inch depth for both TCS and TCC. As shown in Figure 2.9. The concentration of TCS and TCC was highest at top 0-6 inch depths for treatment 3, which had the highest biosolids application rate during the past 33 years. The concentration TCC in all the four treatments showed the same trend, it decreased sharply after 0-6 inch depth and than was almost the same for all the treatments at 24-48 inch depth. However TCS showed higher concentrations at a depth of 24-48 inches, which suggest higher leaching potential of TCS. Even control plots showed the presence of TCS and TCC, which can be due to cross-contamination from neighboring

plots receiving application of biosolids. Concentrations of TCS and TCC were also measured using HPLC-UV utilizing the same LC conditions with UV absorbance of 280 and 260 nm for TCS and TCC, respectively. As shown in Table 2.8, the concentration obtained with HPLC-UV always gave higher estimation of the concentration of TCS and TCC in the samples. The reason for higher levels of TCS and TCC determined using LC/UV compared to using LC/MS may be due to the presence of interfering compounds displaying absorbance at the same wavelength as the target compounds when using LC/UV. Figure 2.10 a & b, shows the single ion monitoring (SIM) chromatograms of TCS and TCC peaks detected in a soil sample. As can be seen, SIM mode is effective in detecting TCC and TCS in the presence of interfering compounds. The LC-UV chromatogram (Figure 2.10 (c)) however does not shows complete separation of TCS from the interfering peak, which makes quantification of TCS difficult using UV.

The LOQ values for TCS and TCC using LC-MS in soil samples were determined to be 5 µg/kg. For biosolids, LOQ for TCS was 12.5 µg/kg, and for TCC the LOQ was 8 µg/kg. The LC-UV, LOQ in soil samples for TCS was 25 µg/kg and 20 µg/kg for TCC. But quantification of TCS and TCC in several biosolids-applied soil samples was difficult using LC-UV due to overlap with interfering peaks and concentrations below the detection limit after dilution with 50mL ACN, see Table 2.8. The biosolids were not analyzed using LC-UV due to the presence of interference from the matrix (Figure 2.11)

UV detection often lacks the required selectivity and sensitivity. An efficient extraction, cleanup, and pre-concentration step before the HPLC/UV detection is extremely critical for reaching the detection limits that meet the regulatory requirements for screening and monitoring of environmental samples. Developing method for analysis of TCS and TCC in biosolids using LC-UV would require a good clean-up method,

which is not employed in present study. Hence LC-UV cannot be employed for detection of TCS and TCC in biosolids samples.

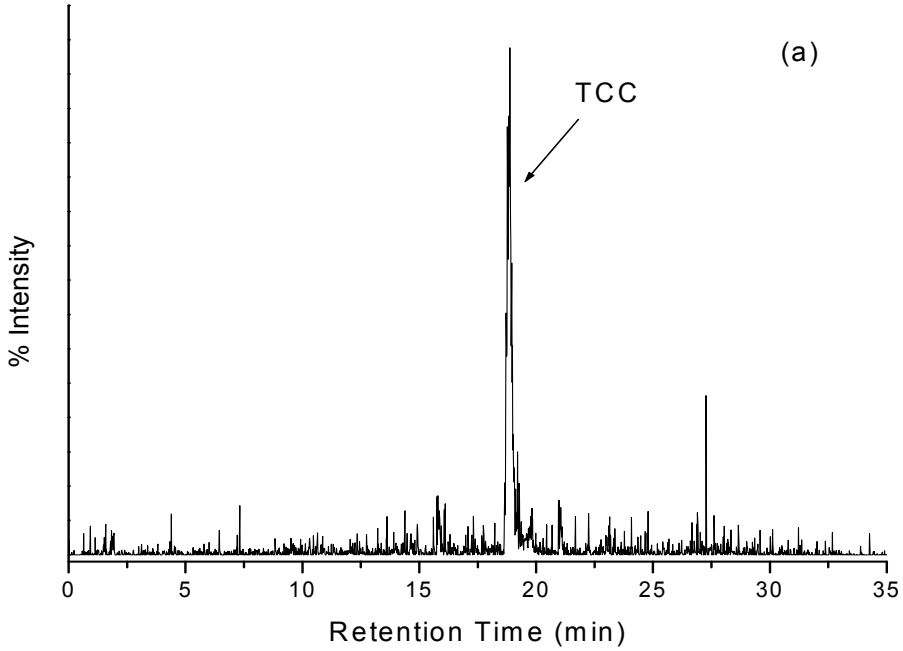


Figure 2.10 SIM chromatogram of TCC (a), SIM chromatogram of TCS (b) and LC-UV chromatogram (c) at 280 nm for biosolids-applied soil # 04-1.

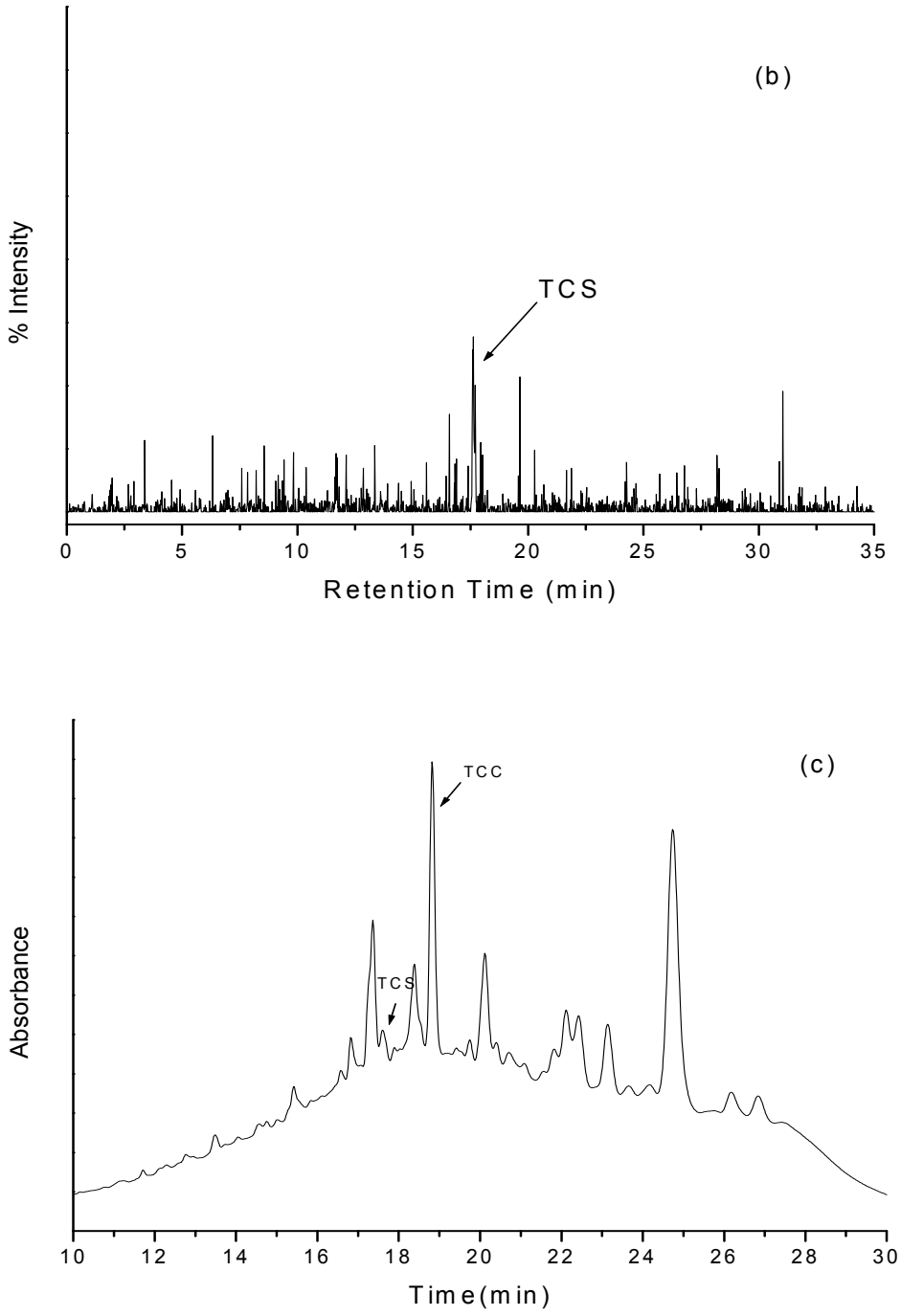


Figure 2.10 (continued)

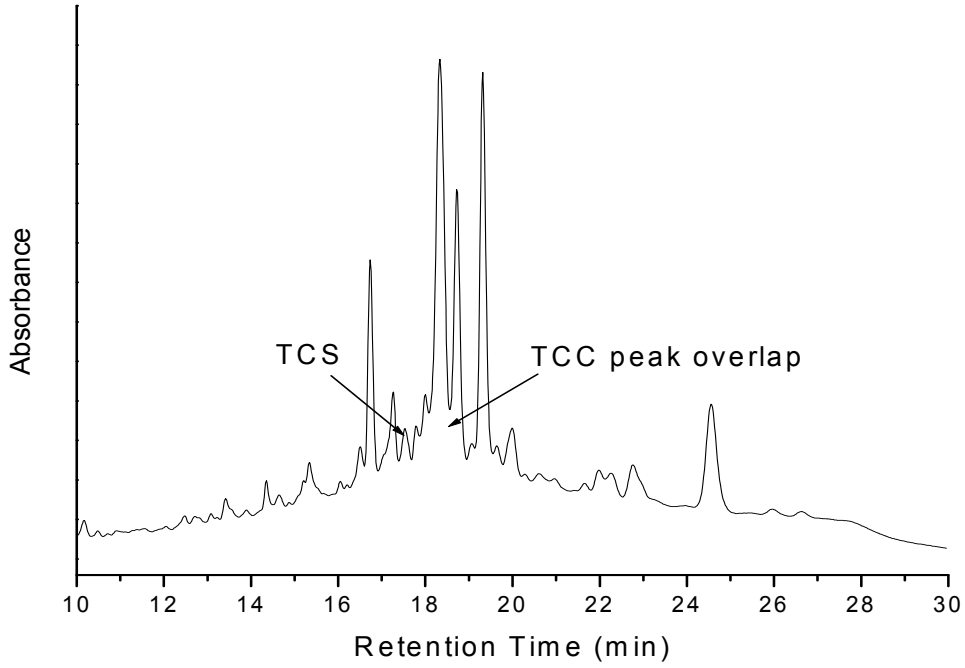


Figure 2.11 LC-UV chromatogram at 280 nm for biosolids sample.

Table 2.8 Concentrations of TCS and TCC in biosolids-applied soil samples determined by LC-UV and LC-ESI-MS method (n=3).

Sample name	LC-UV (µg/kg)		LC-MS (µg/kg)	
	TCS	TCC	TCS	TCC
01-01	102.1± 3.1	1287.7 ± 7.5	51 ± 1.5	881 ± 21
01-02	29.7 ± 6.6	177.3 ± 45.6	18 ± 7.1	283 ± 2.8
01-04	N/D	N/D	1.5 ± 2.1	108 ± 8.5
02-01	145.5 ± 0.7	1443.3 ± 149.28	50 ± 5.7	1086 ± 144
02-02	7.2 ± 2.7	176.0 ± 21.7	7.0 ± 1.4	287 ± 25
02-04	23.7 ± 2.1	N/D	7.0 ± 1.4	84 ± 20
03-01	35.5 ± 4.2	530.3 ± 56.2	17 ± 6.4	863 ± 2.8
03-02	N/D	46 ± 6.0	4.0 ± 0.0	171 ± 3.5
03-04	N/D	N/D	14 ± 0.7	24 ± 15.6
04-01	203 ± 12.7	2355.0 ± 93.6	81 ± 1.4	1035 ± 30
04-02	80.4 ± 5.2	746.7 ± 70.7	25 ± 1.4	469 ± 11
04-04	N/D	N/D	15 ± 2.1	29 ± 2.1
13-01	119.7 ± 4.3	1413.3 ± 25.6	45 ± 5.6	1244 ± 87
13-02	10.4 ± 0.5	132.0 ± 30.7	7 ± 0.0	288 ± 22
13-04	N/D	N/D	N/D	38 ± 3.5
14-01	122.5 ± 7.8	1222.3 ± 297.7	38 ± 5.7	903 ± 24
14-02	14.69 ± 4.9	263.7 ± 15.6	6.5 ± 2.1	220 ± 4.9
14-04	N/D	N/D	13 ± 3.5	32 ± 2.8
15-01	43.9 ± 5.7	1081.7 ± 222.6	23 ± 0.7	689 ± 65
15-02	N/D	892.7 ± 202.4	24 ± 2.8	273 ± 2.8
15-04	N/D	N/D	23 ± 10.6	18 ± 2.8
16-01	44.2 ± 5.9	984.0 ± 44.3	25 ± 7.8	625 ± 67
16-02	16.1 ± 1.8	110.0 ± 17.3	1.5 ± 0.7	149 ± 4.9
16-04	N/D	N/D	17 ± 3.5	14 ± 4.2

Table 2.9 Concentrations of TCS and TCC in biosolids samples determined by LC-ESI-MS method (n=3).

Sample name	Biosolids (mg/kg)	
	TCS	TCC
Calumet 1	4.4 ± 0.4	27.4 ± 0.8
Calumet 2	3.9 ± 0.9	21.3 ± 1.9
Stickney 1	7.4 ± 0.6	24.7 ± 2.1
Stickney 2	9.4 ± 1.3	15.4 ± 0.4
1	N/D	9.80 ± 0.1
3	11.9± 1.1	11.2 ± 0.4
4	2.78 ± 0.2	0.25 ± 0.1
5	10.1 ± 1.0	14.5 ± 2.4
6	1.81 ± 0.2	8.35 ± 0.5
7	1.91 ± 0.1	8.61 ± 0.8
8	4.59 ± 1.3	19.1 ± 1.4
9	1.61 ± 0.2	4.01 ± 0.7
10	2.41 ± 0.6	18.7 ± 3.7
11	11.5 ± 1.3	43.1 ± 0.4
12	0.75 ± 0.1	11.1 ± 0.7
13	0.61 ± 0.0	6.78 ± 0.5
14	7.64 ± 0.7	20.8 ± 1.6
15	N/D	1.24 ± 0.1
17	0.62 ± 0.1	6.07 ± 1.3
18	1.96 ± 0.0	62.3 ± 6.1
19	0.33 ± 0.2	0.23 ± 0.2
20	13.6 ± 0.6	17.1 ± 2.7
21	9.14 ± 0.7	12.3 ± 1.2
From Co-A	61.2 ± 0.35	55.2 ± 2.1
From Co-B	18.8 ± 0.6	17.7 ± 0.9

2.5.9 Analysis of TCS and TCC in biosolids samples

A large number of biosolids collected from sixteen WWTPs in the United States were analyzed using the developed method. The information regarding the biosolids and biosolids compost samples collected is given in Table 2.4. As shown in Table 2.9, TCS and TCC were detected in all the biosolids samples analyzed. Triclosan was detected above the detection limit in most of the biosolids samples analyzed. The highest concentration detected was 61 mg/kg and the lowest was 0.33 mg/kg for TCS. Triclocarban was also detected at concentrations higher than the analytical detection limits, the highest TCC concentration detected was 80 mg/kg and the lowest was 0.23 mg/kg.

No significant difference was observed between the concentrations of TCS and TCC in WWTPs employing aerobic or anaerobic treatments. All compost biosolids showed reduced concentrations of TCS and TCC, which suggest that transformation of TCS and TCC, can occur in the biosolids compost.

2.6 Conclusions

In this study, a method using sonication extraction coupled with LC-ESI-MS detection was developed to simultaneously quantify TCS and TCC concentrations in soils and biosolids. The sonication extraction method does not require using expensive extraction instrument such as ASE that is used by method developed by Kinney et al [155] and Chu et al.[88] Until now there are only two research articles for simultaneous determination of TCS and TCC in biosolids-applied soil and biosolids. Chu et al., developed a method for extraction of TCS and TCC in municipal biosolids in the Canada, they used ASE extraction for TCS and TCC, followed by SPE clean up and analysis using LC-ESI-MS/MS.[88] In this method they were not able to achieve complete

separation of TCS and TCC. The method developed in this study was able to completely chromatographically resolve both TCS and TCC. No additional clean up of the sample extract was required for the LC/MS analysis. In their studies they detected TCS and TCC in sludge and treated biosolids collected from four WWTPs in Canada and found their concentrations to be in the range of 0.62-11.55 mg/kg for TCS and 2.17-5.97 mg/kg and TCC.[88] The method developed by Cha et al., for analysis of TCS and TCC in agricultural soil amended with biosolids was also based on ASE extraction, no clean-up method was employed after the extraction, and TCS and TCC were ionized by two separate ionization sources using LC/MS for detection. Triclosan was ionized using atmospheric pressure chemical ionization (APCI) and TCC was ionized with ESI. They also found that TCC was present in higher concentration compared to TCS. They detected TCC in biosolids at concentration range of 4.8 mg/kg – 9.3 mg/kg, while TCS concentrations ranged from 0.09 mg/kg – 7.1 mg/kg.[85]Ying et al., determined concentration of TCS in biosolids collected from 19 Australian WWTPs. The concentration of TCS ranged from 0.09 mg/kg – 16.79 mg/kg. They used GC-MS method for detection of TCS in wastewater effluents and biosolids. TCS was extracted from wastewater using C_{18} SPE and sonication extraction followed by C_{18} SPE for biosolids samples.[84] The results obtained in this study are in agreement with Ying et al. In another study Kinney et al., determined the concentration of TCS in biosolids samples collected from seven WWTPs across the US. They used LC-ESI-MS and GC-MS for analysis of TCS, the biosolids samples were extracted using ASE extraction and SPE cleanup method. The concentration of TCS determined in the collected biosolids ranged from 1.2 to 33 mg/kg.[155] In our study the highest concentrations observed for TCS and TCC in biosolids were 61 and 80 mg/kg, respectively. The concentrations observed in our studies are higher than found in other

studies. This difference can arise because of different WWTPs from which biosolids are collected. WWTPs located in areas which large populations can receive higher input of TCS and TCC leading to higher detection concentrations. Also the different treatment employed in WWTPs can result in different removal efficiencies of these compounds from wastewater influents, resulting in higher or lower concentrations of these compounds in biosolids.

Studying the fate of TCS and TCC in environment is necessary because of the concerns of these compounds leaching in the ground water supplies and surface runoff during rainy weather. Another consequence is the bioaccumulation of these compounds following the widespread practice of biosolids land application. Our studies found that TCS and TCC are almost ubiquitous in all the biosolids applied soil and biosolids samples. Lower concentration of TCS and TCC in 24-48 inch depth compared to top soil layer suggest that both compound tend to accumulate in soil surface. Triclosan and TCC were also detected in lower concentration in biosolids compost, which suggest that the significant transformation of TCS and TCC can occur in biosolids compost. In this study, detected concentrations of TCC were much higher than that of TCS, which suggest that TCC might be more persistent in the environment when compared with TCS. A lot of information is available regarding transformation and bioaccumulation of TCS but not much data exists for TCC. There is a much higher need to direct attention towards the toxicity, transformation and bioaccumulation of TCC. Previous studies suggested that half-life of TCS in aerobic soil is about 18 days, while it is 108 days for TCC, under anaerobic conditions the degradation slows down ever further.[103] Longer half-life of TCC can be the reason for the higher concentrations of TCC found in all the samples analyzed in our studies.

The results obtained in this study were comparable with other studies. The developed method is easy, sensitive, and fast and no cleanup is required before LC/ESI/MS analysis. Due to the complexity of the biosolids matrix expensive instruments such as LC/ESI/MS and GC/MS are the methods that are currently employed for determination of TCS and TCC. Instruments like LC/MS provides a sensitive method for the detection of these compounds in the presence of interfering compounds present in matrix. LC-UV lacks sensitivity, and interfering compounds from matrix makes detection and quantification of these compounds difficult. A method that is able to achieve good sensitivity and quantification using LC-UV would be an economical solution for analysis of TCS and TCC.

CHAPTER III

ANALYSIS OF TRICLOSAN AND TRICLOCARBAN IN BIOSOLIDS-APPLIED SOIL AND BIOSOLIDS USING MOLECULARLY IMPRINTED SOLID PHASE EXTRACTION (MISPE) COUPLED WITH HPLC-UV

3.1 Introduction

To this day, trace levels of TCS and TCC in the environmental samples such as wastewater, soil, and biosolids have been analyzed using expensive instruments such as liquid chromatography-tandem mass spectrometry (LC/MS/MS) [88] or gas chromatography-mass spectrometry (GC/MS).[81] No method exists in the literature which utilizes less expensive liquid chromatography coupled with ultraviolet detection (HPLC/UV) to detect those compounds in complicated environmental samples. This is largely due to the fact that there is a lack of efficient extraction and cleanup methods to remove the matrix compounds that chromatographically interfere with the target compounds analysis at trace levels.

Chu and Metcalfe developed a LC/MS/MS method for simultaneous quantification of TCS and TCC in biosolids using stable isotopes of TCS and TCC as internal standards.[88] The target compounds were first extracted by pressurized liquid extraction and then cleaned up using Oasis^{TM} HLB solid phase extraction (SPE) before analyzed on LC/MS/MS using negative electrospray ionization mode. The method developed by Chu and Metcalfe provided mean recovery of 98 ± 6 % for both TCS and TCC-spiked biosolids and method detection limits of 1.5 and 0.2 μg kg^{-1} for TCS and

TCC, respectively. The method used by Halden and Paull [82] to analyze TCS and TCC in surface water samples was similar to that developed by Chu and Metcalfe [88]. Recovery rate of 95 ± 9 % was achieved with method detection limits ranging from 3 to 50 ng L^{-1} depending on the matrix.[81, 82, 88] McAvoy et al, developed methods for extracting and cleanup of TCS from wastewater samples using C_{18} SPE and extraction of TCS from digested sewage sludge samples using supercritical fluid CO_2 without further cleanup.[81] The TCS in the extracted samples was derivatized and analyzed using GC-MS. Recovery rates of 79 ± 21 % and 84 ± 21 % were achieved for TCS-spiked wastewater and sewage sludge, respectively, with limits of quantitation at 100 ng L^{-1} for the wastewater and 70 μg kg^{-1} for the sewage sludge. In a method developed by Ying and Kookana [84], TCS was extracted and cleaned up from surface water and wastewater samples using C_{18} SPE and from biosolids using sonication followed by C_{18} SPE cleanup. The TCS in the final extracts was further derivatized into its trimethylsilyl derivatives and analyzed using GC/MS. The limits of quantitation of this method was 3 ng L^{-1} and 5 μg kg^{-1} for the water samples and biosolids, respectively, with recoveries of 101–104 % for surface water, 89–96 % for wastewater, and 73–74 % for biosolids.

When a mass spectrometry detector is coupled with GC or HPLC to analyze TCS and TCC in complex environmental samples [81, 84, 88], matrix interferences can be corrected mainly by isotope dilution method with ^{13}C-labeled internal standards and by using single ion monitoring mode when collecting mass spectra. Therefore, when GC/MS or LC/MS are used, effective extraction and cleanup of samples is not as critical for sensitive analysis of trace level analytes as when using GC or HPLC coupled with detectors other than mass spectrometry detectors.

The sorbent used in Oasis™ HLB SPE cartridges is a macroporous copolymer made from a mixture of a hydrophilic monomer (N-vinylpyrrolidone) and a lipophilic monomer (divinylbenzene) to provide both polar and non polar interactions.[166] The sorbent used in C_{18} SPE cartridges consists of octadecyl (C_{18}) functional groups bonded to the silica surface to provide non polar interactions.[166] The principle of both types of sorbents is based on hydrophobic or hydrophilic interactions between the analytes and the sorbent and rather non-analyte specific in nature. However, when used for extraction and cleanup of trace level analytes in complex environmental samples, co-extraction of interfering compounds with similar hydrophobicities to the target analytes is likely to happen, making it difficult for analysis using chromatographic instruments coupled with less expensive non-mass spectrometry detectors such as HPLC with UV detection. Because of wide spread use of TCC and TCS in a variety of consumer products, both compounds are frequently detected in environmental samples. Instruments such as GC/MS and LC/MS are fairly expensive and rarely available in a common environmental research or monitoring laboratory. Therefore, for the routine environmental monitoring purpose, inexpensive instrumentation such as HPLC/UV is highly desirable. Because UV detection often lacks the required selectivity and sensitivity, an efficient extraction, cleanup, and pre-concentration step before the HPLC/UV detection is extremely critical for reaching the detection limits that meet the regulatory requirements for screening and monitoring of environmental samples.

One approach to increase extraction and cleanup efficiency for trace level analytes in environmental samples is to employ analytes selective and specific sorbents for SPE. In recent years, molecular imprinting polymers (MIPs) have shown a great potential to be used as selective sorbents for target analytes in environmental samples.

Various MIPs have been developed as compound selective SPE sorbents for extraction and cleanup of pesticides [167], pharmaceutical compounds [168, 169], polycyclic aromatic hydrocarbons [170], mycotoxins [171], and methylmercury [172, 173] in environmental samples.

The objective of the present study was to develop a MIP sorbent for selective extraction, cleanup, and pre-concentration of trace levels TCS and TCC in complex environmental matrices such as biosolids-applied soil and biosolids prior to their analysis using HPLC-UV.

3.2 Molecularly imprinted polymers

Wulff in 1970's introduced molecularly imprinted polymers, which were later prepared by Mosbach in the 1980's and since then it has proved to useful in preparing tailor made polymers for various applications which require selective analyte recognition.[174]

Molecular imprinted polymers are cross linked macromolecules bearing "tailor-made" binding sites for target molecules.[166, 172] They are prepared by polymerizing functional and cross-linking monomers around a template molecule which is a target analyte or a compound physically and chemically similar to the target analytes, leading to a highly cross-linked three-dimensional network polymer.[172, 175]

The presence of complementary interactions, noncovalent or reversible covalent, between the template molecules and the functional monomers used in the polymerization process is the base for the molecular imprinting.[175] Upon removal of the template molecule from the polymer network, specific recognition sites that are complementary to the target analyte in terms of their size, shape, and functionality are established. The resulting imprinted polymers are stable, robust and resistant to a wide range of pH,

solvents and temperature [172]. Factors such as functional monomer, polarity of the porogen solvent and template molecule, polymerization conditions affect the specificity and efficiency of the MIPs constructed.[176]

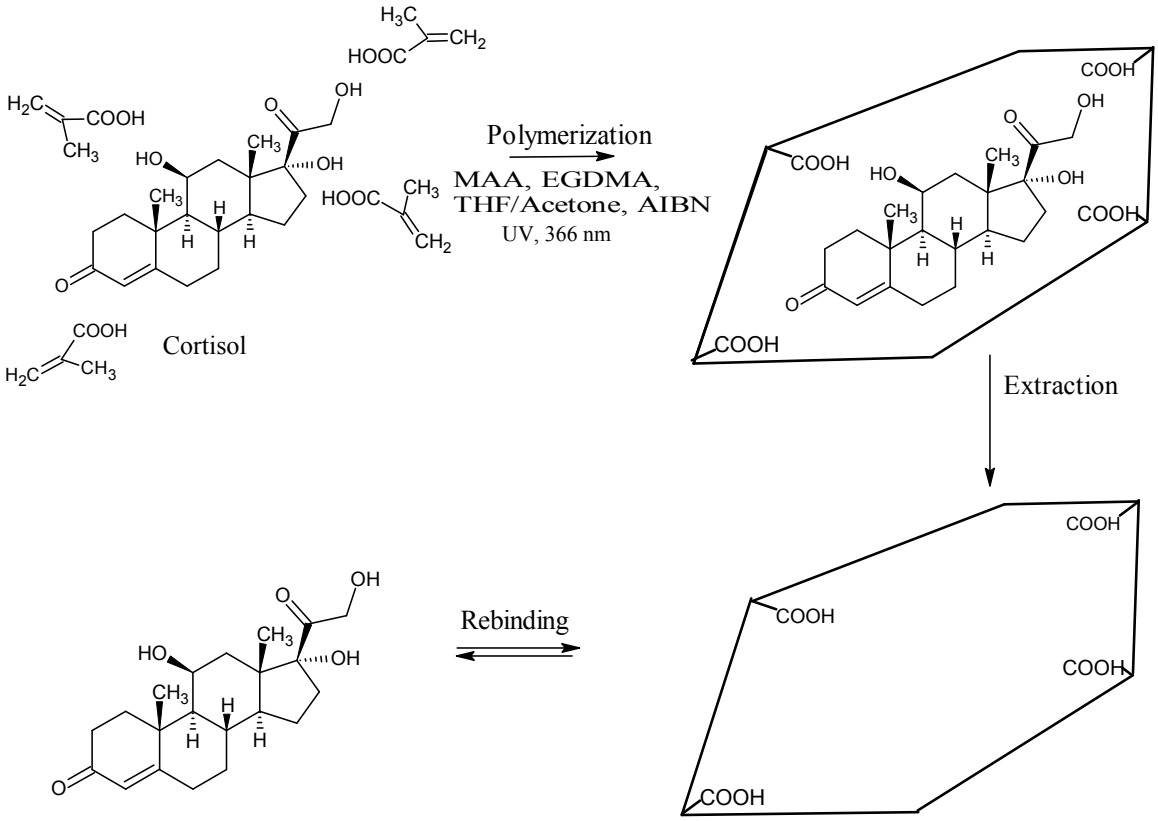


Figure 3.1 A schematic representation of the non-covalent approach for synthesis of MIP for Cortisol , using porogen, tetrahydrofuran or acetone; functional monomer , MAA; cross-linker, ethylene glycol dimethacrylate (EGDMA) and initiator, azobisisobutyronitirile (AIBN). Reproduced from reference.[177]

3.3 Molecularly imprinted polymer synthesis

MIP synthesis can be carried out by either covalent or non-covalent interactions between the template molecule and the functional monomer.[178]

3.3.1 Covalent imprinting

Covalent imprinting was first intoduced by Wulff and coworkers, but is not used frequently. It requires the analyte of interest to form reversible covalent bonds with the functional monomer, which limits the use of this apporach.[176] This approach yeilds highly specific MIPs selective to the target analyte. After polymerization the template needs to be chemically cleaved from the highly crosslinked polymer. Covalent imprinting yeilds highly selective MIPs but has more limitations than advantages.

- Slow binding kinetics is not suitable for chromatographic separations
- Reversible covalent interactions does not necessarily happen between the template and the functional monomer.[178]

3.3.2 Non-covalent imprinting

Non-covalent imprinting was introduced by Mosbach and coworkers, and is the most employed imprinting approach to date.[179] It relies on non-covalent interactions such as hydrogen bonding, hydrophobic interactions, and electrostatic interactions. This approach offers several advantages such as ease of preparation, because the template molecule forms complexes with functional monomers by self-assembly during the pre-polymerization step using non-covalent interactions.[178] The template removal step is easier compared to covalent imprinting, which involves several washing steps with appropriate washing solvent. The possibilities are endless with non-covalent imprinting because of the large number of templates capable of forming non-covalent bonding with the functional monomers. Despite all the attractive advantages non-covalent imprinting has to offer, it does suffers from several limitations.[176]

- A hydrophobic environment is required for the formation of self-assembly non-covalent complexes between the template and functional monomer. A

polar environment disrupts the non-covalent interactions responsible for this type of imprinting.

- The large number of functional groups on the template, the better is the interaction between the template and functional monomer, which leads to high molecular recognition.

Figure 3.1, shows the general approach for non-covalent imprinting used in MIP synthesis. In non-covalent imprinting the first step involves formation of the monomer and template complex by self-assembly process. The self-assembly process employs interactions such as hydrophobic, ionic and hydrogen bonds. In the next step polymerization is carried out using thermal or UV initiation followed by extraction of the template from the polymer using extensive washing with solvents. Molecular recognition is based on rebinding of the target compound selectively to the cavities formed during polymerization.[180] Molecular recognition diminishes in polar solvents, which interfere with complex formation. Also to ensure complexation of all the target molecule functional groups excess quantities of functional monomers are used. As a result functional groups of the polymer used are not always involved in complex formation with the template and gives rise to non-specific interaction.[181]

3.4 Methods

Reagents and chemicals

- *Triclosan (TCS)* – 97 % purity (CAS # 3380-34-5), Sigma-Aldrich (Stylus, MO).
- *Triclocarban (TCC)* – 99 % purity (CAS # 101-20-2), Sigma-Aldrich (Stylus, MO).
- *4, 4'-dichlorobenzophenone (4,4'-DCB)* – 99 % purity (CAS # 90-98-2), Alfa Aesar (Ward Hill, MA).
- *4-vinylpyridine (4-VP)* – 95 % purity (CAS # 100-43-6), Sigma-Aldrich (St.Louis, MO).
- *Ethylene glycol dimethylacrylate (EGDMA)* – 98 % purity (CAS # 97-90-5), Sigma-Aldrich (St.Louis, MO).
- *Azobis(isobutyronitrile) (AIBN)* – 98 % purity (CAS # 78-67-1), Sigma-Aldrich (St.Louis, MO).
- *Acetonitrile, acetone, methanol, dichloromethane, toluene and water* -- HPLC grade, Fisher Scientific (Fair Lawn, NJ, USA).

All the reagents were used in our experiments without further purification.

3.4.1 Apparatus

- *Double beam UV spectrometer* - Model Lambda 25, (Perkin Elmer, Shelton, CT).
- *Rotary vacuum evaporator*. – Rotavapor RE 121 (Büchi, Schweiz, Switzerland)

- *Solid phase extraction* **(***SPE)*. – BAKERBOND SPE Octadecyl C18, 1000 mg, 6 mL (J.T.Baker, Phillipsburg, NJ, USA);
- *Accelerated solvent extractor (ASE)*. **–** ASE 200 (Dionex, Sunnyvale, CA, USA).
- *LC system***. –** The Waters Alliance 2695 Separations Module (Waters, Milford, MA, USA) is equipped with quaternary, low pressure mixing pump and inline vacuum degasser and a photodiode array detector with a wavelength range of 190-800 nm.
- *MS detector*. – Waters Quattro-Micro Mass spectrometer (Micromass, Manchester, UK) is a triple quadrupole mass spectrometer equipped with Electrospray ionization source (ESI).
- *Analytical column*. **–** Xterra (C_{18} reversed phase, particle size 5µm, 4.6 × 150 mm id, Waters, Milford, MA, USA).

3.4.2 Samples

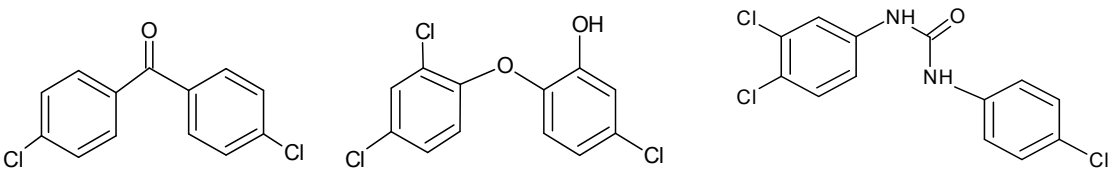
- *Soil* – surface soil (top 20 cm) was collected from Oktibbeha County, Mississippi (Marietta fine loam, organic matter content (wt %): 1.8%; pH (1:1 soil: water): 7.8; CEC (meq/100 g): 17.3; sand (wt %): 40; silt (wt %): 34; clay (wt %): 26).
- *Biosolids* - obtained from a secondary wastewater treatment plant with aerobic digester in California.
- *Spiked soil and biosolids* – 0.5 mL of 100 ppm and 1000 ppm standard containing both TCC and TCS dissolved in acetonitrile was homogenously mixed with 50 g soil and biosolids, respectively to achieve a spiked

concentration of 1 and 10 mg kg^{-1} for each compound in the soil and biosolids, respectively. The spiking level for the biosolids was higher than that in the soil because the biosolids control already had certain amount of TCS and TCC.

3.4.3 Polymer preparation

The imprinting effect is generally a result of non-covalent or reversible covalent interactions between a target analyte or template and functional monomers.[182] Functional monomers that do not complex with a template will result in MIP with non-specific binding sites for a target analyte, while complexing functional monomers will create highly selective specific binging sites in the MIP.[183] Hence, in the current study, the optimum functional monomer to be used for constructing the MIP was selected based on the molecular-level interactions between the target analytes and the monomer, revealed by UV spectroscopic studies using method described in the literatures.[175, 182] Briefly, 1 mL TCC or TCS dissolved in chloroform at 0.1 mM was separately mixed with 1 mL of the four tested monomers (Table 3.1) dissolved in chloroform at concentrations of 0.4 mM. After mixing the target analyte solution and the monomer solution, the mixture was allowed to rest in a refrigerator for 30 min. before the UV spectra of the mixture, TCC or TCS only, and monomer only solutions were measured using a double beam UV spectrometer (Lambda 25, Perkin Elmer, and Shelton, CT). UV baseline correction was done with respective concentrations of monomer without the target molecule. Chloroform is typically used as a solvent for testing the existence of hydrogen bonding between monomer and target compound during the UV experiment because chloroform does not interfere in hydrogen bonding.[175]

Since TCS and TCC in environmental samples are normally at trace levels, both compounds are not suitable as template because of trace level template bleeding when using MIP as SPE sorbent. It has shown that traces of template remain in the MIP network even after exhaustive washing by different methodologies.[184] To prevent the template bleeding problem, 4, 4'-DCB, a structural analog of TCS and TCC (Figure 3.3), was used as template. Our previous investigation has shown that 4, 4'-DCB does not interfere with the quantification of TCS and TCC. Molecular imprinted polymer and the non-imprinted polymer (NIP) as control were prepared via thermo-polymerization using procedures modified based on method by Schwarz et al.[185] Briefly, 0.315 g of 4-VP (functional monomer), 0.251 g of 4, 4'-DCB (template), 2.97 g of EGDMA (cross linker), 0.05 g AIBN (initiator), and 3.2 mL of dichloromethane (porogen) were mixed in a glass vial and sparged with N_2 gas for 5 min. The thermo-polymerization was carried out at 60^{o} C for 24 h. Following the polymerization, the glass vial containing the solidified MIP was then crushed. The recovered MIP was wet ground with 1 mL acetone with mortar and pestle and sieved to a particle size of < 45 µm under fume hood. The template was removed from the MIP particles by sonicating the MIP particles in 50 mL of methanol for several times until no visible peak was observed for 4,4'- DCB on HPLC-UV (λ_{max} = 264 nm). After removal of the template, the MIP particles were mixed with 20 mL of acetone and the MIP sediment was collected for later construction of SPE cartridges. As a comparison, the NIP was prepared and cleaned up using the above described protocol for MIP preparation but without template addition.



4, 4'-Dichlorobenzophenone TCS TCC

Figure 3.2 Molecular structures of 4, 4'-DCB, TCS and TCC.

3.4.4 Preparation of molecular imprinting solid phase extraction (MISPE) cartridges

Used empty SPE syringe barrels (capacity 6 mL) were prewashed with methanol. A 20 µm polyethylene frit was placed at the outlet of each empty SPE syringe barrel before it was connected to a vacuum manifold. A quantity of 400 mg of the MIP or NIP was slurry packed with methanol into the syringe barrels under vacuum (-10 mm Hg) and let stand under vacuum condition for 1 min. to ensure tight packing. Upon completion of packing, a second 20 µm polyethylene frit was placed on the surface of the polymer packed in each syringe barrel. Each prepared MISPE or non-imprinting SPE (NISPE) cartridge was rinsed without vacuum with methanol (2 volumes of SPE) and allowed to slowly drip.

3.4.5 Evaluation of the effect of loading solvents on TCS and TCC binding to the MIP

The loading solvent plays an important role in allowing the re-binding of the target analytes to the specific sites in MIP. If the complex formation between the template molecules and monomers during the polymerization is driven by hydrogen bonding, using polar solvents during the loading process can have diminished memory effect on the binding capacity of the imprinted polymer.[185] Usually, the solvent in which the polymer is prepared, also referred as porogen solvent, exhibits the most binding effect

for target analytes although deviation from this rule has been reported and is also observed in our experiments (see, results and discussion). To test the solvent effect on the loading capacity of the MIP and NIP, separate 1 mL of dichloromethane, toluene, or acetonitrile each containing TCS and TCC was mixed with 50 mg of MIP or NIP at loading capacities of $2x10^{-4}$, $1x10^{-3}$, $2x10^{-3}$, and $1x10^{-2}$ mg analyte/mg polymer in a 2 mL HPLC vial and equilibrated for 1 hr. Chloroform was not tested as a loading solvent because it caused significant swelling in the polymer, resulting in clogging in the MISPE. After 1 hr, the vials were centrifuged and supernatant was analyzed for TCS and TCC by HPLC/UV to determine the bound efficiency (% bound) of the two compounds to the MIP and NIP using the following equation:

$$\% \text{ bound} = \frac{\text{amount added - amount measured in supernatant}}{\text{amount added}} \text{ x } 100\ \% \qquad (3.1)$$

3.4.6 Evaluation of TCS and TCC washing and elution conditions from MISPE

Solvents such as hexane, dichloromethane, and acetonitrile-water (70:30, v/v) were evaluated as washing solvents. Non-polar solvents are good solvents for removing organic matter from the extract. Once the loading solvent was chosen from the experiment described in the previous section, analyte standards prepared in 1 mL loading solvent containing 0.002 mg each TCC and TCS were loaded onto a MISPE containing 400 mg of the MIP and washed with 3 mL of the tested washing solution. The washing fractions were collected, evaporated, and reconstituted in 1 mL of acetonitrile and analyzed with HPLC-UV. Similarly, acetonitrile, methanol, and chloroform were evaluated as the eluting solvents, 10 mL of each tested solvent was used to elute TCC and

TCS off the MISPE cartridge and the collected fractions were evaporated and reconstituted in 1 mL of acetonitrile and analyzed using HPLC-UV.

3.4.7 Extraction, cleanup, and pre-concentration of TCC and TCS in spiked and non-spiked soil and biosolids samples using MISPE and C_{18} SPE

The TCC and TCS-spiked and un-spiked soil and biosolids were extracted, cleaned up, and pre-concentrated using MIP. Two g of soil or 1 g of biosolids was weighed into a 25 mL beaker. The target compounds were extracted three times with 10 mL acetone each time in a sonicator (15 min each step). The 3 extracts were combined and centrifuged at 3500 g for 10 min. before collecting the supernatant. The collected supernatant was evaporated to complete dryness using turbo evaporator at 40^{o}C and then re-dissolve in 11 mL mixture of dichloromethane:water:0.5 M $FeCl_3.6H_2O$ (5:5:1, v/v) and hand shaken for 5 min. The $FeCl_3.6H_2O$ was used to remove natural organic matter. After the hand shaking, the dichloromethane layer was collected and another 5 mL of dichloromethane was added to the aqueous phase and hand shaken for 5 min. The dichloromethane layer was collected and mixed with the previous dichloromethane fraction. The combined dichloromethane extract was evaporated to complete dryness, reconstituted in 1 mL toluene and loaded on MISPE. For comparison purpose, parallel extractions with the same samples were conducted except each dried dichloromethane fraction was reconstituted in 1 mL acetonitrile: water (50:50, v/v) and loaded on C_{18} SPE. The conditions used in the C_{18} SPE procedure were carefully optimized before this experiment. The MISPE cartridge was washed with 3 mL of dichloromethane and eluted with 10 mL methanol, while C_{18} cartridge was washed with 3 mL of acetonitrile: water (70:30, v/v) and eluted with 5 mL of acetonitrile. The optimum loading, washing, and eluting solvents for the MISPE were chosen based on the experiments described in

Sections 3.4.6 and 3.4.7. Each eluent was dried to complete dryness and reconstituted in 1 mL acetonitrile and analyzed with HPLC/UV. The results obtained were compared with the results obtained using LC-MS.

3.4.8 HPLC/UV and HPLC/MS conditions for TCS and TCC analysis

The HPLC/UV analysis was performed using Waters Alliance HPLC 2695 system coupled with a Waters Alliance 2990 UV detector (Waters, Milford, MA, USA). The analytical column was reversed phase Xterra (particle size 5µm, 4.6 × 150 mm id, Waters, Milford, MA, USA). The mobile phase was acetonitrile-water (70:30, v/v). The mobile phase flow rate was 1.0 mL min^{-1}. The UV detection wavelengths were 260 nm and 236 nm for TCC and TCS, respectively.

A separate Waters Alliance 2695 HPLC coupled with a micromass quattro micro triple- quadropole mass spectrometer (Micromass, Manchester, UK) was used for the LC/MS analysis. Same analytical column was used at that used for the HPLC/UV analysis. The mobile phases were acetonitrile containing 0.1 % formic acid (A) and water containing 0.1 % formic acid (B). The solvent gradient was set as following: 0 – 2 min, 10 % A, 2 – 25 min 10 % A to 90 % A, 25 – 35 min 90 % A to 10 % A. The mobile phase flow rate was 0.8 mL min^{-1}. For the mass spectrometry, electrospray negative ionization mode was used. The capillary voltage and the cone voltage were set at 3.50 and 22 kV, respectively. The source temperature and the desolvation temperature were set at 80 and 300 ^{o}C, respectively. The cone gas flow and the desolvation gas flow were set at 35 and 410 L h^{-1}, respectively. Ions with m/z 287 ($[M-H]^-$) for TCS and m/z 313 ($[M-H]^-$) for TCC were monitored using selected ion recording (SIR) mode. The UV and MS data acquisition and analysis was performed using MassLynx version 4.0 software.

3.4.9 Determination of detection limits and precision

The method detection limit (MDL) and limit of quantification (LOQ) for TCC and TCS were determined by spiking the soil and biosolids samples with a mixture of TCC and TCS standards. The samples were than extracted, cleaned up, and pre-concentrated using MISPE and analyzed by HPLC/UV. The MDL and LOQ were calculated according to method described in the Code of Federal regulations, Part 136, Appendix B [186]. The intraday precision of the method was tested by performing a set of experiments seven times at different hours of the day. The interday precision was tested by performing the same set of experiments for seven days and the results were reported as percentage relative standard deviation (RSD).

3.5 Results and discussion

3.5.1 Polymer preparation

Figure 3.6, shows that comparing to TCS, TCC, or 4,4'-DCB only or 4-VP only solutions, mixing TCS, TCC, or 4,4'-DCB with 4-vinylpyridine (4-VP) resulted in not only significant peak shift but also significant increase in peak intensity. The UV absorption spectrum for TCS exhibits a band with a maximum at 280 nm, while when TCS was mixed with 4-VP this band shifted to 260 nm and became four times more intense (Figure 3.6a). When TCC was mixed with 4-VP, the UV absorption band for TCC at 265 nm shifted to 260 nm with about 50% increase in intensity (Figure 3.6b). A similar peak shift is also observed when 4, 4'-DCB was mixed with 4-VP compared to 4,4'-DCB only (Figure 3.6c). This change of UV spectra is an indication of molecular interaction between the 4-VP and TCS, TCC, and 4, 4'-DCB. A similar UV spectra

change was also observed for TCS and methacrylamide mixture but not for TCC and methacrylamide mixture (Figure 3.3).

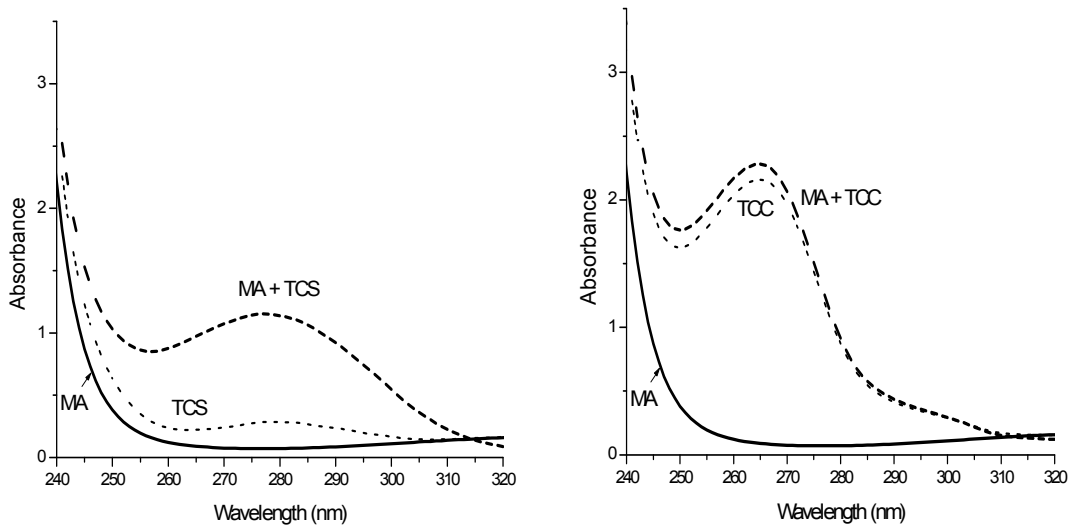


Figure 3.3 UV spectra for TCS, TCC, MA, TCS/MA (0.1 mM/0.4 mM) mixture and TCC/MA (0.1 mM/0.4 mM) mixture

No significant UV spectra change was observed for TCS and TCC when reacted with acrylamide (AA) monomer (Figure 3.4), indicating a lack of complexation between TCS or TCC with those monomers. Similar results were obtained when TCS and TCC were reacted with methylacrylic acid (MAA, Figure 3.5)

Based on the UV study, 4-VP was chosen as the functional monomer for constructing the MIP.

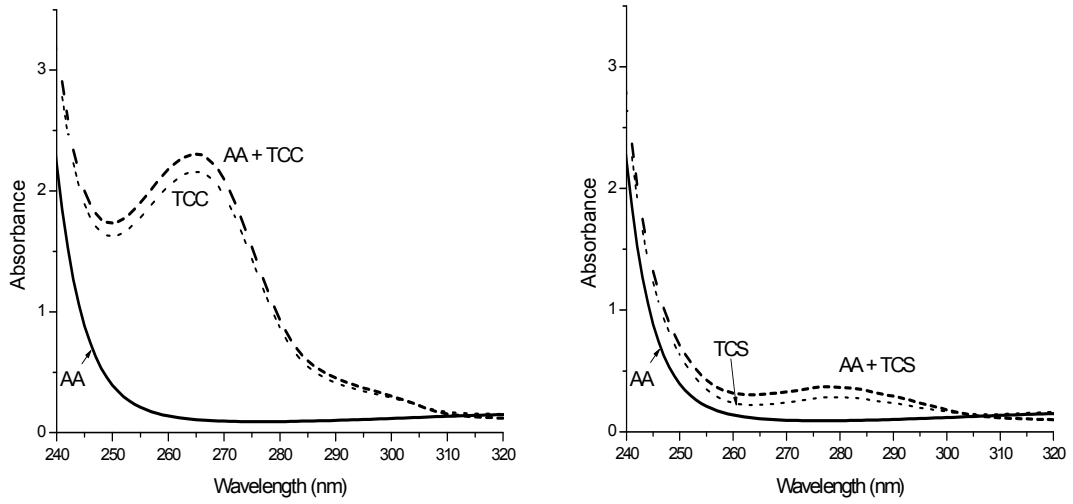


Figure 3.4 UV spectra for TCS, TCC, AA, TCS/AA (0.1 mM/0.4 mM) mixture and TCC/AA (0.1 mM/0.4 mM) mixture

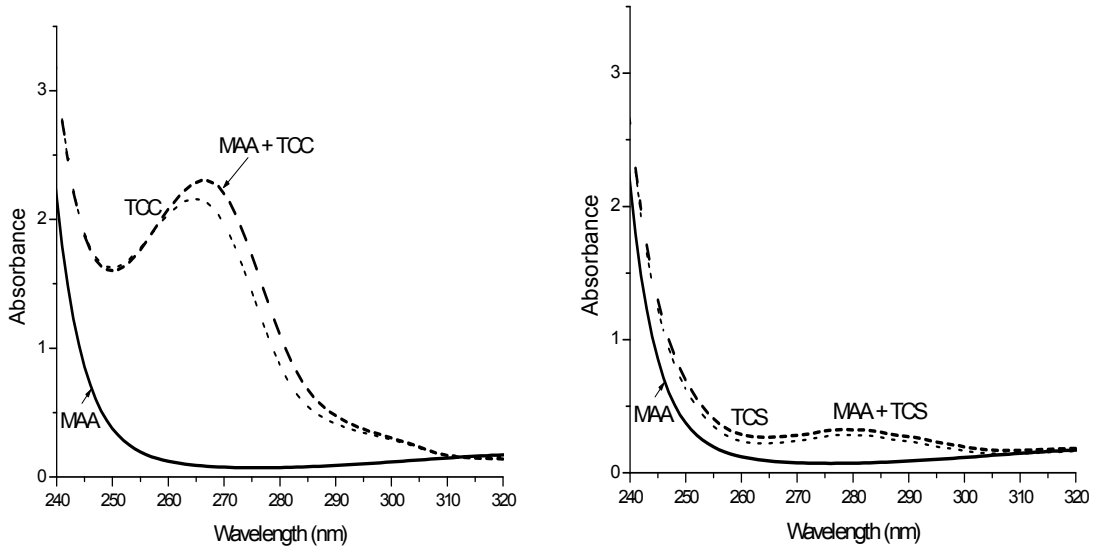


Figure 3.5 UV spectra for TCS, TCC, MAA, TCS/MAA (0.1 mM/0.4 mM) mixture and TCC/MAA (0.1 mM/0.4 mM) mixture

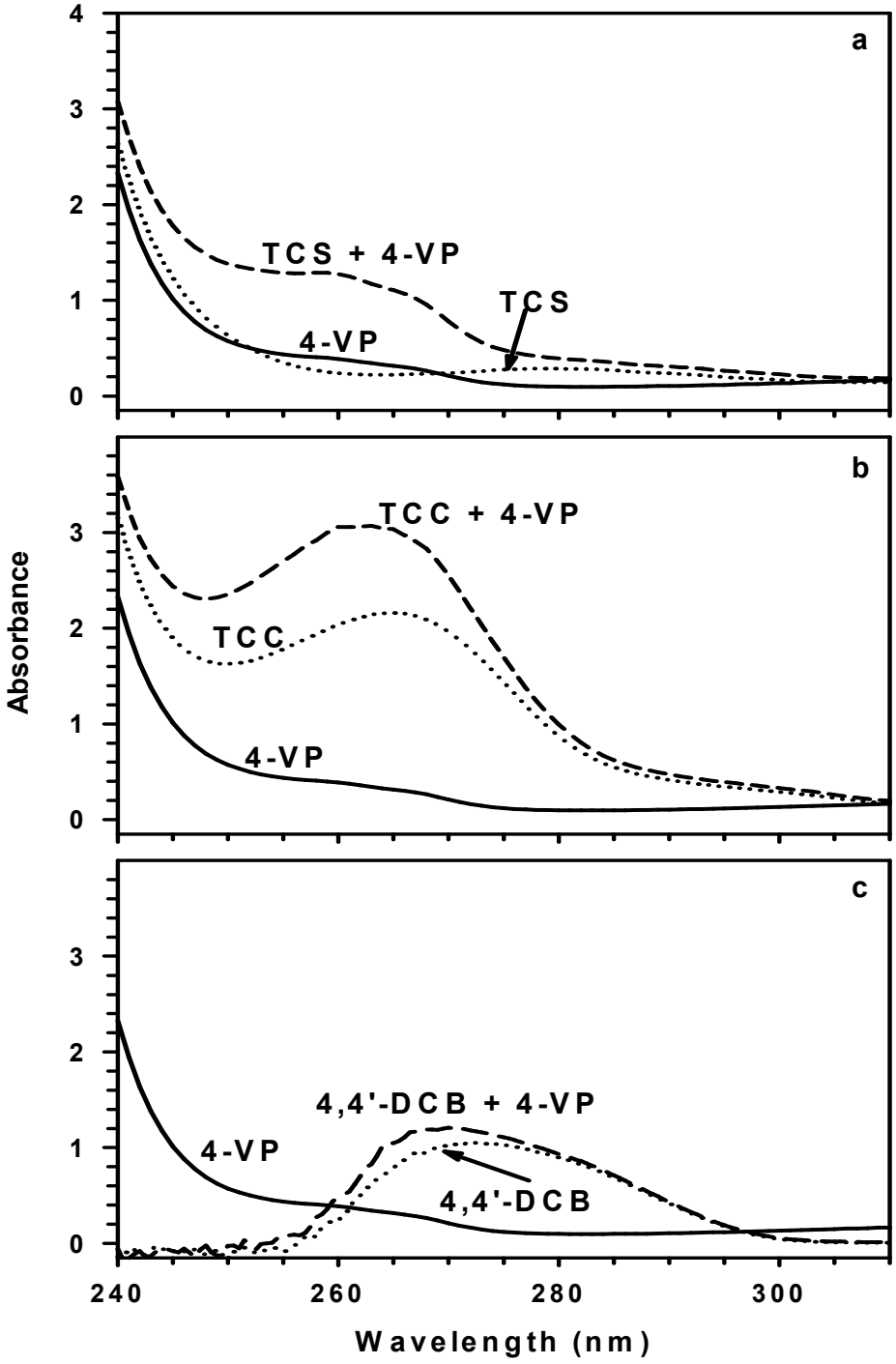


Figure 3.6 UV spectra for TCS, TCC, 4-VP, TCS/4-VP (0.1mM/0.4mM) mixture, TCC/4-VP (0.1 mM/0.4 mM) mixture, and 4,4'-DCB/4-VP mixture (0.1 mM/0.4 mM) mixture

Removal of template 4, 4'-DVB was achieved by continuous washing of MIP polymer with 50 mL of methanol, several wash steps were involved. The MIP was left overnight in the solvent and next day after 1 h of sonication, the solvent was removed by centrifugation at 3500 rpm for 15 mins. Figure 3.7, shows the percent removal of 4, 4;-DCB after each washing step. After eight washing steps 101 % of the 4, 4'-DCB was recovered. LC-UV was used to measure the concentration of template in the washing solution. NIP was also washed two times with methanol.

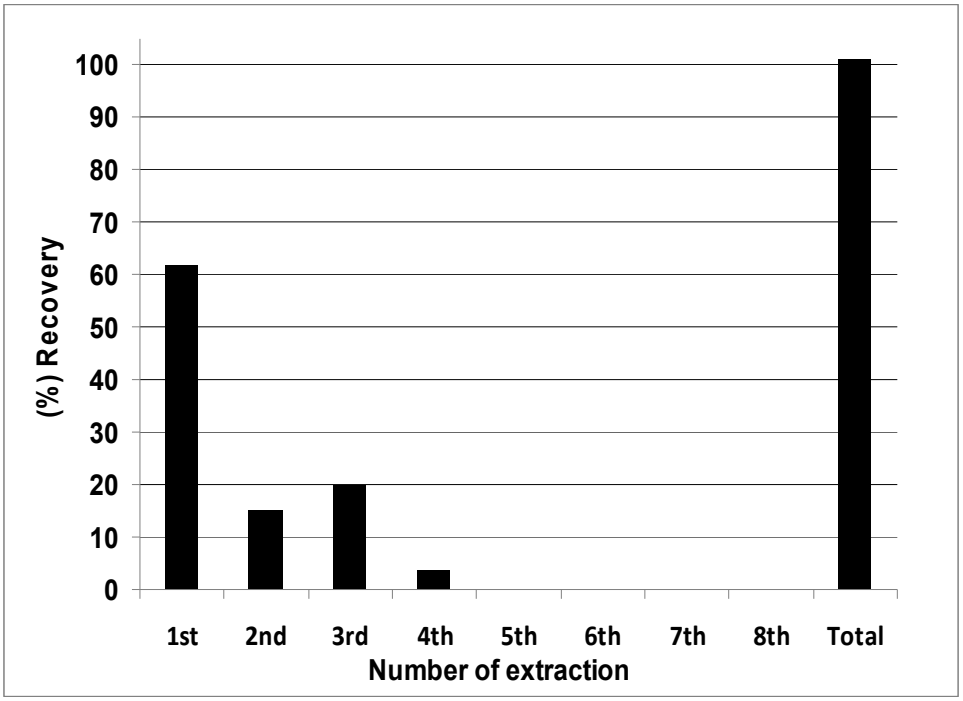


Figure 3.7 Removal of template from MIP.

3.5.2 Optimum loading, washing, and elution solvents for MIP

The optimum loading solvent in which target analytes are dissolved allows maximum rebinding of the analytes to specific binding sites created by the templates in the MIP. The ideal washing solution would remove the non-specifically adsorbed

interference compounds off the MIP without disrupting the analyte-MIP interaction. The elution solvent has to be optimized in order to disrupt the analyte-MIP interaction in order to remove the analyte(s) from the MIP for later instrumental analysis.

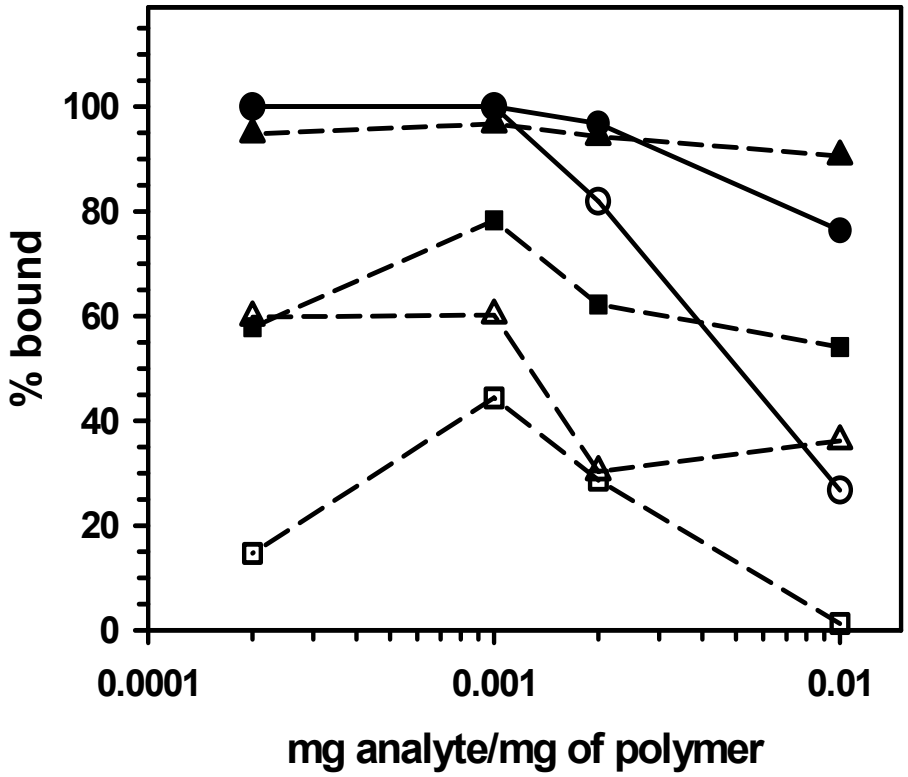


Figure 3.8 Effect of loading solvents toluene (circle), dichloromethane (triangle), and acetonitrile (square) on the retention of TCC (closed symbol) and TCS (open symbol) onto the MIP.

Figure 3.9, shows toluene is the best loading solvent comparing to dichloromethane and acetonitrile. Of the three loading solvents tested, toluene is the only solvent that was able to achieve 100 % bound efficiency for both TCS and TCC. However, the 100 % bound efficiency was obtained for TCS and TCC only when the analyte/MIP ratio was < 0.001 mg analyte/mg MIP. Above this ratio, the bound

efficiency decreased sharply. This indicates that the analyte/MIP ratio is a critical factor to be considered when using MISPE for sample cleanup and concentration. For example, for a MISPE containing 400 mg MIP, the maximum amount of TCC or TCS that can be loaded onto the MISPE would be 0.4 mg. This is equivalent to testing a quantity of 1 g of biosolids sample containing TCC or TCS at 400 mg kg^{-1}, a level much higher than that in a typical biosolids sample. For a sample with higher concentration, dilution of sample extract would be required in order to prevent overloading of an analyte on the MISPE.

Using toluene as loading solution, optimum washing solvent and elution solvent were selected by comparing the recovery rates of TCS and TCC in different washing and elution solvents (Table 3.2). Both TCS and TCC were not removed from the MISPE when using hexane or dichloromethane as washing solvents, while acetonitrile: water solution removed 83.2 % of the loaded TCS from the MISPE during the washing step. Three elution solvents were tested separately to extract TCC and TCS off the MISPE after the TCC and TCS-loaded MISPE was washed with dichloromethane. More than 95 % of the TCC and TCS were eluted with methanol, while poor recovery rates were achieved when using chloroform and acetonitrile as elution solvents (Table 3.2).

Table 3.1 Evaluation of washing and elution solvents for MISPE

Loading solvent 1 mL toluene , 2 ppm std mix		
Analyte	**TCC (% Recovery)**	**TCS (% Recovery)**
Washing Solvent		
Hexane	0.00	0.00
Dichloromethane	0.00	0.00
ACN: H_2O (70:30, v/v)	5.09	83.2
Elution Solvent		
Chloroform	0.00	0.00
Methanol	98.3	95.7
Acetonitrile	3.86	0.00

The performance of MISPE versus NISPE was tested using the optimized loading, washing and elution solvents.

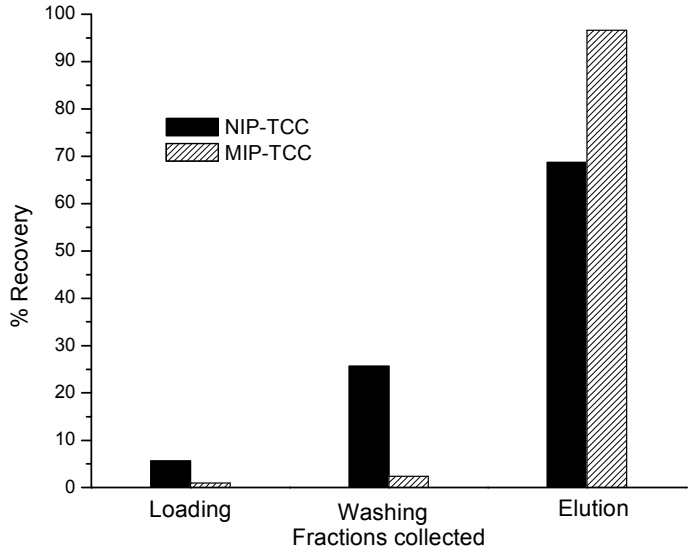


Figure 3.9 MISPE and NISPE of TCC using toluene (loading), DCM (washing) and methanol (elution) solvents.

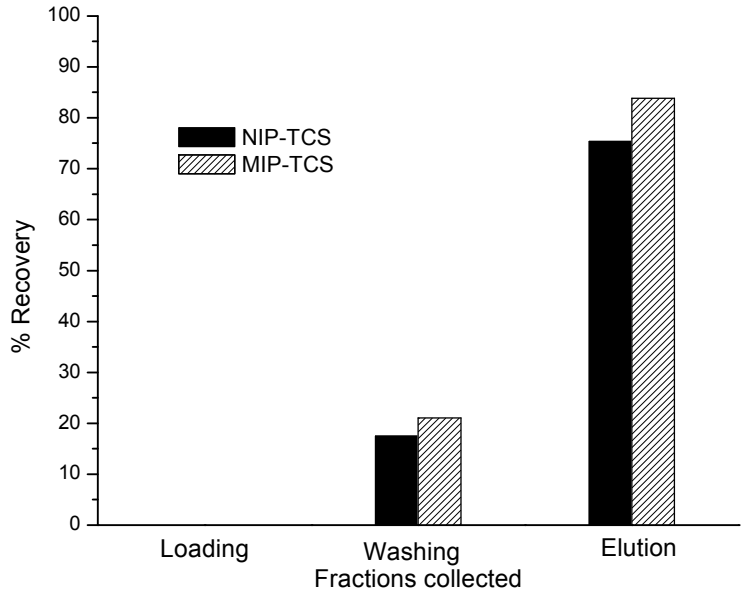


Figure 3.10 MISPE and NISPE of TCS using toluene (loading), DCM (washing) and methanol (elution) solvents.

As can be seen in Figure 3.10 & 3.11 TCC and TCS does not get completely eluted from NIP using polar solvent methanol, which suggests that mechanism of intersection of TCS and TCC with NIP is not similar to MIP. The polar solvent when used in MISPE interferes with hydrogen bonding, electrostatic interaction hence elution of target compounds takes place. However if the mechanism of intersection is not polar interaction the elution of target compounds will not take place as in the case with NIP. The adsorption of TCS and TCC to NIP is non-specific interaction which when eluted with polar solvent does not affect the adsorption.

3.5.3 Recoveries of TCC and TCS using MISPE and C_{18} SPE

The recoveries of TCC and TCS from spiked soil and biosolids samples were tested using MISPE and C_{18} SPE coupled with HPLC/UV. When using MISPE for cleanup of a TCC- and TCS-spiked soil before the HPLC/UV analysis, recovery rates of 88.3% and 83.1% were achieved for TCC and TCS, respectively (Table 3.3). A slightly lower recovery rates were accomplished for both analytes in the same spiked soil sample when using C_{18} SPE for cleanup. Figure 3.11, shows that when using MISPE for cleanup of a soil sample, HPLC/UV chromatograms with less background interference were achieved comparing to using C_{18} SPE for cleanup. For the TCC and TCS spiked biosolids sample, HPLC/UV quantification of TCS in the extract that was cleaned up using C_{18} SPE was impossible due to the appearance of interfering peaks at the same retention time of TCS (Figure 3.12).Only 43.3 % recovery rate was achieved for TCC when C_{18} SPE was used to clean up the extract of the spiked biosolids (Table 3.3). However, for the same spiked biosolids sample, 89.9 % and 82.0 % recovery rates were achieved for TCC and TCS, respectively, when using MISPE for extract cleanup before the HPLC/UV analysis (Table 3.3). The HPLC/UV chromatograms of TCC and TCS in the extract cleaned up with MISPE shows little background interference (Figure 3.12). Longer TCC and TCS retention times shown in Figure 3.12 compared to Figure 3.11 is due to replacement of a damaged column by a new column after the soil analysis.

Table 3.2 Recoveries of TCC and TCS in spiked soil and biosolids samples using MISPE and C18-SPE for cleanup before HPLC-UV analysis

	Mean % recovery (% RSD) for n = 7			
	Soil		Biosolids	
analyte	MISPE	C-18	MISPE	C-18
TCC	88.3 (3.3)	84.1 (6.7)	89.9 (7.7)	43.3 (8.3)
TCS	83.1 (2.7)	76.6 (2.5)	82.0 (8.9)	PO

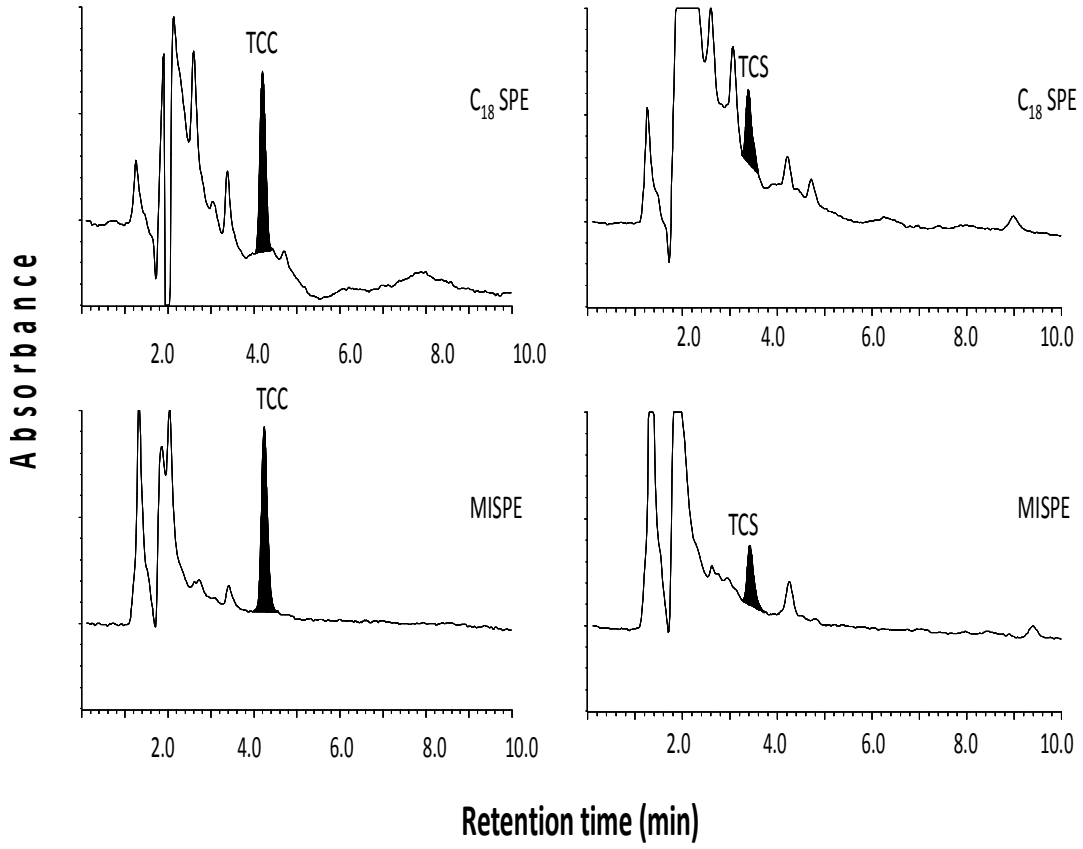


Figure 3.11 HPLC/UV chromatograms of TCC and TCS in extracts of a spiked soil sample (1 mg kg^{-1} for each compound) cleaned up using MISPE and C_{18} SPE before the HPLC/UV analysis. Change in retention time of TCS and TCC compared to other experiments is due to replacement of old damaged column.

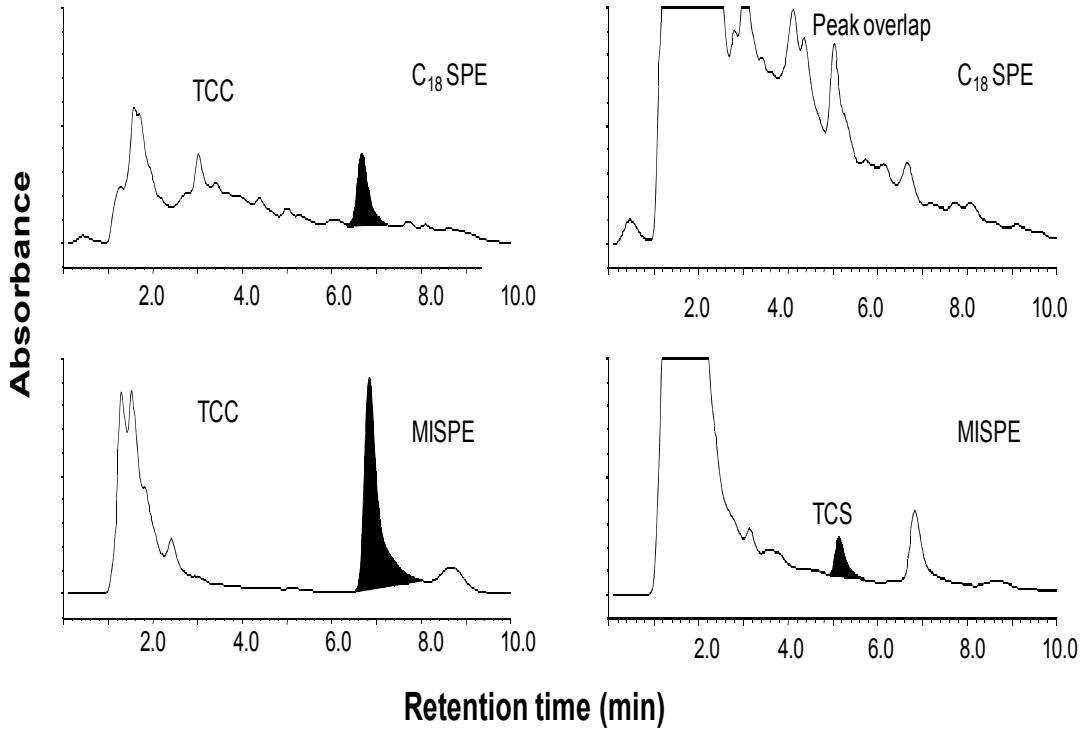


Figure 3.12 HPLC/UV chromatograms of TCC and TCS in extracts of a spiked biosolids sample (10 mg kg^{-1} for each compound) cleaned up using MISPE and C_{18} SPE before the HPLC/UV analysis.

3.5.4 Detection limits and precision

The instrument detection limit for the HPLC/UV was determined to be 20 μg kg^{-1} for both compounds. When using MISPE coupled with HPLC/UV, the LOQ values for both TCC and TCS in the soil samples were determined to be 40 μg kg^{-1}, while in the biosolids samples, the LOQ values were 100 μg kg^{-1} and 300 μg kg^{-1} for TCC and TCS, respectively. Although the LOQ values achieved using the current method of coupling MISPE cleanup with HPLC/UV analysis are higher than those that can be achieved using GC/MS or LC/MS methods, the developed method is less expensive and more feasible for laboratories that do not own expensive GC/MS or LC/MS.[84, 88]

The MISPE cartridges were subjected to 37 consecutive equilibrating, loading, washing, and elution processes without losing sensitivity. This is a big saving comparing to the one-time use of disposable C_{18} SPE or HLB SPE. Table 3.4, shows excellent intraday and interday repeatability of < 5% RSD for TCC and TCS analysis in a soil sample using MISPE coupled with HPLC/UV.

Table 3.3 Interday and intraday repeatability for TCC and TCS analysis in a soil samples using MISPE coupled with HPLC-UV.

Analytes	Interday (% RSD)	Intraday (% RSD)
TCC	4.3	3.3
TCS	2.2	2.7

3.5.5 Application of the MISPE to biosolids-applied soil and biosolids samples

The concentrations of TCC and TCS in biosolids-applied soil and biosolids samples were determined by coupling MISPE cleanup with HPLC/UV analysis. The results were compared with C_{18} SPE cleanup followed by HPLC/UV analysis. In addition, the extracts cleaned up by the MISPE or the C_{18} SPE was also analyzed using LC/MS as comparison. The obtained results are shown in Table 3.5. When the MISPE was used to clean up the extracts of soil samples and biosolids samples, the TCC and TCS concentrations determined by the HPLC/UV were not statistically different from those determined by HPLC/MS (Table 3.5). However, when C_{18} SPE was used for cleanup before instrumental analysis, for some of the biosolids samples there was a large discrepancy between the results determined by HPLC/UV and those determined by LC/MS analysis. For the soil samples, which have less complicated sample matrix than the biosolids, this discrepancy was not significant. The observed difference between the

HPLC/UV and HPLC/MS measurement of the biosolids samples might be caused by the matrix interference during the HPLC/MS analysis due to inefficient cleanup by the C_{18} SPE. Using the current method of coupling MISPE with HPLC/UV, it was determined that the TCC concentrations in the two biosolids-applied soils samples were 1.8 and 2.6 mg kg^{-1} (dry weight), and in the two biosolids samples were 8.6 and 18.3 mg kg^{-1} (dry weight). The levels of TCS in the two soil samples were below the detection limit, while 18.5 and 30.1 mg kg^{-1} (dry weight) were detected TCS in the two biosolids samples. Figure 3.13, shows the HPLC-UV chromatograms of soil samples cleaned up using MISPE and C_{18} SPE. TCS concentration detected was below detection limit using MISPE. However TCS peak showed overlapping when C-18 SPE was used as clean-up method. While TCC peak observed with HPLC-UV analysis after MISPE showed better recovery percentage and better cleanup when compared to C_{18} SPE. HPLC-UV chromatogram of biosolids sample is shown in Figure 3.14. Several peaks are observed when C-18 SPE is used as clean-up method. All the peak come later in the chromatogram which suggest that all the non-polar compounds are retained on the C-18 SPE and get eluted along with TCS and TCC during the elution step. While large discrepancy was observed between the LC-UV and LC-MS data when C-18 SPE was used for clean-up. However when MISPE was employed for clean-up of biosolids one large peak at the beginning of chromatogram is observed. This suggests that polar compounds are retained on the column by non-specific interaction, but organic compounds besides TCS and TCC are not retained. Hence only three peaks are observed after elution of polar compounds in the chromatogram. Two of the three observed peaks are for TCS and TCC and one unidentified peak is observed at retention time 7.50 min.

Table 3.4 Concentrations of TCC and TCS in soil and biosolids samples determined using MISPE or C_{18} SPE coupled with HPLC-UV and HPLC-MS

Sample	TCC				TCS			
	MISPE		C_{18} SPE		MISPE		C_{18} SPE	
	HPLC-UV	HPLC-MS	HPLC-UV	HPLC-MS	HPLC-UV	HPLC-MS	HPLC-UV	HPLC-MS
Biosolids-applied Soil (ppm,(% RSD))								
1	1.8(8.8)	1.8(15)	1.4(8.8)	1.3(5.1)	Nd	Nd	Nd	0.04(12.5)
2	2.6(6.5)	2.2(0.9)	0.9(1.2)	0.3(1.0)	Nd	Nd	Nd	0.02(10.0)
Biosolids (ppm, (% RSD)								
1	8.6(19.5)	8.2(15.0)	11.4(14.0)	55.1(3.7)	18.5(17.3)	17.6(11.2)	19.8(10.4)	61.1(0.57)
2	18.3(2.13)	19.9(8.5)	9.3(7.5)	17.7(5.14)	30.1(5.9)	28.9(6.3)	16.9(7.0)	18.8(2.9)
3	3.3(0.4)	2.9(13.7)	0.4(17.5)	1.2(9.2)	0.09(6.7)	Nd	Nd	Nd

The results obtained using MISPE and analysis using LC-UV and LC-MS agreed with each other (Table 3.4).

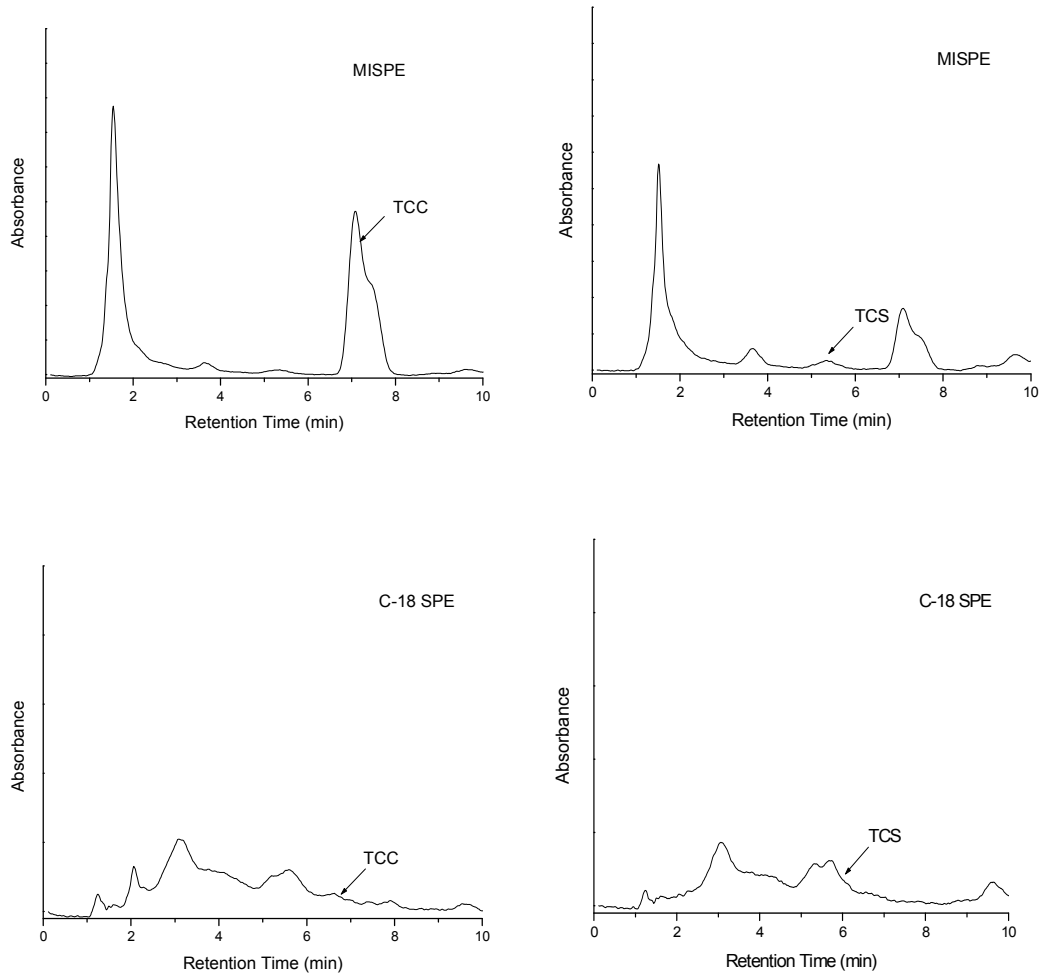


Figure 3.13 LC-UV chromatogram of MISPE and C_{18} SPE clean-up of soils samples at 260 nm for TCC and 280 nm for TCS.

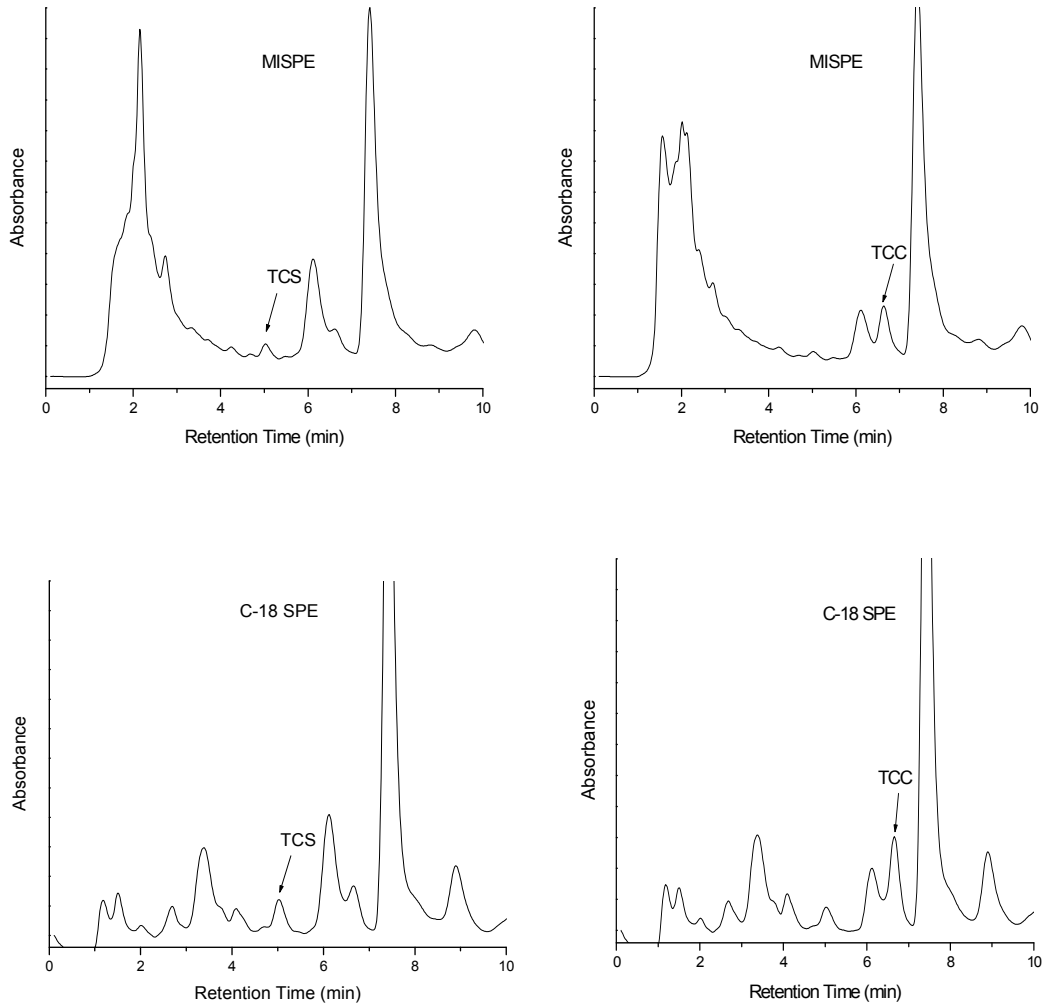


Figure 3.14 LC-UV chromatogram of MISPE and C_{18} SPE clean-up of biosolids samples at 260 nm for TCC and 280 nm for TCS.

3.6 Conclusions

A molecularly imprinted polymer (MIP) able to selectively bind triclosan (TCS) and triclocarban (TCC), commonly used antibacterial agents in many consumer products, was prepared using non-covalent molecular imprinting methods. The prepared MIP was evaluated as a selective sorbent in solid phase extraction for sample cleanup before HPLC/UV analysis of TCS and TCC in soil and biosolids samples. The MIP was also

compared with the commercially available C_{18} SPE sorbent. The molecular imprint solid phase extraction (MISPE) developed in this study was more efficient, compared with C_{18} SPE, to clean up extracts of soil and biosolids samples for analysis of TCC and TCS using HPLC/UV. The LOQ values for both TCC and TCS in the soil samples were determined to be 40 μg kg^{-1}, while in the biosolids samples, the LOQ values were 100 μg kg^{-1} and 300 μg kg^{-1} for TCC and TCS, respectively. Comparing to the C_{18} SPE, using MISPE for sample cleanup may result in significant reduction on analytical cost because: 1) one MISPE can be reused up to 35 times; and 2) HPLC/UV instead of HPLC/MS can be used for instrumental analysis following sample cleanup with the MISPE.

The results of this study proved that the MIP developed was adequate to be used for the solid phase extraction of soil and biosolids samples for analysis of TCC and TCS using HPLC/UV. Intraday and inter-day repeatability of < 5 % RSD was achieved using this method.

CHAPTER IV

OXIDATIVE TRANSFORMATION OF ESTROGENIC HORMONES BY FE (III) - SATURATED MONTMORILLONITE IN AQUEOUS ENVIRONMENTS

4.1 Introduction

There is an increased awareness amongst the society towards the risk that both natural and synthetic chemicals can pose by interfering with human and wildlife reproduction and development.[187] Limited information on how hormones transform in the environment is available, as are the identity and persistence of environmental transformation products. Recently, remediation methods have been developed for estrogens using various catalysts. Most of these methods produce transformation products that pose the same or higher environmental risks when compared to the parent compounds.[117, 151, 188] Ozonation and UV radiation are commonly employed, but are not practical when in the natural environment. Even though UV radiation emitted from sunlight is known to transform various organic pollutants in environment, low water solubility of estrogens and high water-octanol partition coefficient makes photo transformation difficult in aquatic environment, due to sorption to organic matter.[116] Bila and co-workers identified by – products such as 10ε-17β-dihydroxy-1,4-estradieno-3-one (DEO), 2-hydroxyestradiol and testosterone when ozonation of 17β-estradiol in aqueous solutions was carried out.[117] Similar transformation products were observed after 4 hrs of reaction between17β-estradiol and TiO_2.[149] TiO_2 is a photocatalysts known to oxidize most organic compounds to CO_2.[149] Another mineral used for oxidative transformation of

several organic pollutants is MnO_2. Jiang et al. used MnO_2 for transformation of E2 in aqueous solutions. Transformation products such as E1 and 2-hydroxyestradiol were produced.[151] Several factors affect the performance of oxidizing minerals, of which humic acid, pH and metal ions are a few of the important ones.[116, 147, 151, 189] Direct photolysis of E1 and E2 was carried out by Liu and group, in aqueous solutions. No products were reported, but a conclusion was drawn that the benzene ring in estrogen breaks down to produce carbonyl containing compounds, these results were based on the obtained IR spectrum.[145]

Estrogens are known to undergo the nitration process; several studies have shown biotic and abiotic nitration as important degradation process.[190, 191] The ammonia oxidizing bacteria (AOB) has been suggested to play an important role in the degradation of estrogens. This technique can be useful in wastewater treatment facilities because some WWTPs use nitrifying activated sludge treatments to remove various organic compounds. Gaulke et al, claimed that AOB oxidizes NH_4-N to NO_2-N , which than can react with estrogens to produce nitro estrogens.[192] Nitro-estrogens are shown to have lower estrogen activity compared to the parent estrogens.[115]

The clay-mediated organic synthesis is well documented in the literature.[193, 194] Yadav et al., carried out 4+2 cycloaddition reaction using montmorillonite clay for synthesis of pyrano and furanoquinolines. Yields higher than 85% were obtained. The advantage of using clay as heterogeneous catalyst is that milder reaction conditions, great selectivity, low cost, high yields and clean product extracts are achieved.[194] Nitration of estrogen with metal nitrates on clay offers a mild synthesis conditions. Cornellis et al., carried out nitration of E1 on clay-supported ferric nitrate and

synthesized 2-nitroestrone.[195] Hormones are known to undergo nitration and clay can be used as a catalyst for nitration or other oxidative transformation of hormones.[195-197]

The main objective of this project was to study the oxidative transformation of estrogenic hormones in aqueous system when exposed to Fe (III)-saturated montmorillonite (Fe (III) – SM) with and without the presence of nitrite.

4.2 Montmorillonite

There are two types of clays: cationic and anionic. Cationic clays are negatively charged alumino layer silicates and anionic clays are positively charged hydroxides. The charge on the interlayer of those clays are neutralized by cations or anions depending on the type of the clay.[198] Montmorillonite is a cationic clay. Montmorillonite was found in France near Montmorillon in 1874, hence the name Montmorillonite came into existence.[199] Montmorillonite comes from smectite group; it is a 2:1 layered aluminosilicate. Al, Mg and Fe are present inside the octahedral site which is the central layer of each clay sheet. Si atoms are present in tetrahedral sites on the either sides of the central octahedral layer. The overall charge on the sheet is negative, which is neutralized by cations.[200]

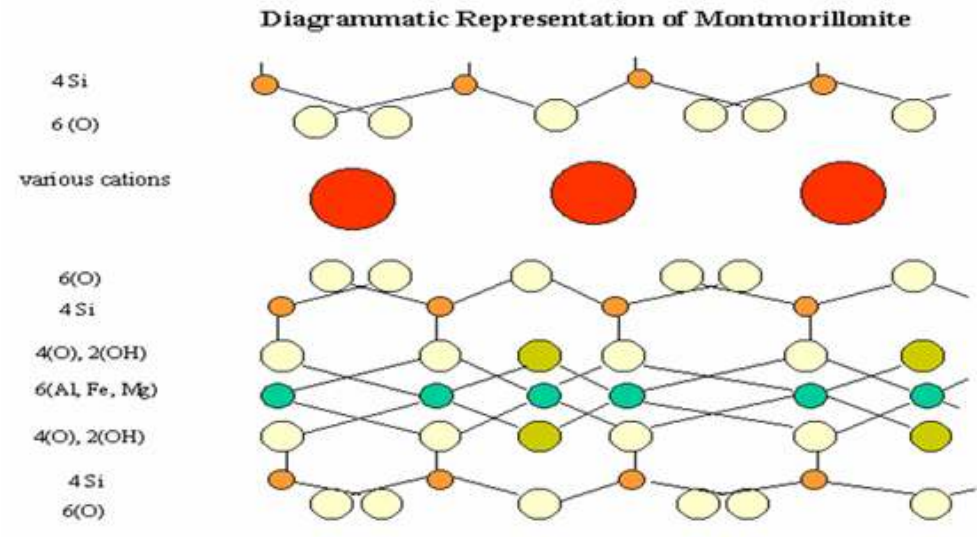


Figure 4.1 Montmorillonite structure.[201]

Figure 4.1, shows the interlayer structure of montmorillonite clay. An abundance of montmorillonite in nature makes it an attractive candidate for use as catalysts. The use of clays as heterogeneous catalysts started from the time when the concept of heterogeneous catalysis was introduced in the first half of 20th century.[199] Due to its high cation exchange capacity and high surface area montmorillonites are used as absorbents, catalysts and ion exchangers.[198]

Smectites such as montmorillonite when exchanged with transitions metals such as Fe (III) can cause formation of radical cations from various organic molecules via single electron transfer to exchangeable interlayer cations (see Figure 4.2). Leading to several reactions based on the chemistry of the molecule including oxidation, polymerization and dechlorination.[202, 203]

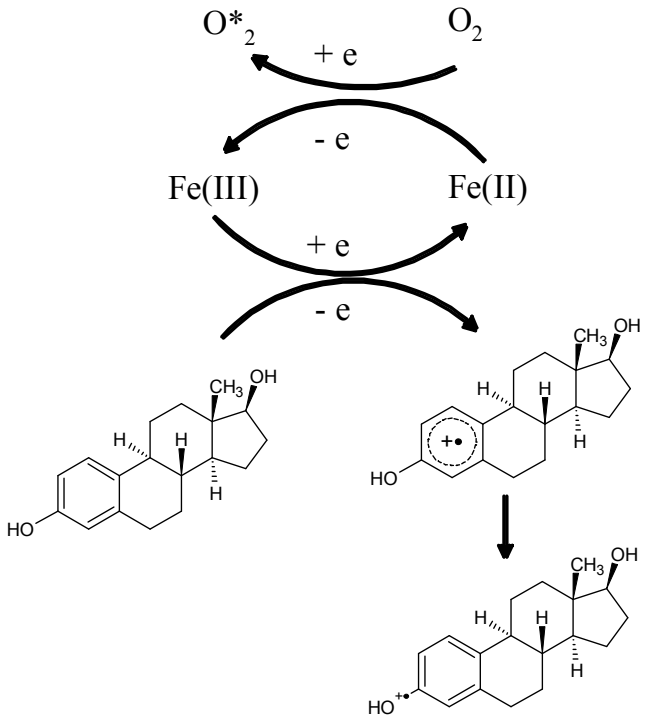


Figure 4.2 Proposed mechanism for radical cation formation on Fe(III)-saturated montmorillonite.[204]

Gu et al., studied the formation of octachlorodibenzodioxin(OCDD) on Fe(III)-SM.[202] They found that when pentachlorophenol (PCP) was reacted with Fe (III)-SM, OCDD was produced. The proposed pathway for formation of OCDD was via single electron transfer leading to formation of PCP radical cation. PCP radical cation can further undergo dimerization, dechlorination and ring closure reactions catalyzed by Fe(III)-SM to form OCDD.[202] A mechanism for formation of OCDD is shown in Figure 4.3.

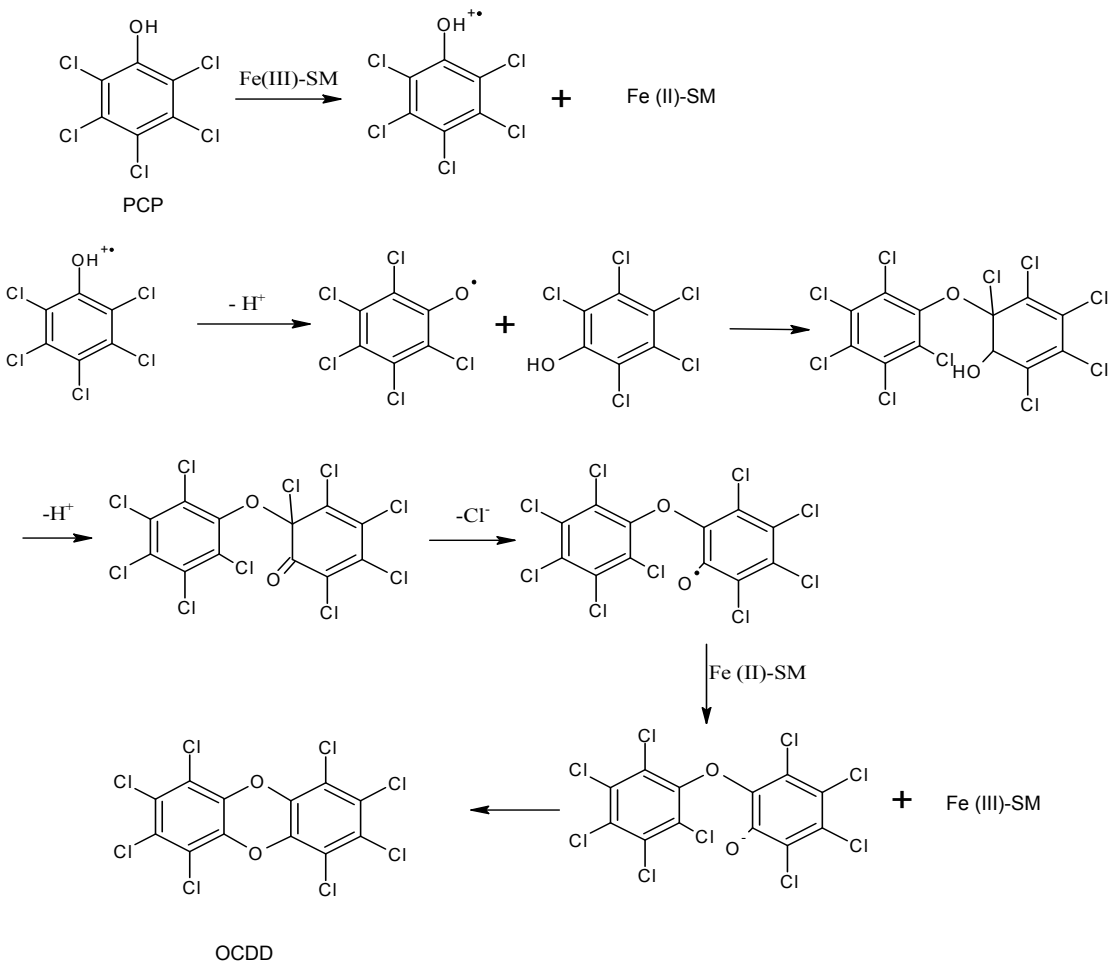


Figure 4.3 Proposed mechanism for OCDD formation when PCP was reacted with Fe (III)-SM.[202]

Liyanapatrina et al., studied the transformation of triclosan by Fe (III)-SM. They observed the formation of 2, 4-dichlorophenol, 3-chlorophenol, 2, 4 – dichlorophenol dimer, chlorophenoxy phenols, TCS dimers and trimers. In their study the predicted pathway for formation of transformation products was also via single electron transfer. TCS phenoxy radical cations were formed and lead to formation of various transformation products.[205] The mechanism by which various TCS transformation products were produced is shown in Figure 4.4. The reaction occurring on the Fe (III)-SM is oxidative in nature was shown by Liyanapatirana et al. They showed that the

concentration of Fe (II) ions increased in the reaction mixture after several days of incubation. This suggests that TCS was oxidized by Fe (III).[205]

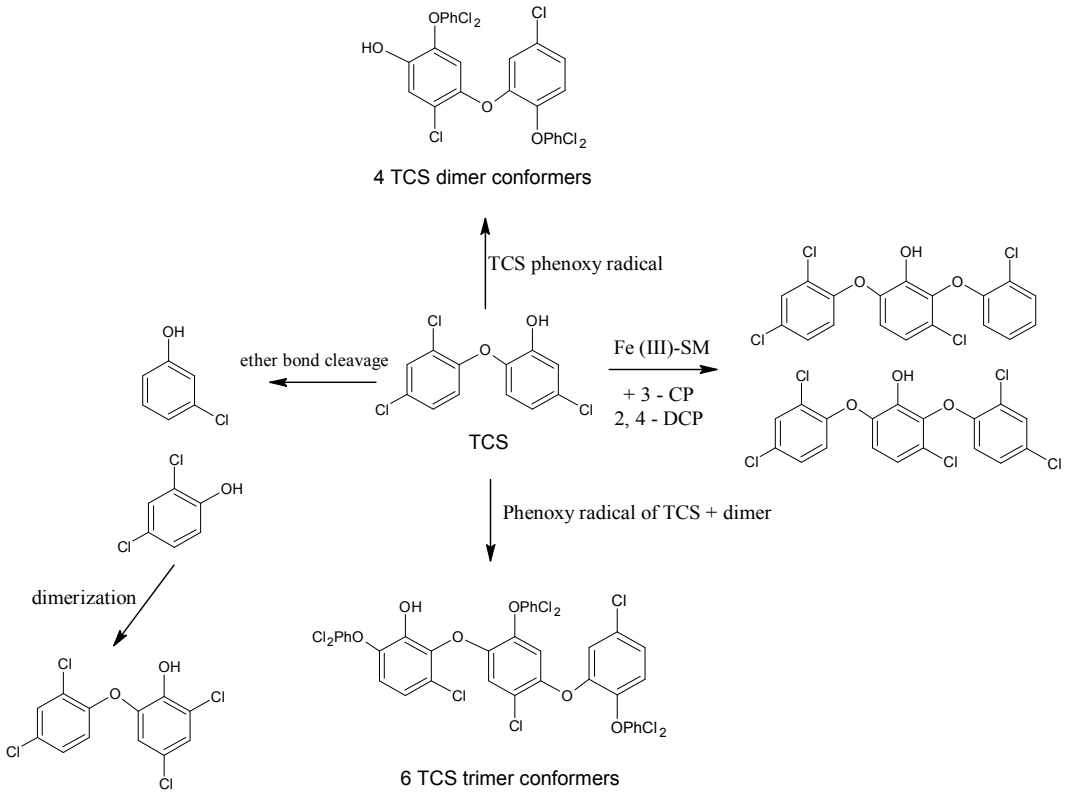


Figure 4.4 Proposed mechanism for formation of TCS transformation products by Fe (III)-SM.[205]

Polubesova et al., investigated adsorption and oxidative transformation of phenolic acids by Fe (III)-SM. They observed production of dimers, trimers and tetramers of ferulic acid by Fe (III)-SM. A 94 % transformation of ferulic acid by Fe (III)-SM was observed. While syringic, p-courmaric and vanilllic acid showed 60 %, 35 % and 25 % transformation by Fe(III)-SM.[206]

4.3 Experimental

4.3.1 Reagents and chemicals

- 17β-Estradiol, 98 %(CAS # 50-28-2), Estriol, 99 % (CAS # 50-27-1), 17α-ethynylestradiol, 98 % (CAS # 57-63-6), Estrone, 99 % (CAS # 53-16-7), Testosterone, 98 % (CAS # 58-22-0) - Sigma-Aldrich (Stylus, MO).
- *$FeCl_3$, Fe_2O_3, MnO_2*- Sigma-Aldrich (Stylus, MO).
- *$NaNO_2$* – J.T.Baker Chemicals Co (Phillipsburg, NJ,USA)
- *KNO_3* – Fisher Scientific (Fair Lawn, NJ, USA).
- *Acetonitrile, acetone, methanol, ethyl acetate and water* - HPLC grade, Fisher Scientific (Fair Lawn, NJ, USA).
- *Na*-montmorillonite – Na (II)-saturated montmorillonite (NM) (SWy-2, Crook County, Wyoming USA) was obtained from the Source Clays Repository of the Clay Minerals Society (Purdue University, West Lafayette, IN). The cation exchange capacity and theoretical surface area of SWy-2 are 82 cmole kg^{-1} and 31.82 ± 0.22 $m^2 g^{-1}$, respectively.
- *Lake Water*: Lake Water was collected at Mississippi state university. The water was collected in an amber colored bottle, filtered with 0.45 μm filter after brought into the lab and used immediately. The pH and the UV absorbance of the lake water were measured.

All the reagents were used in the experiments without further purification.

4.3.2 Apparatus

- *UV Lamp* – Model UVGL-58, Multiband UV- 254/366 nm, 115V, 60Hz - Fisher Scientific (Fair Lawn, NJ, USA).

- *Double beam UV spectrometer* - Model Lambda 25, (Perkin Elmer, Shelton, CT).
- *LC system***. –** The Waters Alliance 2695 Separations Module (Waters, Milford, MA, USA) is equipped with quaternary, low pressure mixing pump and inline vacuum degasser and a photodiode array detector with a wavelength range of 190-800 nm.
- *MS detector*. – Waters Quattro-Micro Mass spectrometer (Micromass, Manchester, UK) is a triple quadrupole mass spectrometer equipped with Electrospray ionization source (ESI).
- *LC system***. –** The Waters Alliance 2695 Separations Module (Waters, Milford, MA, USA) is equipped with quaternary, low pressure mixing pump and inline vacuum degasser and a photodiode array detector with a wavelength range of 190-800 nm.
- *Analytical column*. – µBondapack C18 (particle size 5 µm, 4.6 x 250 mm i.d, Waters).

4.3.3 Preparation of hormone standards and Fe (III)-saturated montmorillonite (FE (III)-SM)

The 17β-estradiol, estriol, 17α-ethinylestradiol, estrone and testosterone standards were prepared in acetone, which were later stored at 4^{o} C until further use. The Fe (III)-SM was prepared as described by Arroyo et. al.[207] Briefly, 10 g of NM was mixed with 400 mL of DI water and stirred for 24 h. After which the pH of the slurry was adjusted to 6.8 using 0.1 M Na acetate buffer (pH 5), over the period of 2 h the pH needed to be adjusted several times, once the pH became constant, 3 g of NaCl was added. The slurry was than centrifuged at 4500 rpm for 20 min. The obtained clay was than mixed with 400

mL of 0.1 M $FeCl_3.6H_2O$ solution and left on a shaker at 120 rpm for 8 h, the slurry was centrifuged again and mixed with 400 mL of 0.1 M $FeCl_3.6H_2O$. The process was repeated six times before the final step of intensive washing with DI water for removing trace chlorine ions was carried out. The washing step was repeated until no precipitate was observed when $AgNO_3$ was added to the supernatant. After which the Fe (III)-SMl was frozen, freeze dried and stored for later use.

4.3.4 Source and optimization of nitration conditions

To find out if nitrite or nitrate anion caused nitration of hormones the following experiments were setup. A 200 µL of 100 mM solutions of KNO_3 or $NaNO_2$ was added to the experimental setup containing 2 mL water, E2 and Fe (III) or Na-saturated montmorillonite. The control setup did not contain any mineral. The vials were than shaken with a mechanical shaker for 2 h.

To optimize the concentration of nitrite used for nitration, 200 µL of $NaNO_2$ with concentrations ranging from 0.05 – 100 mM were added separately to the set-up as described above.

4.3.5 Effect of different minerals and Fe (III) cation on transformation of E2

Fe (III)-SM, NM, $FeCl_3$ and Fe_2O_3 were mixed with the target compounds in order to investigate the effect of minerals and Fe (III) on E2 transformation. In a 5.5 mL glass vial 50 mg of all the mineral was weighed, and 2 mL of DI was added. An appropriate amount of E2 was added to achieve 0.009 mmol E2/g of mineral and left on the shaker for 10 days. The E2 concentration was determined in regular intervals within 10 days. All experiments were ran as triplicates.

4.3.6 Transformation experiments for E2

In a glass vial, 50 mg of Fe (III) and Na- saturated montmorillonite were weighed. In the experiments carried out just with water 2 mL of DI water was used. Two different concentrations of $NaNO_2$ (1 mM and 10 mM) stock solutions were prepared, 200 uL of these stock solution was added to the vial containing mineral and water, such that the final concentration of $NaNO_2$ in each vial was 0.1 mM or 1 mM. The concentration of all the hormones chosen for these experiments represents 50 % of the total coverage of the mineral surface, which turns out to be 0.009 mmol E2 /g of mineral and roughly the same concentrations were used for other hormones too. This concentration is much higher than that found in the environment, but transformation studies usually require higher concentrations in order to identify the metabolites produced. For the experiments with the lake water a similar procedure as described above was used, but instead of DI water lake water was used.

For the pH studies, the pH of the water was adjusted with 0.1 M HCl to pH 4, with 0.1 M NaOH when pH 8 was required. Further the influence of presence of other cation was also tested; 2 mL of 0.1 M $CaCl_2$ solution was used for the experiments instead of DI water.

4.3.7 Transformation experiments for estriol (E3) and 17α-ethinylestradiol (EE2)

In a glass vial, 50 mg of Fe (III) and Na- saturated montmorillonite were weighed into this vial 200 µL of 10 mM $NaNO_2$ solution was added to achieve final concentration of 1 mM in 2 mL DI water. E3 or EE2 standard solution was added to the set to achieve a final concentration of 0.009 mmol/g of mineral.

Similar experiments were set-up but instead of Fe (III)-SM, MnO_2 and Fe_2O_3 were used. All experiments were run as triplicates.

4.3.8 Transformation experiments for estrone (E1) and testosterone

For studying transformation of estrone and testosterone, UV irradiation was also used. The decision to use UV radiation to accelerate transformation was taken after the slow transformation of E1 and testosterone was observed with all the catalysts used in the study. Also testosterone does not undergo nitration reactions due to the absence of –OH group at the C-3 atom of the molecule. The experimental setup was the same, but instead of using $NaNO_2$ to accelerate the transformation process, UV light at 365 nm was used. The UV lamp was placed 20 cm away from the samples inside a house made chamber. The temperature inside the chamber was recorded using a thermometer. The temperature stayed constant at 27^{o} C. Transformation was also studied with MnO_2 and Fe_2O_3 using UV light. The experimental procedure is similar as described in the previous section but no $NaNO_2$ was used to study the transformation of estrone and testosterone.

4.3.9 Sample extraction

After the reaction was completed 3 mL of ethyl acetate was added to the vials and were vigorously shaken on a vortex shaker. After which the vials were subjected to centrifugation at 3500 g for 5 min. The top organic layer was carefully removed, which was later evaporated with a gentle stream of nitrogen. Dried extract was then reconstituted with 1 mL of ACN and analyzed with LC/UV for quantification and LC/MS for metabolite identification.

4.3.10 HPLC/UV and HPLC/ESI/MS analysis

A Waters Alliance HPLC (model no 2695) equipped with a reversed phase analytical column (μBondapack C_{18}, particle size 5 μm, 4.6 x 250 mm i.d, Waters) was used for separation of analytes of interest for both the UV and MS analysis. The mobile

phase consisted of (A) acetonitrile and (B) water. The mobile phase flow rate was 1 mL / min. The mobile phase gradient was: 0 - 10 min 50 % A; 10-20 min from 50 % A to 70 % A; 20 – 23 min 70 % A; 23 – 25 min 70 % A to 90 % A; 25 – 30 min 90 % A, 30 - 33 min from 90 % A to 50 % A, 33 - 35 min 50 % A.

An injection volume of 30 µL was used for the HPLC/UV and HPLC/MS analysis. For the HPLC/UV analysis, a Waters Alliance 2990 UV detector (Waters, Milford, MA, USA) was used. The UV detection wavelength for all the hormones expect for testosterone was 210 nm, testosterone showed maximum absorbance at 240 nm. For the LC/MS analysis, a micromass Quattro Micro triple- quadrupole mass spectrometer (Micromass, Manchester, UK) was used. Electrospray negative ionization mode was employed. The mass spectrometer was setup using these parameters: capillary voltage 4 kV, cone voltage 30 V, source temperature 100° C, desolvation temperature 300° C, cone gas flow 45 L/h, and desolvation gas flow rate 418 L/h. The mass scan range was 50 - 1000 m/z.

4.4 Results and discussion

4.4.1 Role of Fe (III)-SM on E2 transformation

The fastest transformation of E2 was achieved with Fe (III) – SM, ~ 99 % E2 was transformed after 10 days. NM showed ~ 50 % E2 transformation after 10 days (Figure 4.5). When $FeCl_3$ and Fe_2O_3 were used, no significant transformation was observed even after 10 days. The control (E2 + H_2O) showed minimal transformation. This clearly demonstrates the role of mineral surface, Fe (III) by itself, in a solution is not an efficient catalyst to cause oxidative transformation of E2. It can be said that the transformation of E2 is greatly enhanced at the interlayer surface of montmorillonite. This can be explained

by a phenomenon described by Polubesova et al, they studied oxidative transformation of phenolic acids by Fe(III)-SM.[206] The mechanism of electron transfer in $FeCl_3$ solution occurs via an outer-sphere mechanism. While an inner-sphere complex is formed between the organic compound and Fe (III) on the clay surface, and the formed complex is strong due to the reduction of aqueous dielectric permittivity on the clay surface. This strong complexation between organic molecules and Fe (III)-SM has been suggested for enhanced electron transfer, oxidation, transformation and polymerization of organic compounds on a Fe(III)-SM surface.[206] Our results support this hypothesis since no significant transformation of E2 was observed in $FeCl_3$ and NM.

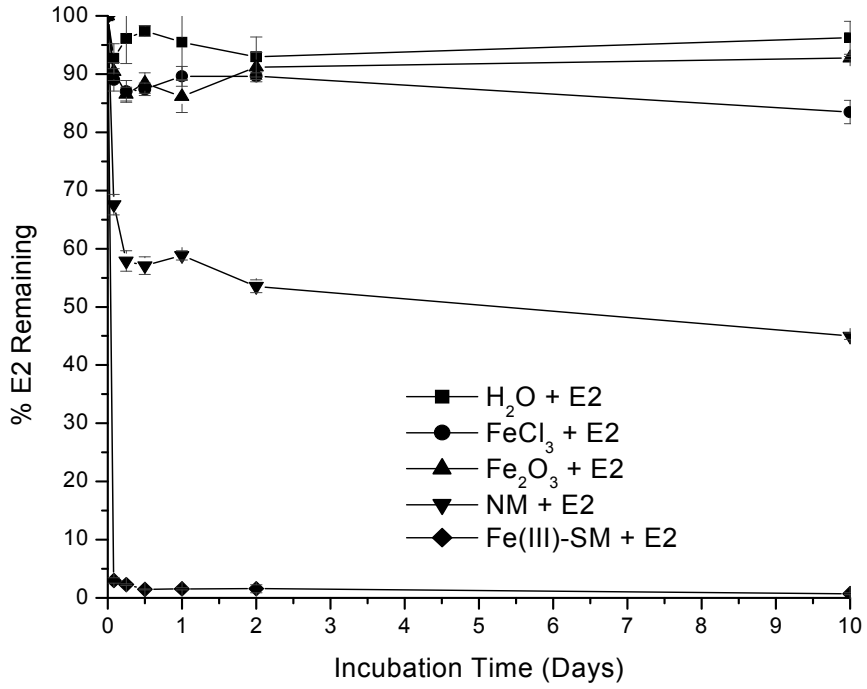


Figure 4.5 Effect of different Fe (III) ion sources on transformation of E2

No transformation of E2 is observed by Fe_2O_3, it can be due to the band energy gap of Fe_2O_3. The band energy gap required to activate Fe_2O_3 is 2.2 eV.[208] The experiments were performed in room conditions. No light source was present to provide enough energy for activation of Fe_2O_3 to cause oxidative transformation of E2. Therefore no transformation of E2 is observed when Fe_2O_3 is used as a catalyst.

4.4.2 Source and optimization of nitration conditions

Estrogenic hormones have been shown to undergo nitration. In wastewater treatment plants biotransformation via nitration is considered as an option for fast removal of estrogenic hormones.[192] Nitro derivatives of estrogens have been synthesized using nitrate and nitrite.[193, 195, 196]

This part of the experiment was done to find out which ion is a better catalyst for E2 nitration when used along with Fe (III)-SM. The combination of NO_2^- + Fe (III)-SM proved to be more potent for E2 transformation than NO_3^- + Fe (III) – SM as shown in Figure 4.6. The control in this case was Fe (III)-SM, which showed more E2 transformation than NO_3^- + Fe (III) - SM.

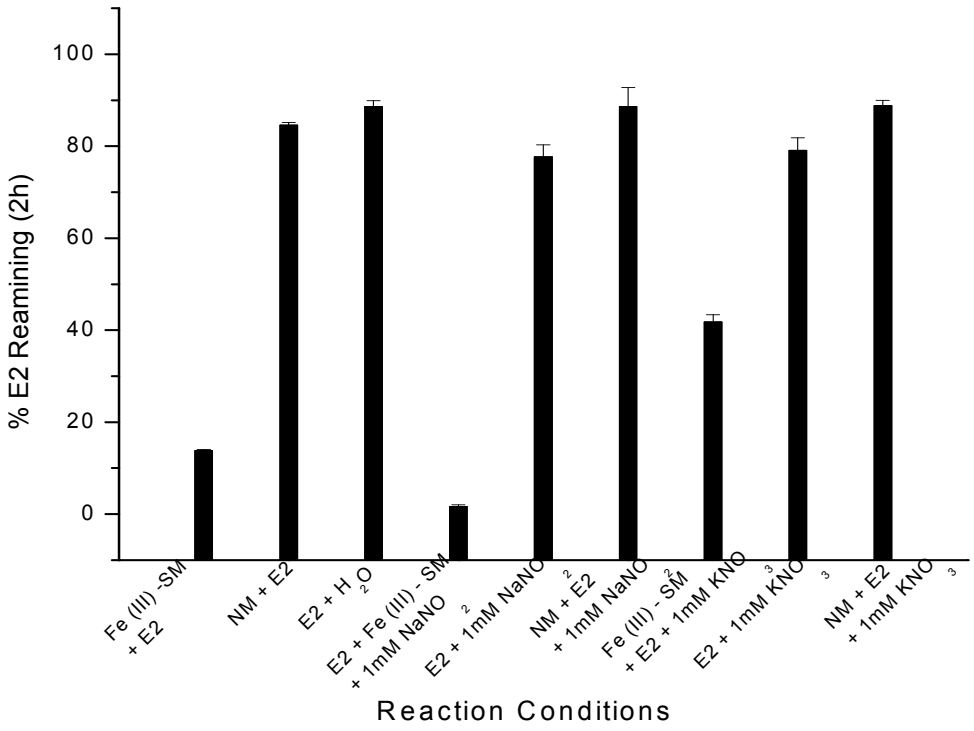


Figure 4.6 Transformation of E2 in presence of nitrate and nitrite by Fe (III) - SM or NM.

The results observed can be due to the fact that Fe(III) present in mineral react with NO_3^- to form $Fe(NO_3)_3$. This can reduce the availability of Fe (III) to carry out transformation of E2. Transformation of E2 in Fe (III)-SM without addition of NO_2^- or NO_3^- was higher than that observed for E2 transformation with NO_3^- + Fe (III)-SM. This suggests that availability of Fe (III) ions for transformation of E2 is decreased in the reaction mixture containing NO_3^- ion. The formation of NO_2^+ from KNO_3 on the mineral surface is required for nitration of E2.[195] Meshram et al., described that nitrate decompose in acidic clay to NO^+, which in presence of $HClO_4$ forms nitrosyl perchlorate. Nitrosyl perchlorate reacts with arenes to produce nitroso arenes which are further oxidized to nitro arenes.[209] This suggests that the presence of strong oxidizing agent such as

perchlorate is required for nitration using nitrate. In our study no such oxidizing agent is present hence nitrate does not decomposes; instead it occupies the reactive site making Fe (III) less assessable to the E2 molecule. This results in a reduced transformation rates of E2 when KNO_3 + Fe (III) –SM are used for transformation of E2.

The concentration of nitrite used in the experiment was optimized; concentrations varying from 0.01 mM $NaNO_2$ to 100 mM $NaNO_2$ were used along with Fe (III)-SM. The reaction rate observed for control (Fe (III)-SM containing no $NaNO_2$) was comparable with samples containing concentrations of $NaNO_2$ up to 0.05 mM. Concentrations at 0.1 mM $NaNO_2$ and 1 mM $NaNO_2$ showed significant acceleration of the transformation process see Figure 4.7. But concentrations higher than 1mM $NaNO_2$ showed decrease in the transformation process. Also using high concentrations of nitrite is not practical considering the fact that the lower concentration < 1 mg/L of nitrite is present in the WWTPs employing nitrification using nitrifying activated sludge.[192] The lowest concentration (0.1 mM and 1 mM $NaNO_2$) showing most enhancement in reaction rate was chosen for the experiments.

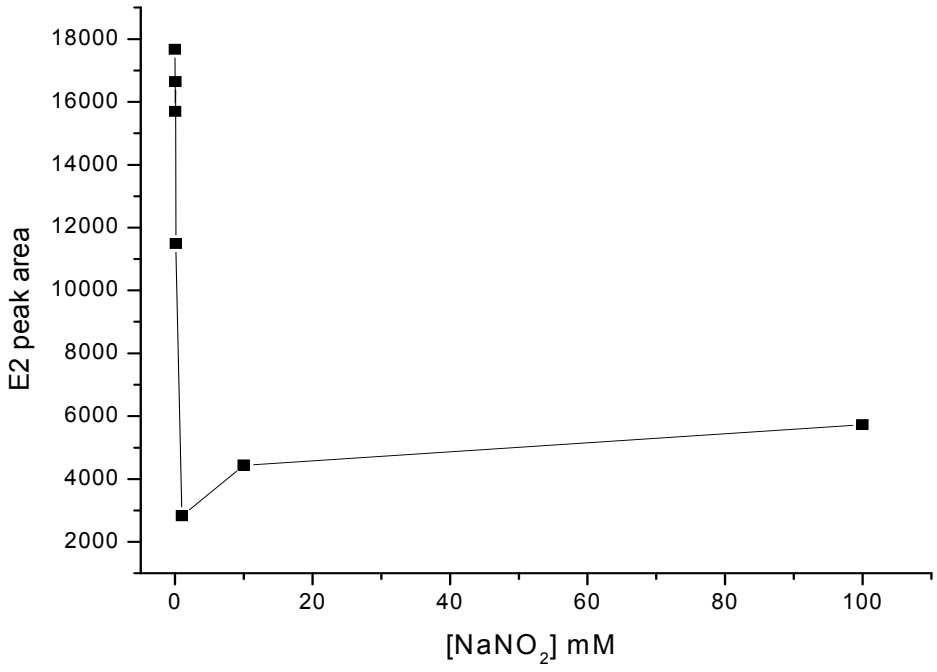


Figure 4.7 Effect of $NaNO_2$ concentration on transformation of E2 by Fe (III)-SM for 2 h.

4.4.3 Abiotic transformation of E2 with Fe (III) -SM, NM at different pH, in presence of interfering cation and organic matter

The transformation experiments were carried at room temperature and in room light (bright during day and dark at night). Transformation of E2 was monitored with HPLC/UV at 210 nm. Complete transformation of E2 was observed by Fe(III)-SM + 1 mM $NaNO_2$, with half-life value of 4.99 min and reaction rate constant of 22.37 $(mmol/g)^{-1}min^{-1}$. While in reaction mixture containing 0.1 mM $NaNO_2$ + Fe (III)-SM and 0 mM $NaNO_2$ + Fe (III)-SM after 4 h 10 % and 20 % E2 remained. And almost 85 % E2 remained in all the treatments employing NM (Figure 4.8).

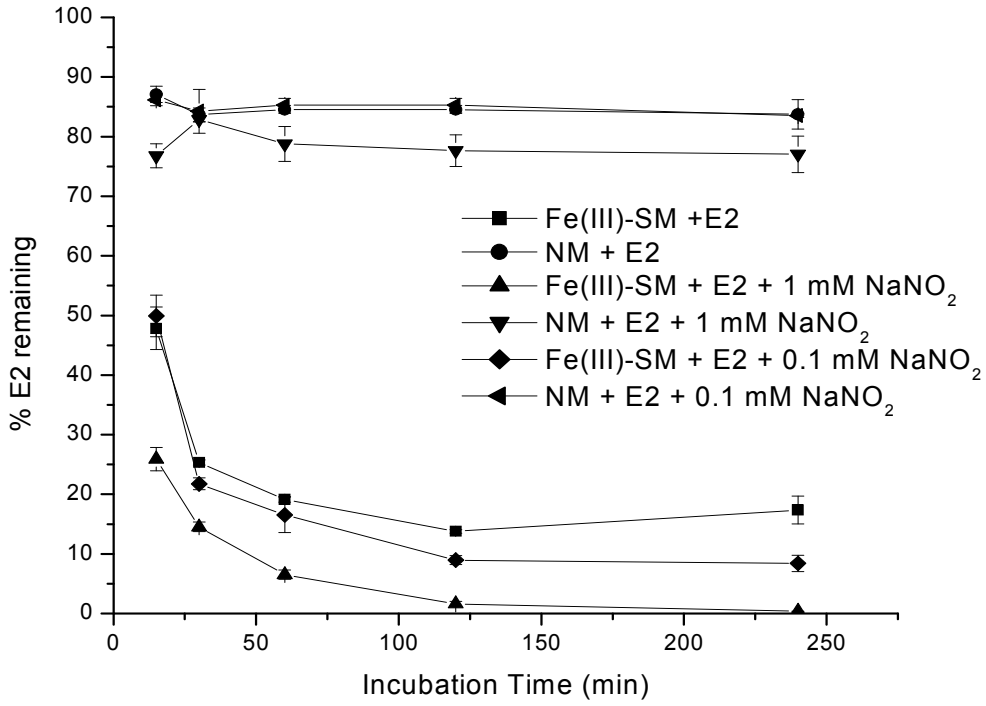


Figure 4.8 Transformation of E2 by Fe(III) - SM and NM in presence of 0 mM, 0.1 mM and 1 mM $NaNO_2$.

Jiang et al., studied oxidative transformation of E2 using MnO_2. According to their study 90 % E2 was transformed by MnO_2 in 8 h. A similar transformation pattern was observed in our study, concentration of E2 decreased sharply during the early stage of the reaction and slowly later on. E1 and 2-hydroxyestradiol were major transformation products produced in the study by Jiang et al.[151] The efficiency of E2 removal using Fe (III) - SM + 1 mM $NaNO_2$ is better compared to MnO_2 as observed in the study by Jiang et al.[151] Zhao et al., developed a photo-Fenton catalysts α-FeOOH loaded resin (α-FeOOHR) for oxidative transformation of E2. In their study the initial concentration of E2 - 272 µg/L decreased to 1.8 µg/L after 22 h.[188] When compared to catalysts developed in our study the efficiency of α-FeOOHR is less. Use of H_2O_2 and UV

irradiation for activation of the catalysts makes the use of this catalyst expensive. Also the concentration of E2 employed in our study is much higher. Fe (III)-SM + 1mM $NaNO_2$ transformation rates are higher even when high concentration of E2 is employed.

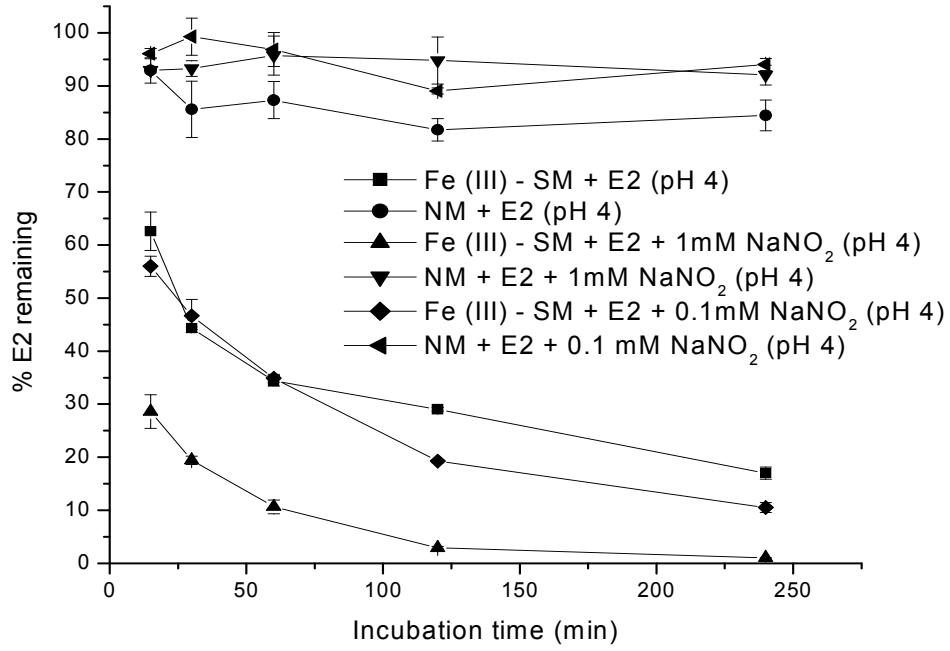


Figure 4.9 Transformation of E2 at pH 4 by FE(III) and NM with and without nitrite

Shappell et al., used another catalysts iron-tetraamidoacrocyclic ligand (Fe-TAML)/peroxide to study transformation of estrogens. The concentration of hormones used was 80 µM, Fe-TAML was 83 nM and that of H_2O_2 was 4mM. Fe-TAML by itself was ineffective in transformation of E2, with addition of H_2O_2 at pH 8, 95 % of E2 had transformed after 15 min. The mentioned catalysts shows fast transformation of E2, but activation of catalysts requires addition of H_2O_2.[118]

The pH of the solution determines the transformation rate of organic compounds by a catalyst. At acidic pH the phenol group will not be deprotonated, while in basic pH

phenol group will exist in phenolate form.[117] To understand the role of pH on the transformation of E2 by Fe (III)-SM, experiments were designed with water pH adjusted at pH 4, 6 and 8. The E2 showed highest transformation with 1mM $NaNO_2$ + Fe (III) – SM catalytic system at all the pH, followed by 0.1 mM $NaNO_2$ + Fe (III)-SM and 0 mM $NaNO_2$ + Fe (III) – SM. The NM (control) showed no appreciable E2 removal at any pH.

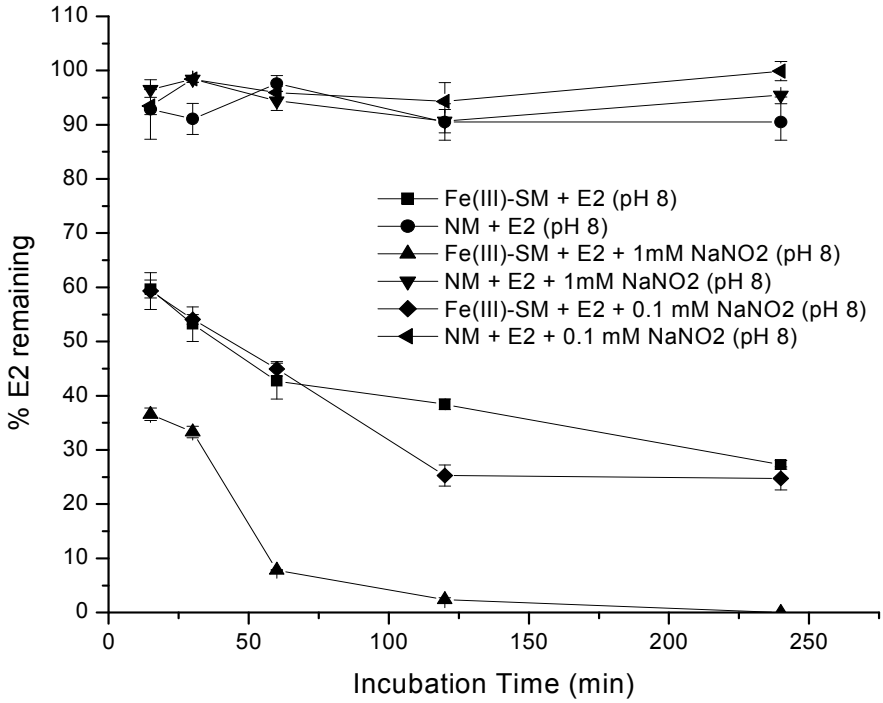


Figure 4.10 Transformation of E2 at pH 8 by FE (III) – SM and NM.

At pH 6 (Figure 4.8) highest transformation of E2 was observed under all the treatment conditions, followed by pH 4 (Figure 4.9) and pH 8 (Figure 4.10). When 1 mM $NaNO_2$ + Fe (III) –SM was used in the treatment no significant influence was observed in transformation rates, complete transformation was observed at all the pH (Figure 4.13). But in the treatments with 0.1mM $NaNO_2$ + Fe (III) –SM and Fe (III) – SM a significant

difference was observed. Bila et al., observed the highest transformation rate of E2 by ozonation at pH 11 and the slowest at pH 3. The results obtained in our studies does not agree with the results obtained by Bila et al.[117] This can be due to the fact the Fe (III) – SM requires formation of radical cations for the transformation of organic compounds which can be better achieved at acidic pH. While Bila et al., used ozonation for the transformation of E2, which requires formation of O_3 and hydroxyl radicals which are abundant at basic pH.[117] In another study by Xu et al., MnO_2 was used for transformation of E2. They studied the effect of pH on the transformation rates and found that an acidic pH increased the transformation rate. One unit decrease in pH from 4 to 5 resulted in significant decrease in E2 transformation. They concluded that oxidation using MnO_2 required proton production which is favored at acidic pH.[116] The results observed in our study correspond well with that of Xu et al. Higher transformation of E2 is observed at an acidic pH and the transformation rate decreases in basic pH.

The results observed in this study suggests that this Fe(III)-SM can be used as a catalysts at wide pH ranges which can imply for various aqueous samples found in the environment without losing its efficiency. The products formed at all the pH's remained the same. The products formed during the reaction will be discussed in later sections.

Another experiment was designed to see the influence of the presence of cations. Since soil and water contain various cations in the environment, the presence of these cations can accelerate or decelerate the transformation process via altering the surface properties of clay such as surface acidity.[210] For the experiments in this study $CaCl_2$ (Ca^{+2}) was used, since it is widely present in the environment. Instead of water, 0.01 M $CaCl_2$ solution was added to the reaction mixture. The presence of $CaCl_2$ increased the

adsorption of E2 on the NM (Figure 4.11). Almost 20 % of E2 stayed adsorbed on the NM, but no decrease in E2 concentration beyond that point was observed.

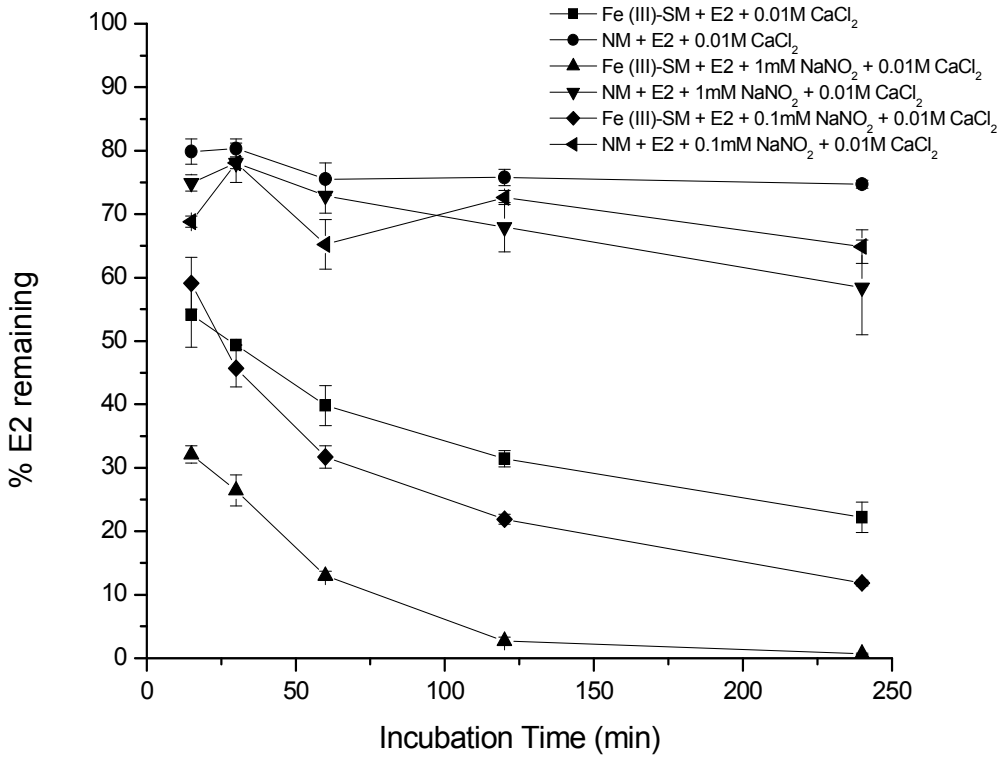


Figure 4.11 Transformation of E2 in presence of interfering cations Ca^{+2}.

The highest transformation was observed in treatment with Fe (III)-SM + 1mM $NaNO_2$ followed by Fe (III) –SM + 0.1mM $NaNO_2$ and Fe (III)-SM. No transformation was observed with NM. Half-life values obtained with $CaCl_2$ treatments were similar to those observed at pH 4, see Table 4.1.

Xu et al., studied the effect of metal ions on E2 transformation by MnO_2. They found that in presence of metal ions Zn (II), Cu (II), Fe (III) and Mn (II), transformation

of E2 was severely affected. The reason for the decrease in the transformation rates was believed to be adsorption of uncomplexed metal ions to the reactive sites via formation of inner-sphere complexes.[116] In our studies no severe effect was observed in transformation rates, but increased adsorption of E2 was observed on NM mineral. This can be due to alteration of surface properties of NM. Our study suggests that the developed Fe (III)-SM catalysts can perform well in the presence of interfering cations.

In the environment, water bodies contain organic matter.[211] Organic matter is known to reduce the oxidative capacity of the catalysts by exhausting the oxidative species and undergoing transformation.[116] Dissolved organic matter can also indirectly participate in the photolysis of estrogenic compounds. Organic matter can absorb solar energy and produce free radicals which in turn can cause the oxidative transformation of estrogens.[147] In this section experiments were designed to shows that combination of Fe (III) – SM and NO_2^- is effective in the transformation of E2, despite the presence of organic matter. Water was collected from a lake and used for experiments to show the influence of organic matter on transformation of E2 by Fe (III) - SM. As shown in Figure 4.12**,** 0 mM, 0.1 mM and 1 mM $NaNO_2$ was used along with Fe (III) - SM for the transformation of E2 in lake water. At the end of 4 h, 65, 85 and 99 % E2 was transformed in Fe (III)-SM, 0.1 mM $NaNO_2$ + Fe (III)-SM and 1 mM $NaNO_2$ + Fe (III)-SM respectively.

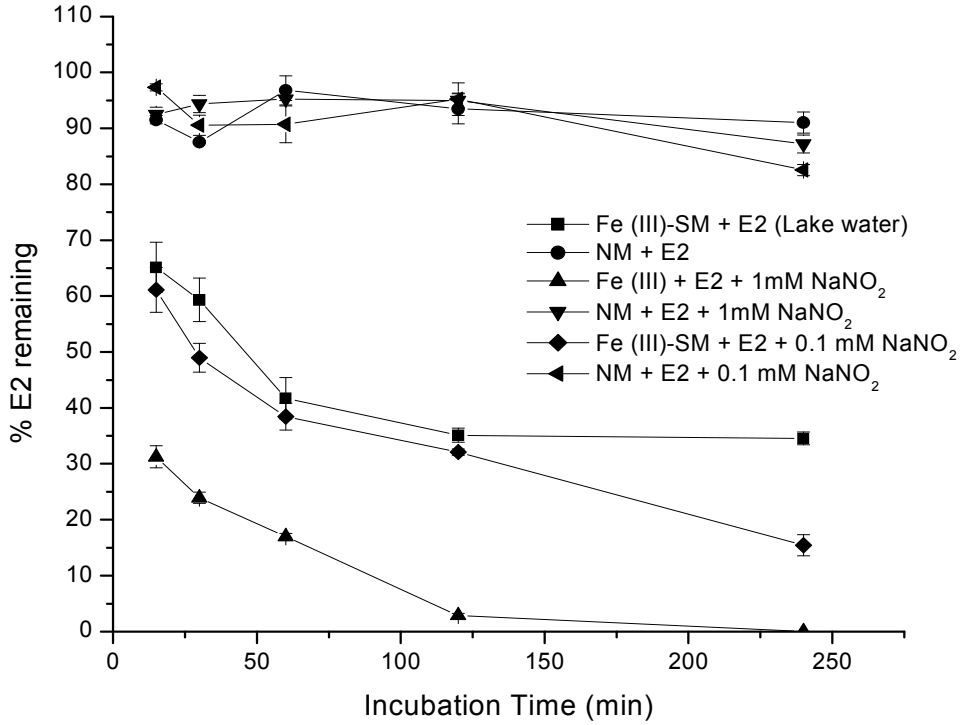


Figure 4.12 Transformation of E2 in lake water when treated with different concentrations of $NaNO_2$ by Fe (III)-SM or NM.

This suggests that the transformation of E2 is not affected severely by the presence of organic matter. As the amount of $NaNO_2$ decreased in the reaction mixture the percent of E2 removal also decreased. While use of NM showed no appreciable removal of E2 after 4 h, ≤ 15 % (Figure 4.12).

Leech et al., studied the influence of dissolved organic matter on transformation of E2 by stimulated sunlight. In the study they observed positive influence of presence of humic acid on the transformation of E2. Although the positive influence was only observed in light, in dark no increase in transformation of E2 was observed. Transformation rate increased with increase in the concentration of humic acid (HA).[147] Atkinson et al., observed an inverse relation between the E1 transformation rate and

dissolved organic matter when photo-transformation of E1 was carried out.[211] While in the research conducted by Xu et al., tannic acid (TA) showed reduction in transformation of E2 by MnO_2. The reason behind the reduction in transformation rates was believed to be competitive oxidation of TA by MnO_2. While HA showed an increase in E2 transformation by MnO_2.[116] In our study, the transformation of E2 in lake water was slowest. This decrease in the transformation can be due to the pH of the lake water which was measured to be 7.3. The transformation rates of E2 observed at pH 8 were similar to that observed for lake water. From this it can the concluded the effect of organic matter on E2 transformation by Fe (III) – SM was not pronounced.

The results of this study suggest that Fe (III) – SM and $NaNO_2$ plays a very important role in the transformation of E2. The presence of organic matter, cations and different pH did affect the transformation rates of E2 to some extent, but still provided efficient transformation rates. Fe (III) – SM was proven to be an efficient catalysts for transformation of E2.

4.4.4 Half-lives and reaction rates

The half-life values and reaction rate constants were calculated by plotting time against 1/[C]. The slope of the obtained line gave the reaction rate constant as shown in equation 4.1.

$$\frac{1}{[C]} = \frac{1}{[C_0]} + kt \qquad (4.1)$$

where C_0 and C are concentrations of hormones at time zero and concentration at time t, k is second order rate constant. The half-life of the reaction was determined using equation 4.2.

$$t_{1/2} = \frac{1}{k\,[C_0]} \qquad (4.2)$$

In our experiments, E2 was found to follow the similar transformation pattern at all pH solutions. The half-life of the reactions at all the pH values are listed in Table 4.1. The half-life at pH 6 was the smallest of all the half-life values, followed by pH 4.

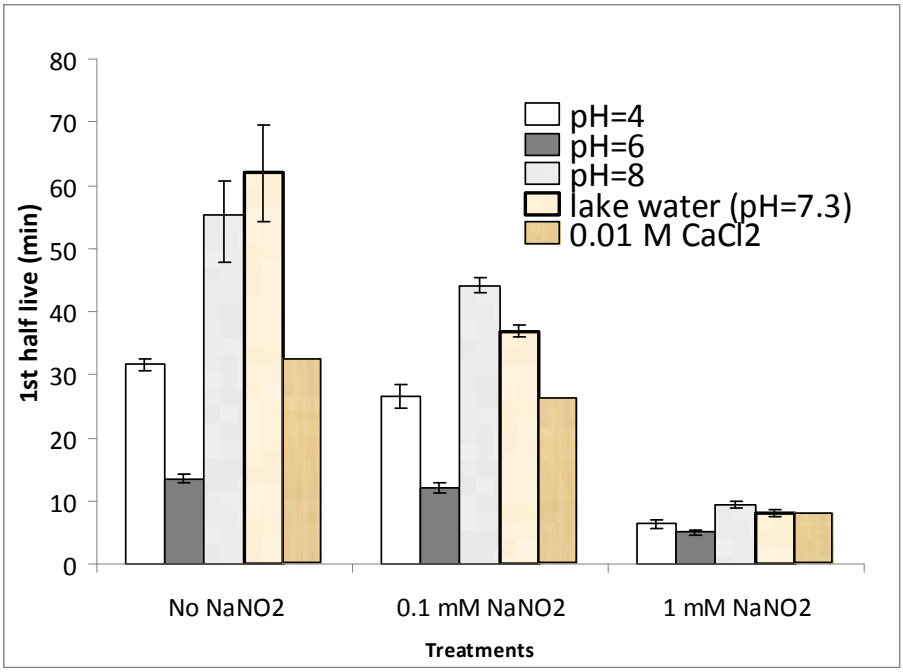


Figure 4.13 Effect of pH and Ca^{+2} on the half-life of E2 transformation reactions by Fe (III)-SM, 0.1 mM + Fe (III)-SM and 1 mM $NaNO_2$ + Fe (III)-SM in lake water

The lake water had a pH of 7.3. Half-life at pH 8 and 7.3 were comparable (Figure 4.13). At all the pH values treatments with 1mM $NaNO_2$ + Fe (III)-SM showed the smallest half-life and higher rate constant, followed by treatments 0.1 mM + Fe (III)-SM and Fe (III)-SM.

Table 4.1 Half-life values and reaction rate constants at various pH values. Half-life values for E2 degradation in lake water with varying $NaNO_2$ concentrations and in presence of Ca^{+2} cation.

DI water pH	Treatment	$t_{1/2}$ ± stdev (min)	K $(mmol/g)^{-1}min^{-1}$ ± stdev
4	No $NaNO_2$	31.59 ± 0.98	3.610 ± 0.14
	1 mM $NaNO_2$	6.350 ± 0.59	17.59 ± 1.62
	0.1 mM $NaNO_2$	26.48 ± 1.88	4.300 ± 0.32
6	No $NaNO_2$	13.51 ± 0.62	8.260 ± 0.41
	1 mM $NaNO_2$	4.990 ± 0.42	22.37 ± 1.96
	0.1 mM $NaNO_2$	12.02 ± 0.74	9.240 ± 0.58
8	No $NaNO_2$	55.33 ± 5.23	2.210 ± 0.21
	1 mM $NaNO_2$	9.470 ± 0.33	11.74 ± 0.42
	0.1 mM $NaNO_2$	43.94 ± 1.31	2.680 ± 0.10
Lake Water (pH 7.43)	No $NaNO_2$	61.89 ± 7.59	1.960 ± 0.23
	1 mM $NaNO_2$	7.980 ± 0.57	14.00 ± 0.97
	0.1 mM $NaNO_2$	36.86 ± 0.95	3.140 ± 0.11
0.01 M $CaCl_2$	No $NaNO_2$	32.51 ± 3.34	3.650 ± 0.35
	1 mM $NaNO_2$	8.030 ± 0.49	13.89 ± 0.21
	0.1 mM $NaNO_2$	26.33 ± 1.20	4.290 ± 0.88

The half-life in the presence of 0.01 M $CaCl_2$ + Fe (III)-SM was 32.51 ± 3.34 min. This half-life is very similar to half-life at pH 4. Lake water had a pH of 7.3, and had the largest half-life of all the transformation conditions studied in our laboratory.

Most probably the transformation of E2 would follow the transformation rates observed in lake water

4.4.5 LC-ESI-MS analysis of E2 oxidative transformation products

In this study, several dimers were observed as the intermediate products, the intermediate products were a small fraction of the initial concentration of E2.

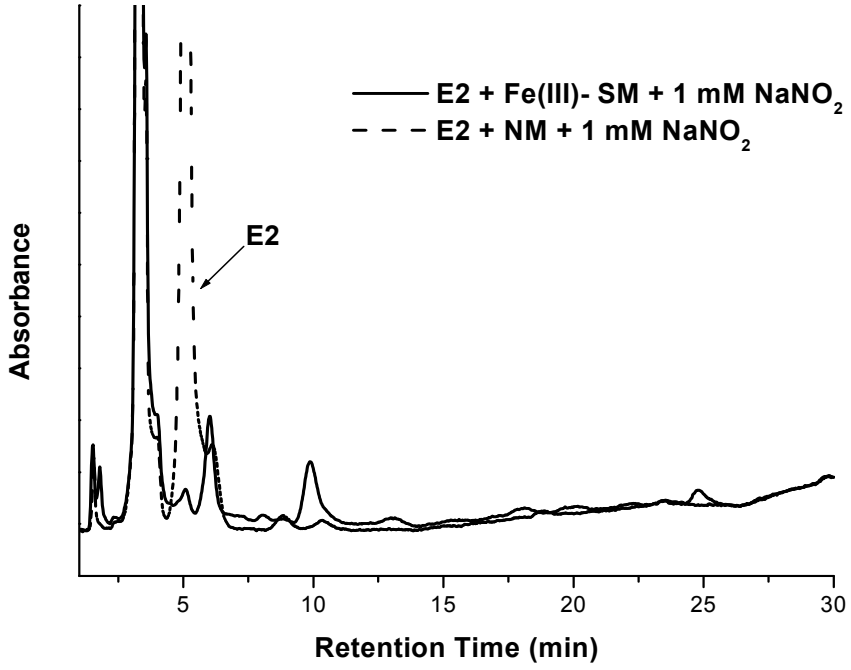


Figure 4.14 UV chromatogram showing transformation of E2 by 1 mM $NaNO_2$ + Fe(III) - SM and 1 mM $NaNO_2$ + NM.

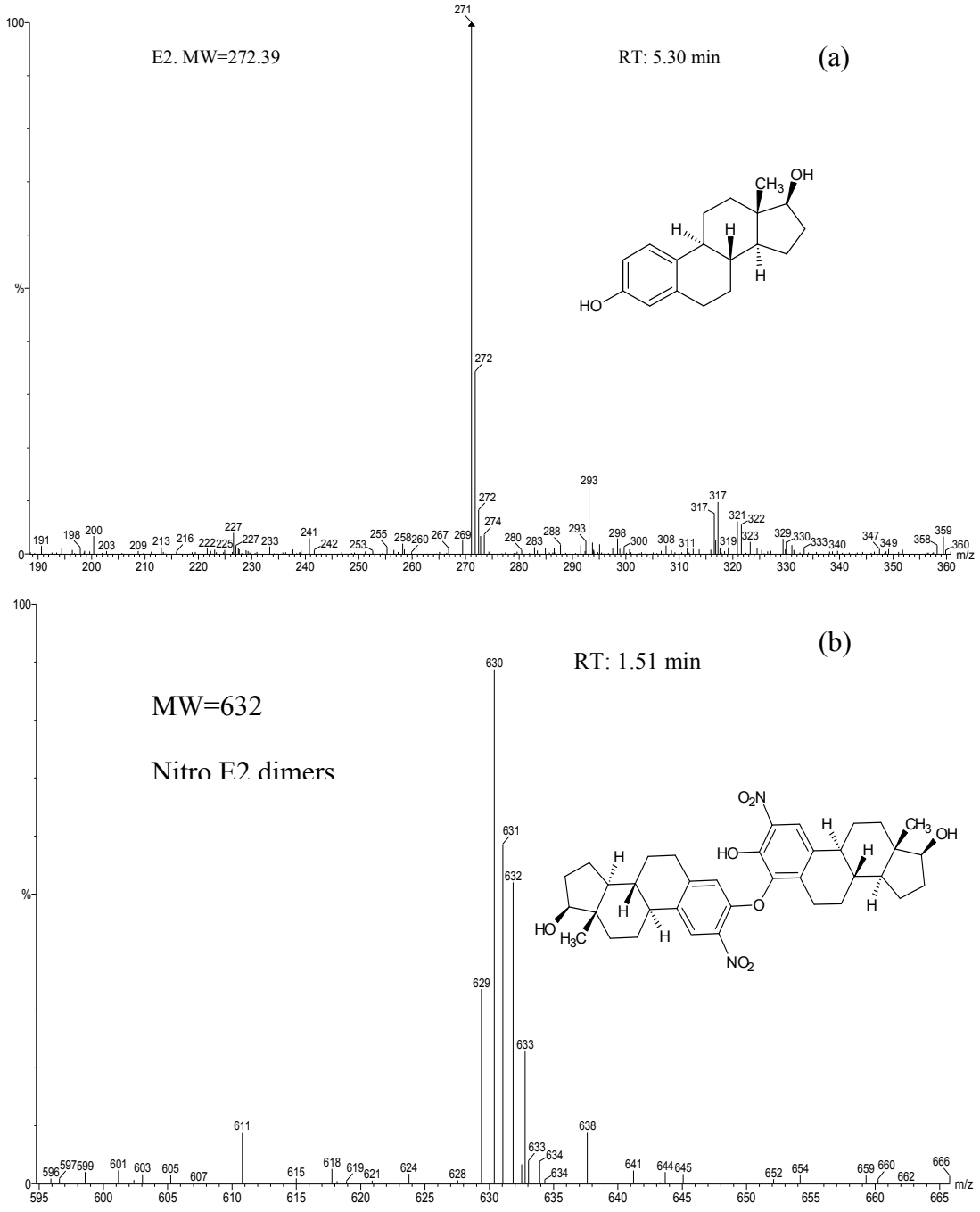


Figure 4.15 Mass spectrum of transformation products produced by transformation of E2 by Fe(III)-SM + 1 mM $NaNO_2$ (a, b, c, d, e)

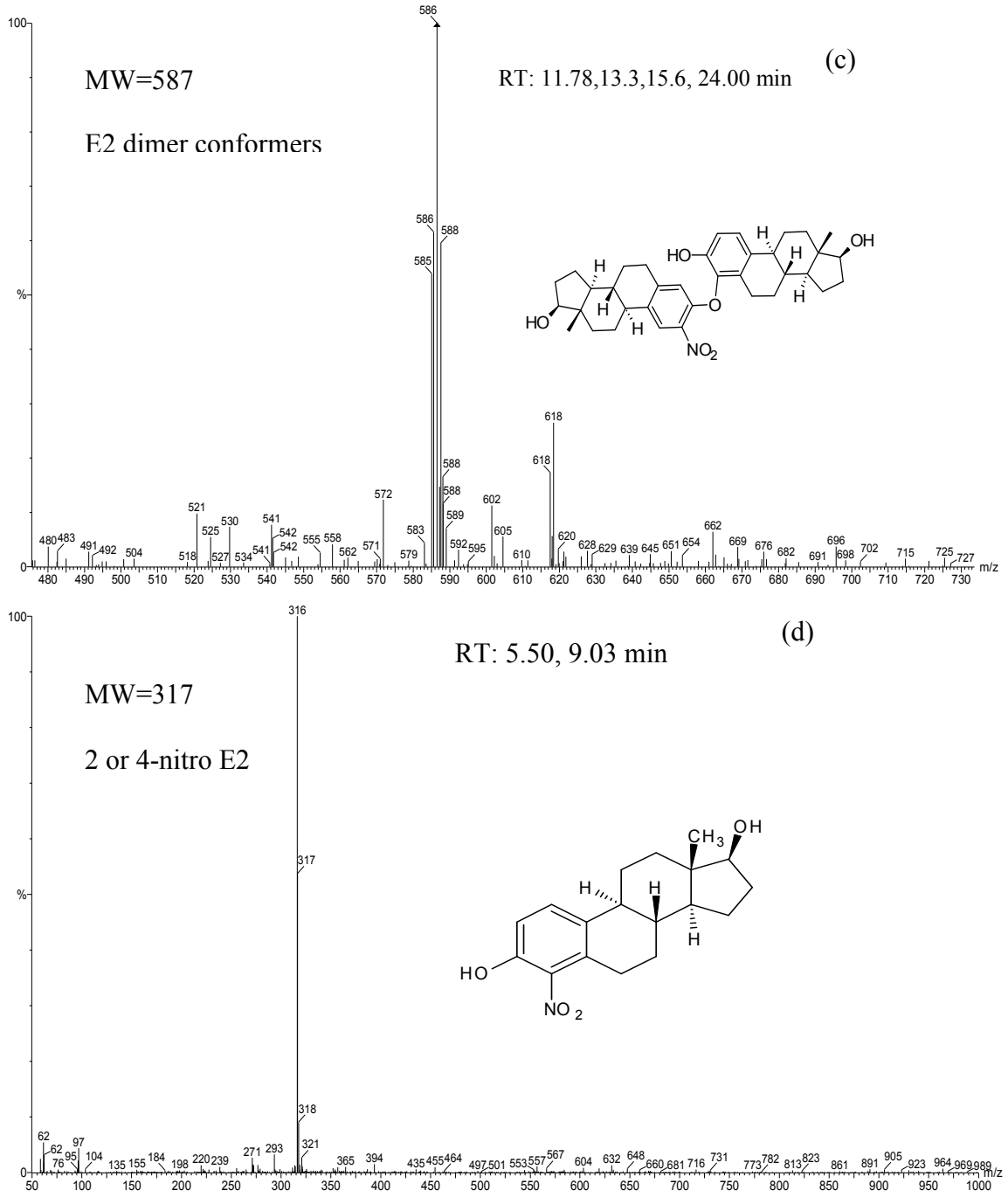


Figure 4.15 (continued)

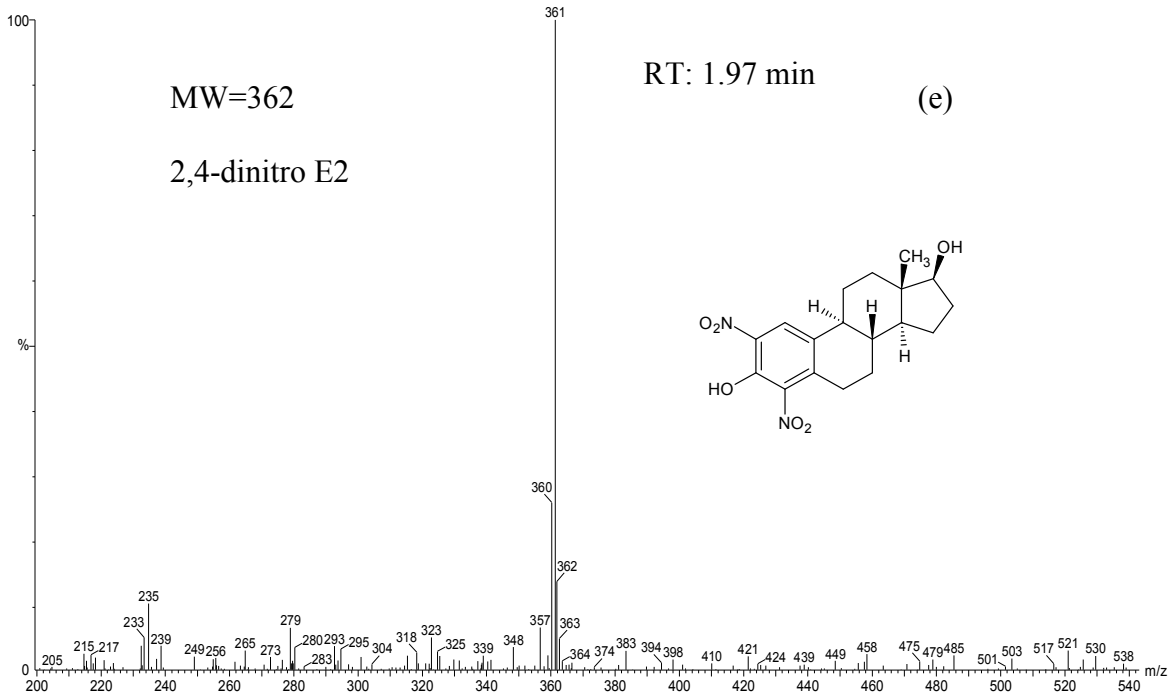


Figure 4.15 (continued)

Figure 4.15 a, shows the mass spectrum of E2. While Figure 4.15.b shows mass spectrum of nitro-E2 dimers at m/z 631 $[M-H]^-$ observed at retention time of 1.51 min produced by nitration of E2 by 1 mM $NaNO_2$ + Fe (III)-SM. Figure 4.15c shows mass spectrum of another dimer, nitro-E2 and E2 dimer formed at m/z 587 $[M-H]^-$. Four peaks were observed for these dimers, which suggest presence of different conformers of the dimers. In the mass spectrum shown in Figure 4.15 d, nitration products of E2 were identified at m/z 316 $[M-H]^-$.Two peaks at this m/z ratio were observed at different retention times, which suggests that two conformers of nitro-E2 were formed. The most probable conformers produced by nitration of E2 are 2-nitro-E2 and 4-nitro-E2. In the next mass spectrum Figure 4.14 e another nitration product was detected at m/z 361 $[M-H]^-$, which can be identified as 2, 4-dinitro E2. Wang et al., also reported production of 2-nitroestradiol and 4-nitroestradiol when photoacatalytic transformation of E2 was carried

out using $FeCl_3/NaNO_2$.[190] Pezzella et al., studied nitration of E2 by peroxidase/H_2O_2/NO_2^-, three main nitration products were produced namely, 2-nitroestradiol, 4-nitroestradiol and 2, 4-dinitroestradiol. The structures of the products obtained in the study by Pezzella et al., were confirmed by NMR.[196]

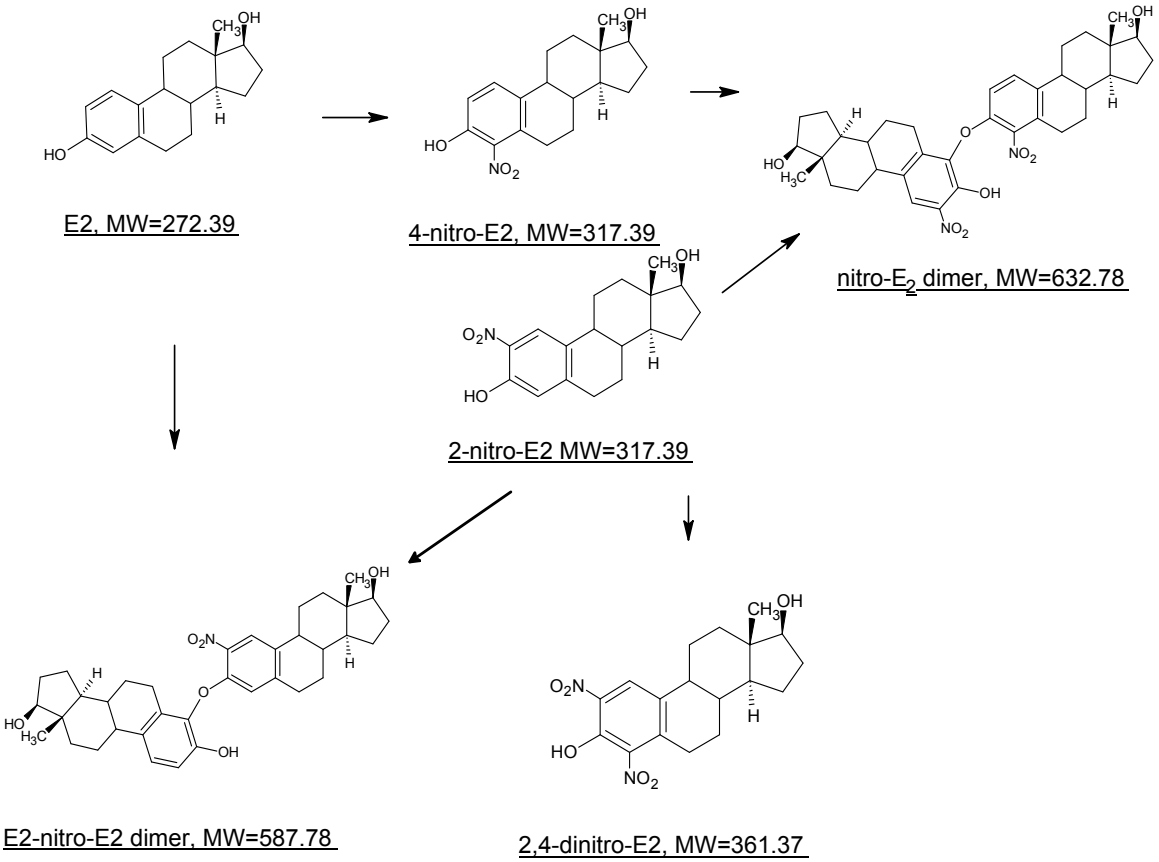


Figure 4.16 Proposed pathway for transformation of E2 by 1 mM $NaNO_2$ + Fe (III) – SM

Based on the products detected in this study by LC-MS, a pathway for the transformation of E2 by 1 mM $NaNO_2$ + Fe (III) – SM is proposed (see Figure 4.16), includes nitration substitution reactions, followed by radical cation formation. Combining

of nitro-E2 or E2 radical cation with other nitro-E2 or E2 molecule leads to production of several dimers.

When the transformation of E2 was carried out by 0.1 mM $NaNO_2$ + Fe (III)-SM, E2 dimers at m/z 541$[M-H]^-$ (Figure 4.18) were obtained. Four peaks with m/z 541$[M-H]^-$ were obtained, which suggest presence of different conformers (Figure 4.17). Nitro-E2 conformers were also observed at m/z 316 $[M-H]^-$. 2, 4-dinitro E2 was not detected in the reaction mixture containing 0.1 mM $NaNO_2$. It seems like production of nitro derivatives of E2 is dependent on the concentration of $NaNO_2$ used along with Fe (III) – SM. Nitro derivatives of E2 were not observed with NM. Hence production of nitro derivatives depends on Fe (III) – SM and $NaNO_2$. When higher concentration of $NaNO_2$ was employed 2 - nitroE2, 4 - nitroE2, 2, 4 - dinitroE2 and nitroE2 dimers were formed by Fe (III) – SM. But when lower concentration of $NaNO_2$ was used only 2 – nitroE2 and 4 – nitroE2 were formed. NitroE2 dimers and 2, 4 – dinitroE2 were not observed. Similar results were obtained by Pezzella et al., when they synthesized nitroE2 using HNO_3 and glacial acetic acid at 70^o C. When lower concentrations of HNO_3 was used 2 – nitroE2 (30 % yield) and 4 – nitroE2 (25 % yield) were obtained as main synthesis products. However when higher concentration of HNO_3 was used 2, 4 – dinitroE2 (12 % yield) was obtained in the reaction mixture.[196]

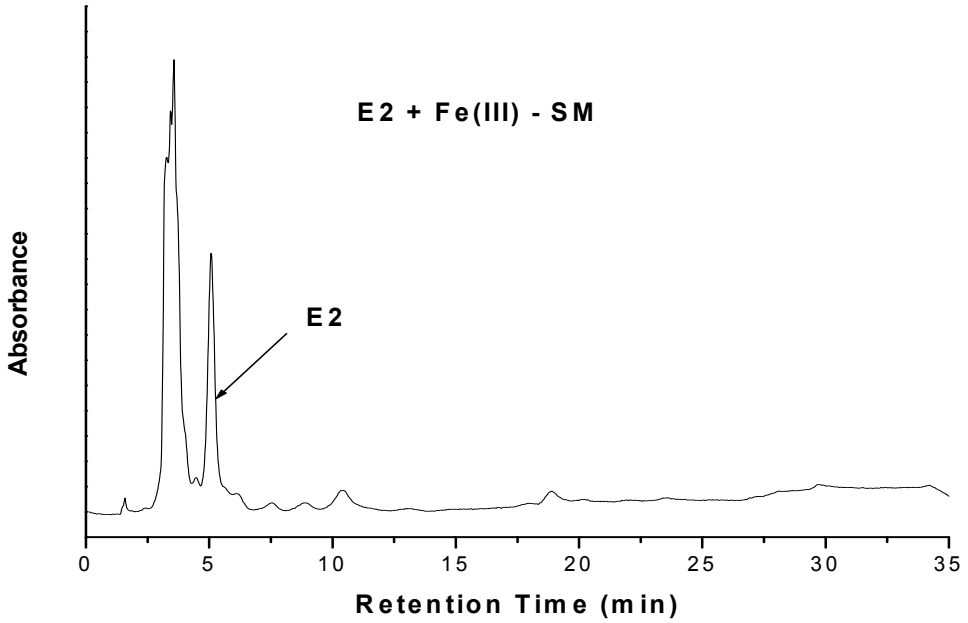


Figure 4.17 LC-UV chromatogram at 210 nm showing transformation of E2 in Fe(III)-saturated montmorillonite

Based on the study by Pezella et al., and products detected in our study using LC-MS a pathway for production of nitroE2 is proposed in Figure 4.19. Production of dimers, trimers, and tetramers is a very common phenomenon observed when oxidative transformation of organic compounds is carried by Fe (III) – SM. Yong et al., observed polymerization of 2, 6 – dimethylphenol (DMP) and o-methylphenol (MP) by Na^{+}, Ca^{2+}, Al^{3+} and Fe^{3+} saturated montmorillonite.[212] In our study, dimers were not observed with NM which is Na^{+} saturated montmorillonite. This can be due to the slow production of dimers by NM. The concentration of dimers obtained might be undetectable by LC-MS. Liyanapatrina et al., and Gu et al., also observed formation of dimers for the compounds used in their study.[202, 205] A mechanism for production of dimers by Fe (III) –SM is given in Figure 4.19. The mechanism is based on the single electron transfer mechanism proposed in the studies mentioned above.

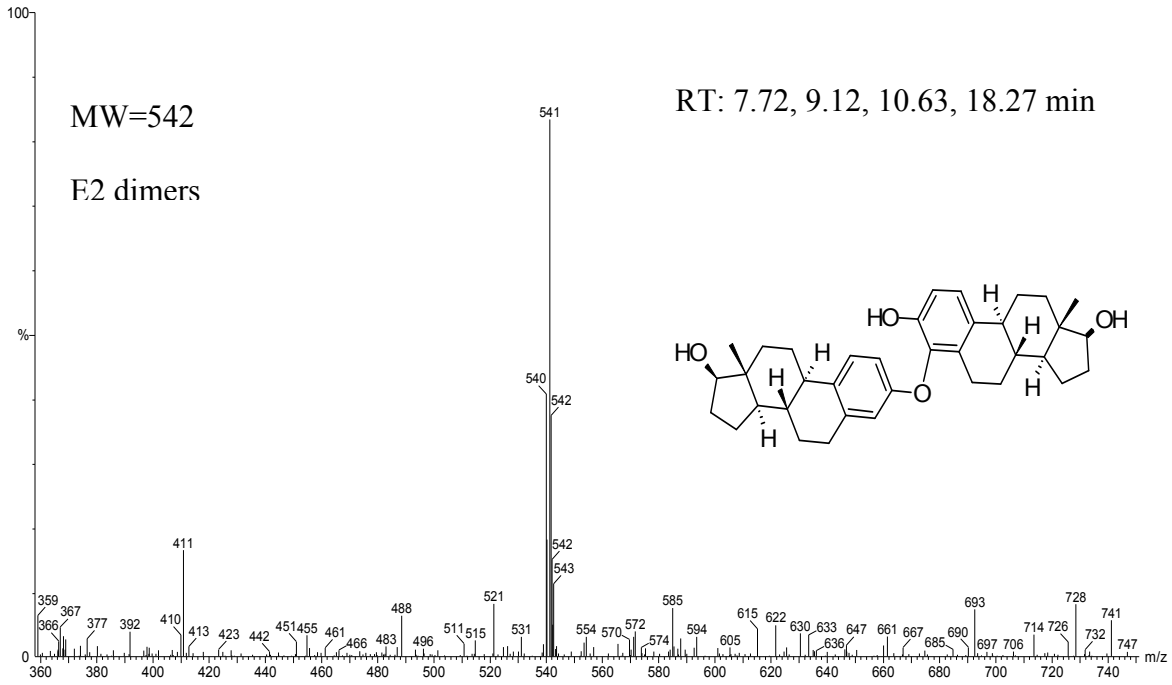


Figure 4.18 Mass spectrum of E2 dimers produced by 0.1 mM $NaNO_2$ + Fe (III)-SM and Fe(III)-SM

When $NaNO_2$ was not present in the system, only E2 dimers were detected. E2 dimers were the most abundant in the system, different conformers were present. Based on the retention time four dimer conformer peaks were observed at m/z 541 $[M-H]^-$. A LC-UV chromatogram is shown in Figure 4.16, for the transformation of E2 in Fe (III)-saturated montmorillonite, showing very few product peaks. Similar mechanism as shown in Figure 4.19 for production of dimers is true for dimers production of E2 by Fe (III) –SM.

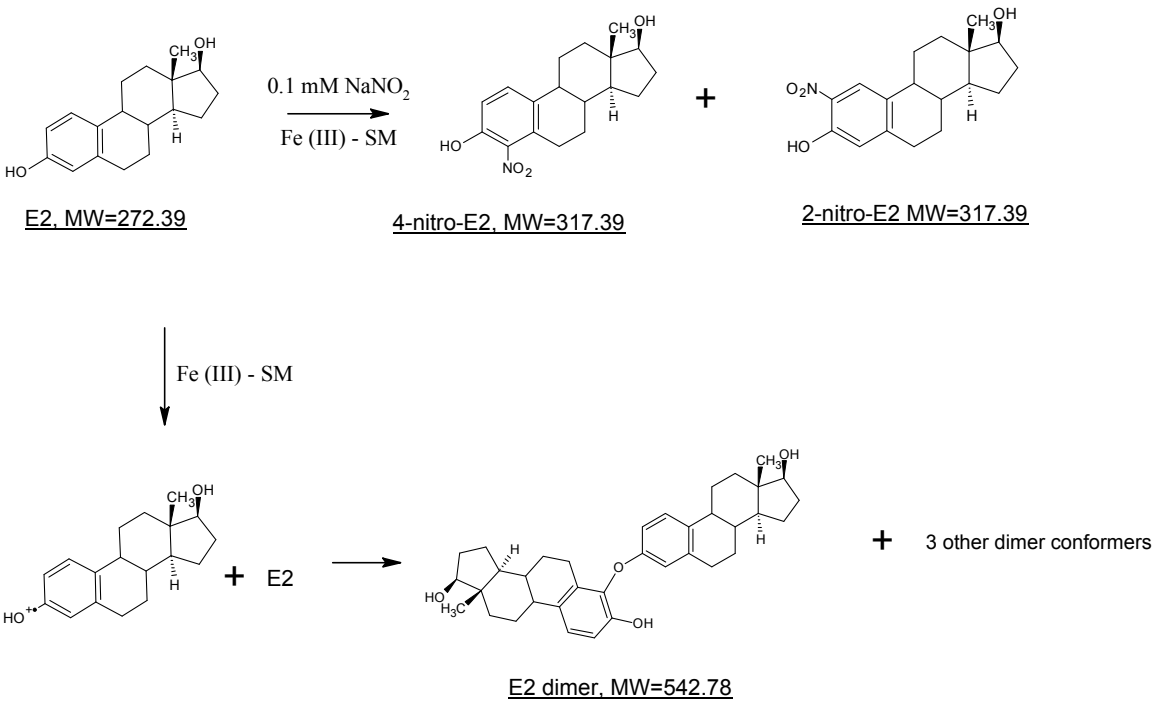


Figure 4.19 Proposed pathway for transformation of E2 by 0.1 mM $NaNO_2$ + Fe (III)-SM

From this study we can conclude that E2 transformation can take place in two ways. When the source of nitrite ion is abundant, nitration can be the dominant mode of transformation, and lead to formation of nitro products and dimers. However when the nitration source is not present Fe (III) – SM mediated oxidation will be the major transformation process. Both transformation processes are pretty fast, 99 % E2 transformation was observed in 4 h. But no products besides nitro-derivatives of E2 and dimers were detected. The products formed were not major fraction of the compound transformed. This leads to a question what happens to the majority of the compound in the reaction mixture. Similar results were observed by Wang et al, when they used $FeCl_3/NaNO_2$ as their catalytic system, they observed the presence of some organic acids such as malonic acid, which eventually disappeared and no products were observed after 30 days of reaction.[190] The catalyst studied in this dissertation is much faster than that

observed by Wang et al., hence it is quite possible that the products produced in this study disappear at an even faster rate than theirs.

4.4.6 Abiotic transformation of estriol by Fe (III) – SM, NM, MnO_2 and Fe_2O_3

Transformation of E3 was carried out by 1 mM $NaNO_2$ + Fe (III) - SM, 1 mM $NaNO_2$ + NM. Transformation was also studied in Fe (III) –SM, NM, MnO_2 and Fe_2O_3. As shown in Figure 4.20, in presence of 1 mM $NaNO_2$ + Fe (III) – SM, complete transformation of E3 was obtained within 1 day. The next best performance in terms of transformation was given by Fe (III) – SM, 50 % of E3 transformation took place within 5 days. At the end of 20 days only 15 % E3 remained in the Fe (III) –SM + E2 reaction mixture. MnO_2 showed slower transformation of E3, 50 % E3 transformation was achieved after 8 days. No transformation of E3 was observed by 1 mM $NaNO_2$ + NM, NM and Fe_2O_3. After 20 days in 1 mM $NaNO_2$ + NM, NM and Fe_2O_3 treatments more than 90 % E3 was still not transformed.

Table 4.2 shows the reaction rate constants and half-life values for transformation of E3 using various catalysts. Highest reaction rate constant was observed by Fe (III) – SM + 1 mM $NaNO_2$ + E3, with a value of 1041.7 $(mmol/g)^{-1}$ days and a half-life of 0.11 days. The reaction rates observed for transformation of E3 by Fe (III) – SM and MnO_2 were almost similar with Fe (III) – SM slightly higher. The half-life values for Fe (III) – SM and MnO_2 were 3.09 and 8.14 days, respectively

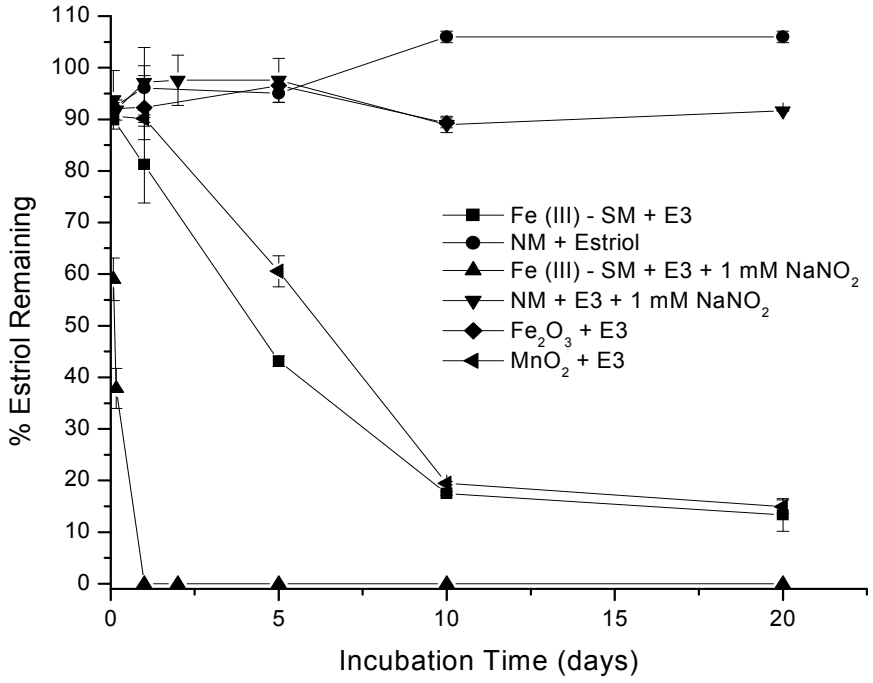


Figure 4.20 Transformation of E3 by Fe(III) – SM and NM in presence and absence of 1 mM $NaNO_2$ and by Fe_2O_3 and MnO_2

Table 4.2 Half-life values and reaction rate constants for transformation of E3 using various catalysts

Treatment	Reaction rate constant K $(mmol/g)^{-1}$ days	$t_{1/2}$ (days)
Fe (III) – SM + E3	36.109	3.09
Fe (III) – SM + 1 mM $NaNO_2$ + E3	1041.7	0.11
MnO_2 + E3	13.649	8.14

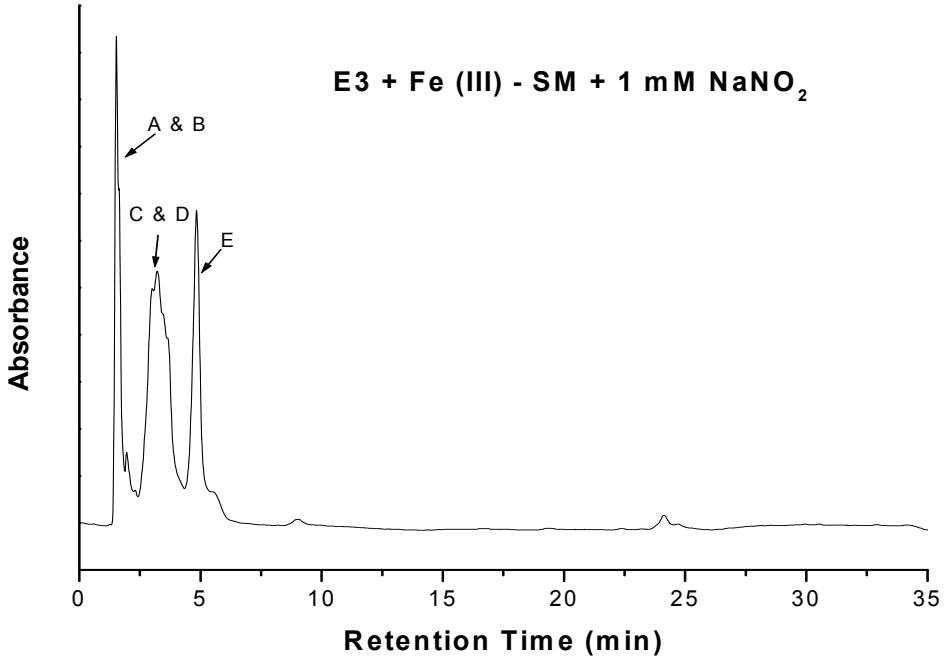


Figure 4.21 LC-UV chromatogram at 210 nm showing transformation of E3 by Fe(III)-SM + 1 mM $NaNO_2$

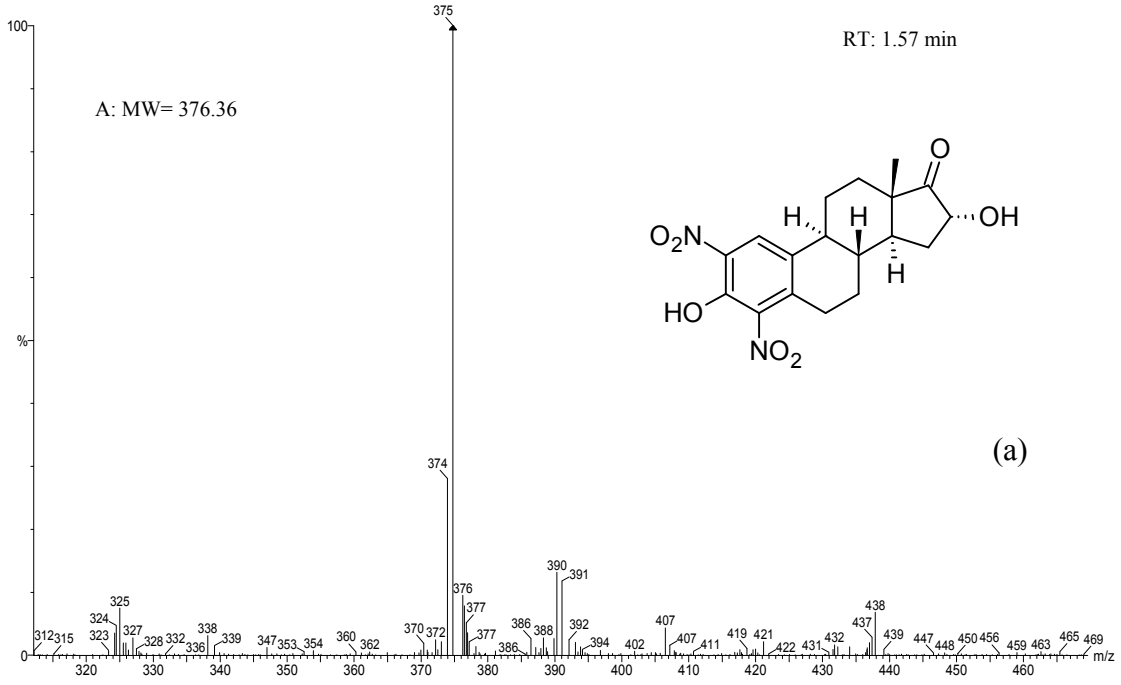


Figure 4.22 Mass spectrum of transformation products of E3 produced by Fe (III) –SM + 1 mM $NaNO_2$ (a, b, c, d, e)

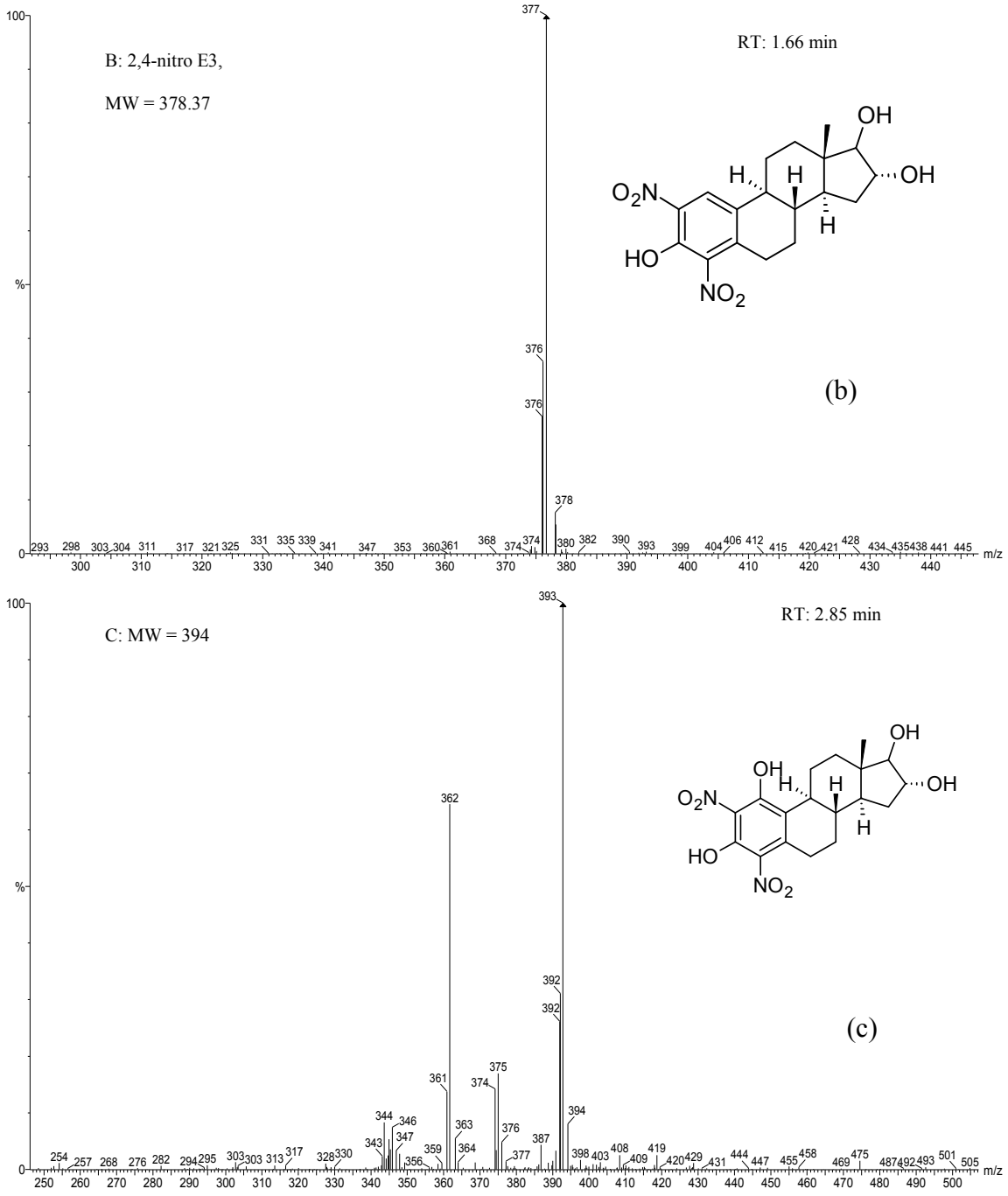


Figure 4.22 (continued)

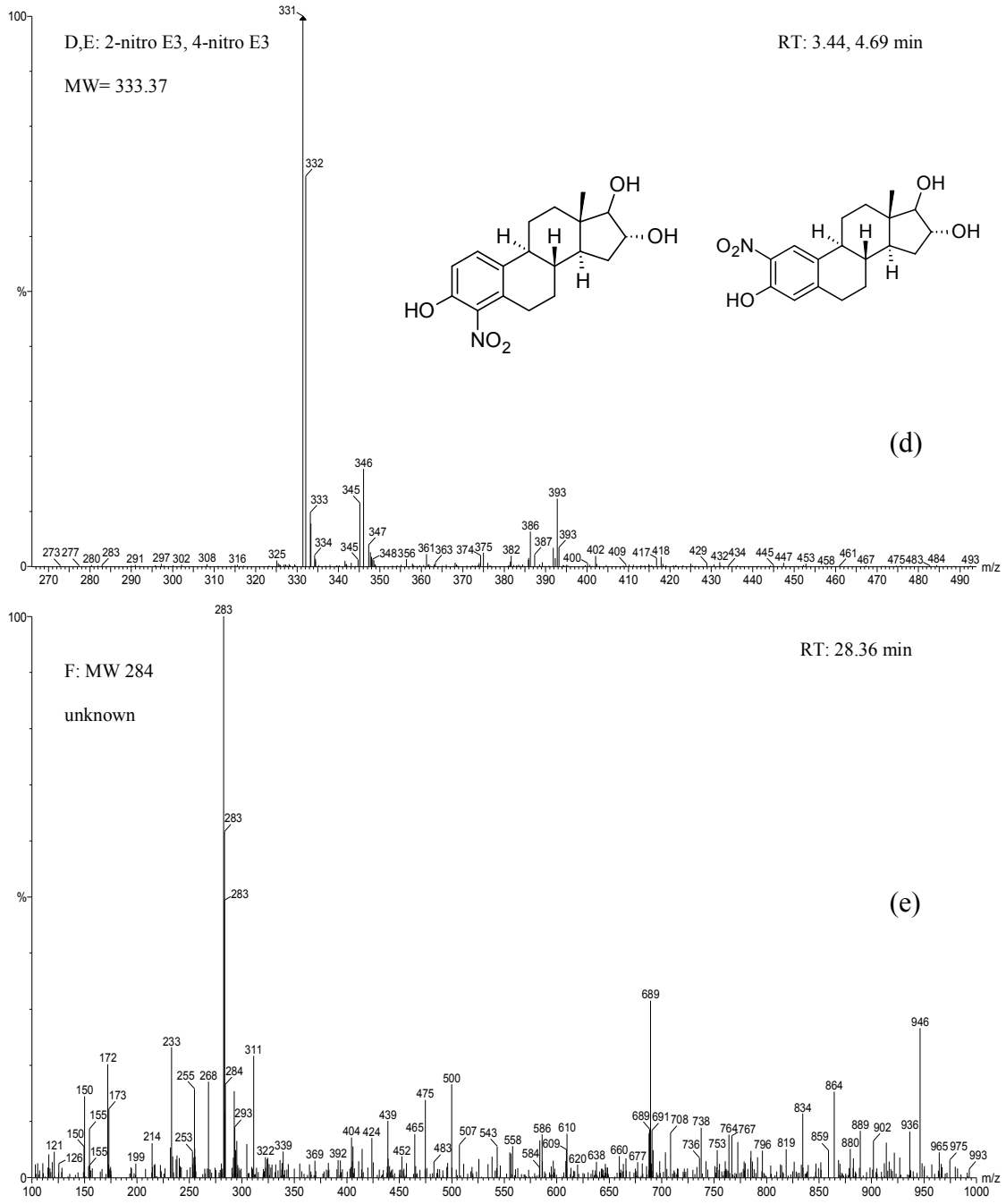


Figure 4.22 (continued)

As shown in Figure 4.21, transformation of E3 by Fe (III)-SM + 1 mM $NaNO_2$ is accompanied by production of overlapping product peaks. Peaks A and B were identified

using LC-MS, these peaks appeared before E3 peak which had a retention time of 3.38 min, this suggests that these peaks are more polar compared to E3. The addition of $–NO_2$ can increase the polarity of E3. The mass spectrum in Figure 4.22 a, shows formation of a nitration product. The –OH group at C-17 position in E3 has been converted to a keto group, and hence a product molecular ion peak at m/z 375 $[M-H]^-$, 2, 4-dinitro, 16-hydroxyestrone is formed. The mass spectrum in Figure 4.22 b, shows a product formed at m/z of 377 $[M-H]^-$, the product was identified as 2, 4-dinitro-E3. Peak c and d showed overlapping when analyzed using LC-UV. LC-MS was used to identify the products formed. The mass spectrum (Figure 4.22 c) showed a product at m/z 394 $[M-H]^-$, which suggests that two $–NO_2$ and two –OH groups has been added to the E3 molecule. The suggested structure of the molecule is one of the several possibilities. To identify the correct position of the attachment of the nitro and hydroxyl group NMR studies are required. The mass spectrum of products D and E (Figure 4.22 d) shows the presence of product formed at m/z 331 $[M-H]^-$, this m/z was assigned to 2-nitro E3 and 4-nitro E3. An unknown product was detected; it was not possible to assign a structure based on the mass spectrum (Figure 4.22e).

Based on the products detected and proposed pathway for chlorination of E2 by Hu et al.,[144] a pathway for transformation of E3 is suggested in Figure 4.23. As shown in the Figure 4.23, nitration products are the primary transformation products produced.

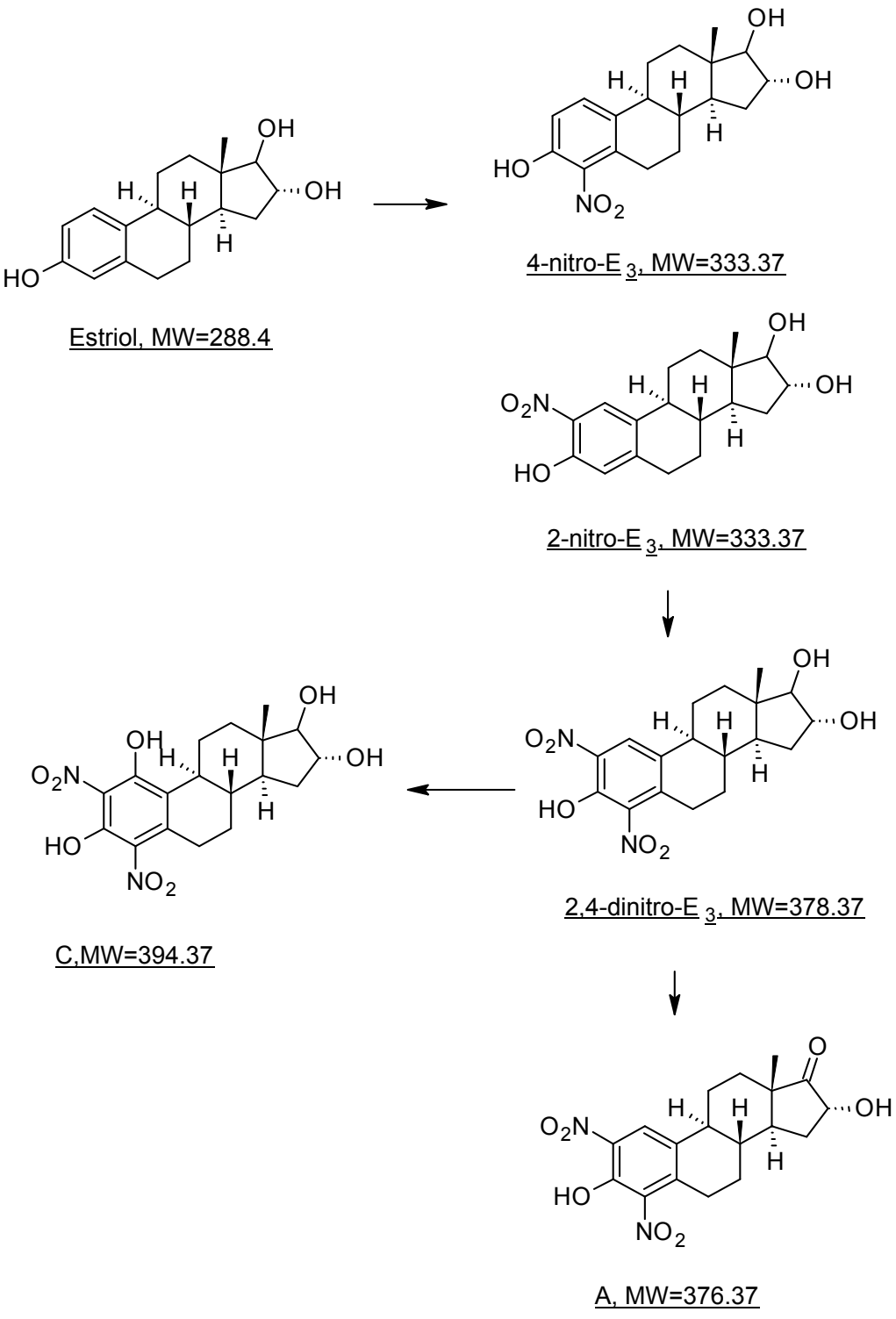


Figure 4.23 Suggested pathway for transformation of E3 by Fe (III) - SM + 1 mM $NaNO_2$

These nitration products are further oxidized to produce keto group at the C-17 carbon position. Hydroxylation of 2, 4-dinitro E3 results in introduction of the –OH group at the C-1 position on the benzene ring. The structures in this study are proposed structures; further studies are required to confirm the identity of the identified transformation products. No dimers were observed, this may be due to the fact, that all the possible sites for dimer formation are substituted via nitration or hydroxylation and steric hindrance would make formation of dimers thermodynamically high in energy.

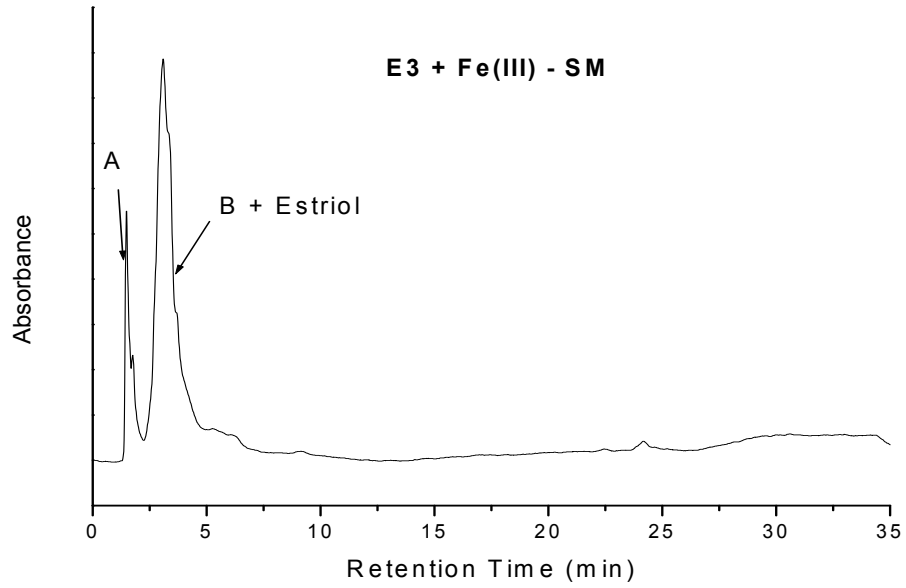


Figure 4.24 LC-UV chromatogram for transformation of E3 by Fe (III) - SM

When transformation of E3 was studied in Fe (III)-SM, only two peaks were observed in both LC-UV and LC-MS. The Figure 4.24, shows the LC-UV chromatogram for the transformation of E3 in Fe (III)-SM. The first peak identified by LC-MS at retention time of 1.42 min, had a m/z of 305 $[M-H]^-$. The structure identified on the basis

of mass spectrum (Figure 4.25 b) shows addition of the –OH group on C-2 position on the benzene ring.

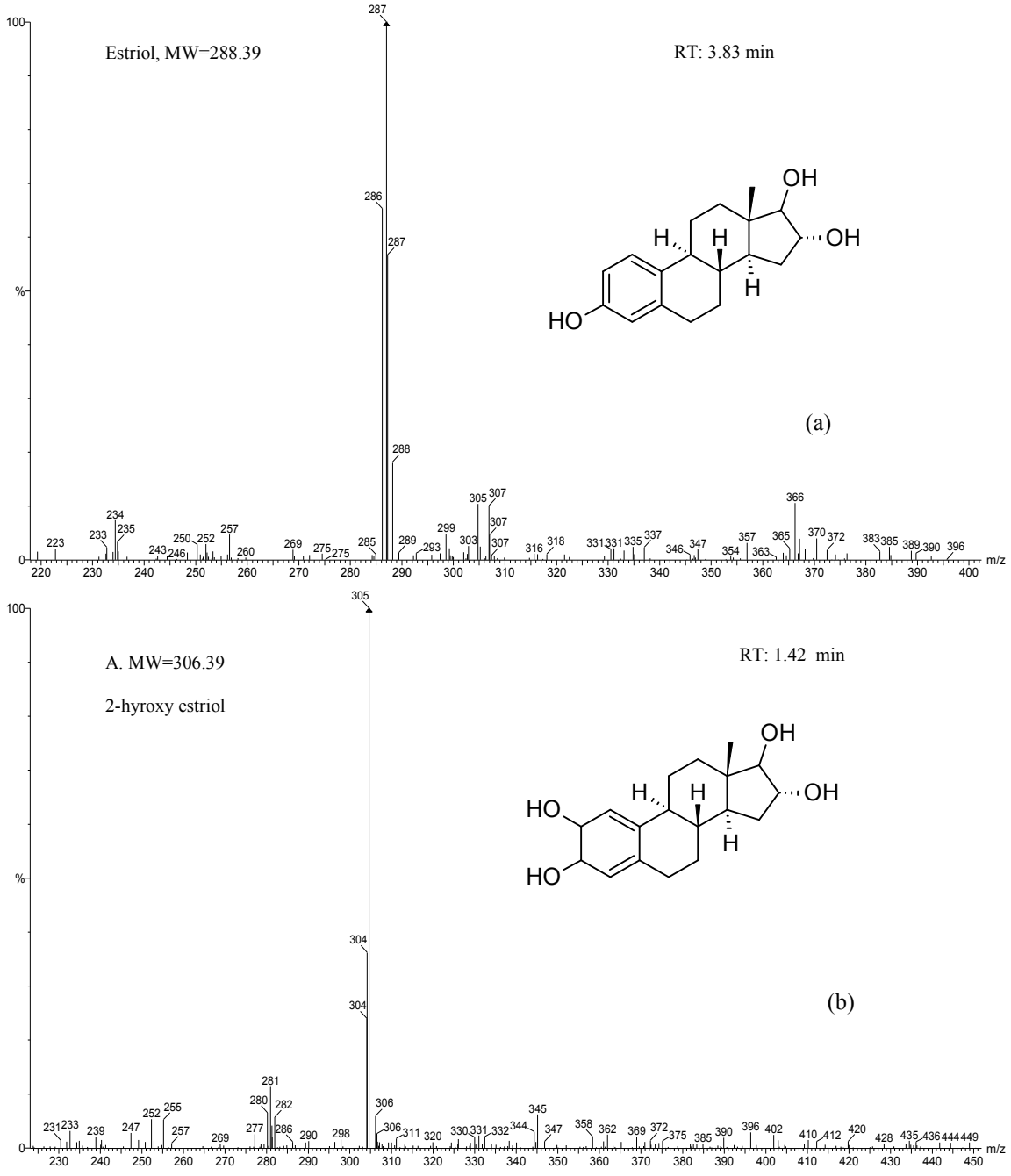


Figure 4.25 Mass spectrum of E3 (a), and E3 oxidation transformation products produced by Fe (III) - SM (b) and (c).

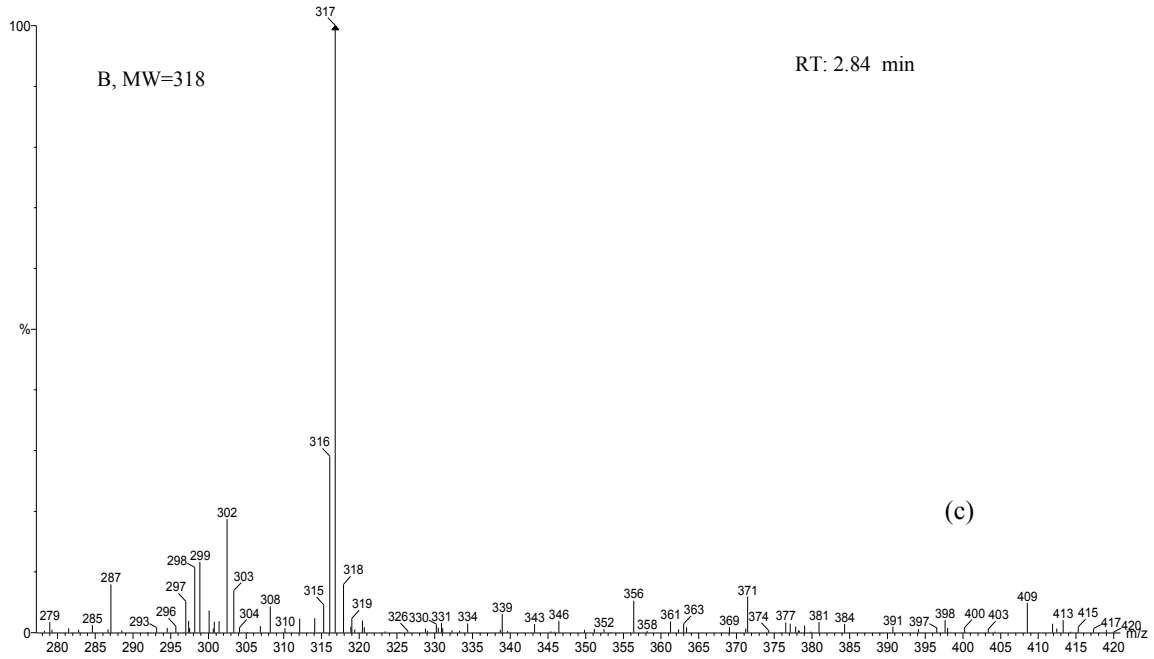


Figure 4.25 (continued)

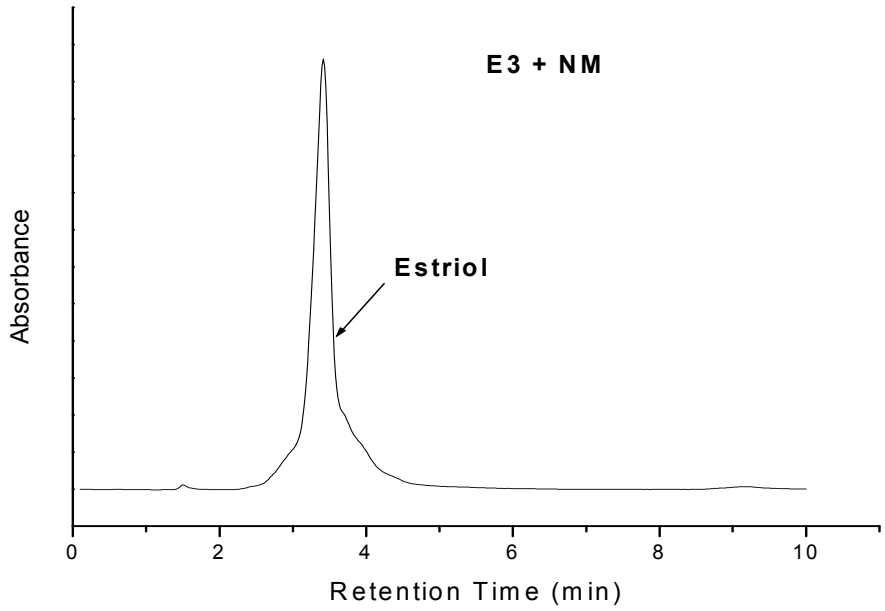


Figure 4.26 LC-UV chromatogram showing E3 peak after being subjected to oxidative transformation by NM

A peak was detected at retention time 2.84 min, in Fe(III)- SM and E3 reaction mixture with a m/z of 317 (Figure 4.25c), but based on the mass spectrum the peak could not be identified. The problem associated with identification of transformation products of hormones is that no information on generated transformation products exists in the literature and no databases exist for identification.

As can be seen in Figure 4.26, only a single peak is observed when E3 was subjected to transformation using NM. No transformation of E3 occurred by NM.

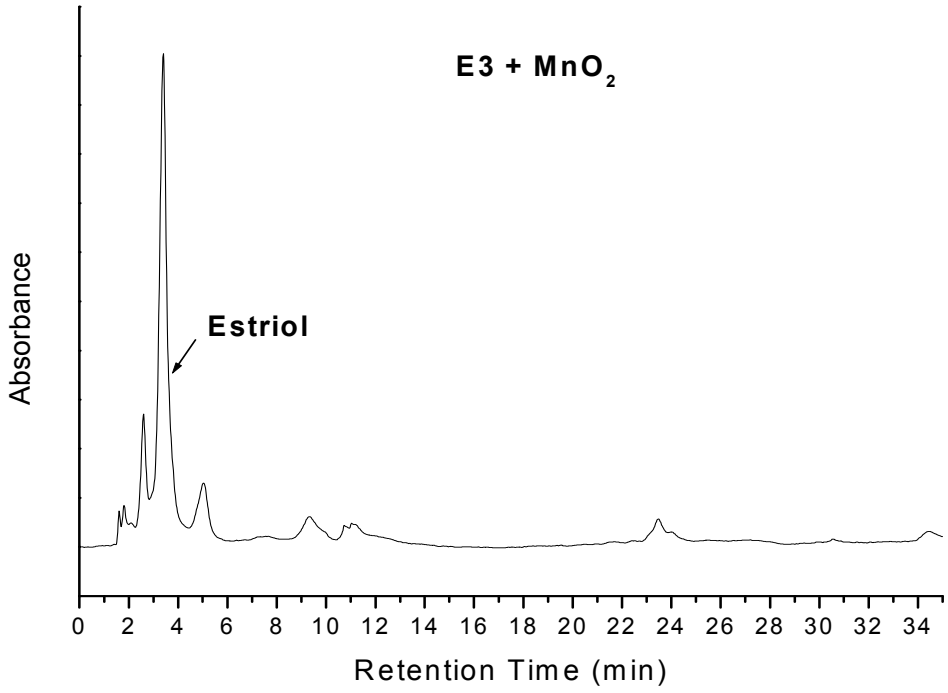


Figure 4.27 LC-UV chromatogram showing peaks generated due to oxidative transformation of E3 by MnO_2

Transformation of E3 was studied using MnO_2. MnO_2 proved to be efficient in removal of E3. The reaction followed the same rate as Fe (III)-SM. Figure 4.27, shows the presence of a few peaks after E3 exposure to MnO_2 in the aqueous environment. Those peaks were identified using LC-MS. According to mass spectrum shown in Figure 4.28 a, product formed at retention time 2.23 min had a m/z of 301 $[M-H]^-$, a quinone type structure was predicted. The next identified peak had a m/z of 285 $[M-H]^-$, the structure predicted is a 16α-hyroxyestrone (Figure 4.27 b). Similar transformation products were identified by Jiang et al., when the transformation of E2 was carried out using MnO_2. In their study two products were identified as the major metabolites of E2, one of the metabolite was E1 and other was 2-hydroxyestradiol.[151]

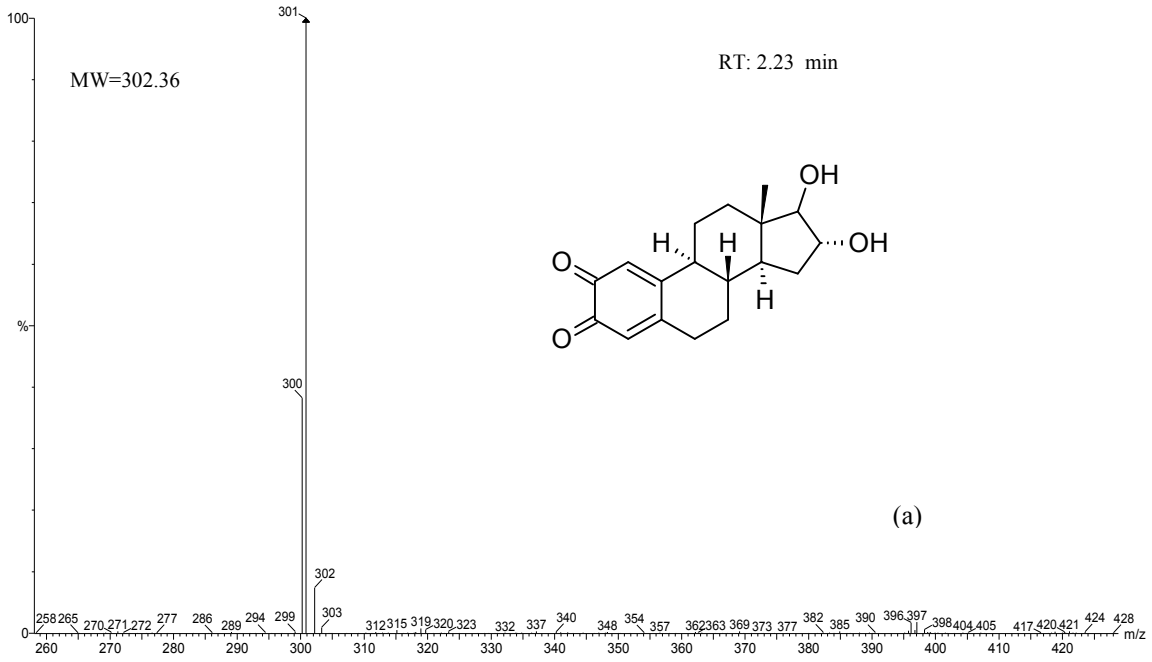


Figure 4.28 Mass spectrum of oxidative transformation products detected when E3 was exposed to MnO_2

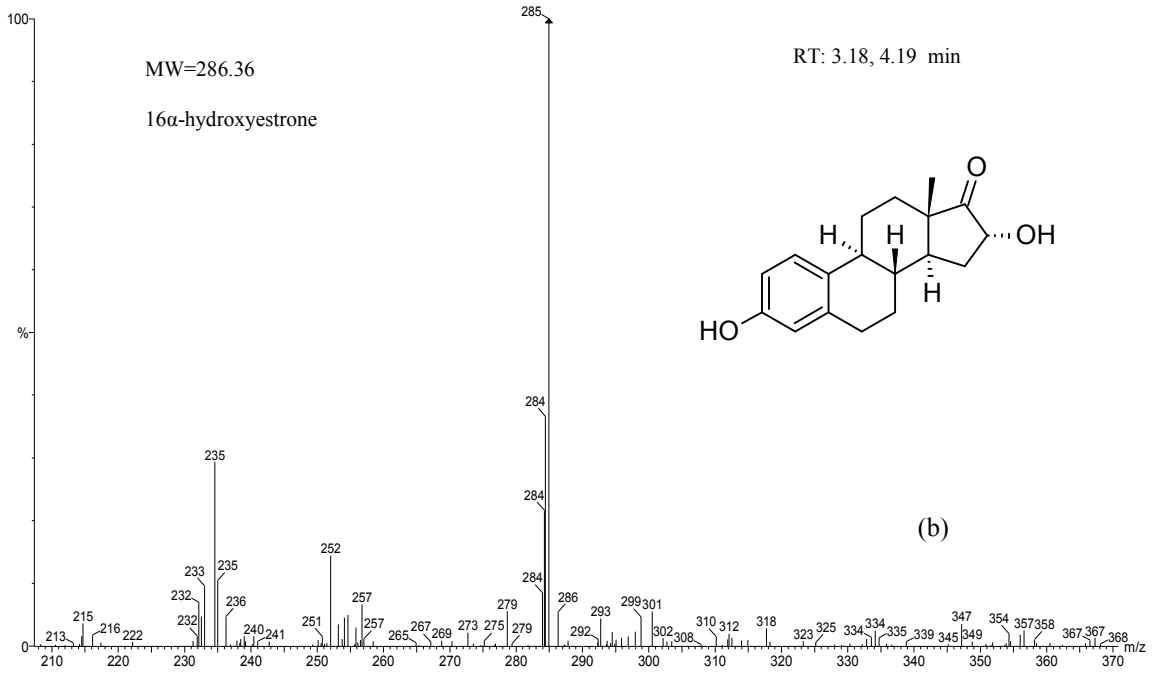


Figure 4.28 (continued)

Haaften et al., also observed the presence of 16α-hydroxyestrone as a transformation product of estriol. In women receiving estriol, 16α-hydroxyestrone was identified as the primary transformation product.[213] 16α-hydroxyestrone is considered a "bad estrogen" and is associated with development of cancerous tumors.[214] The products produced by MnO_2 oxidation of E3 in this study are highly toxic in nature. Use of MnO_2 as a method for remediation of E3 in the environment should be given a thorough consideration. It is very important to confirm the identity of the detected compounds to avoid the risk of releasing toxic compounds in the environment.

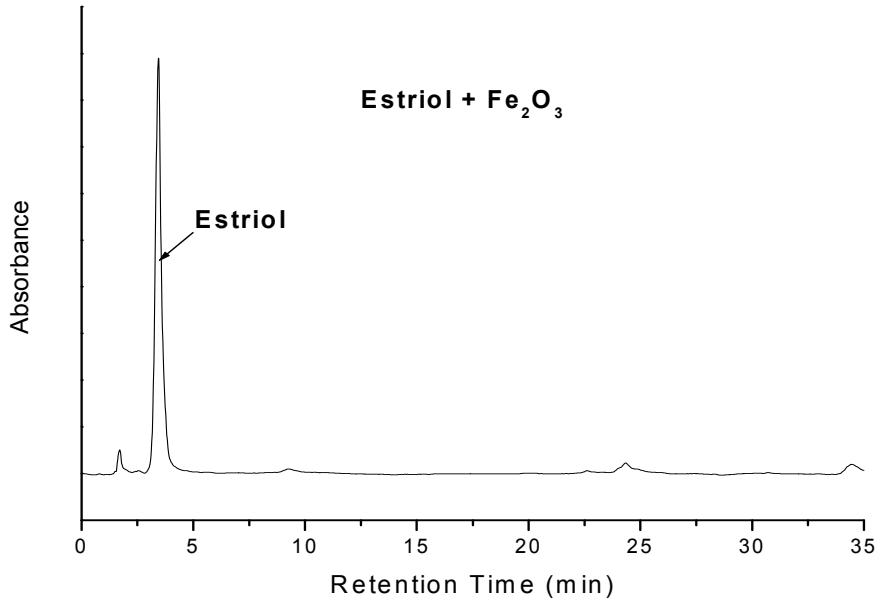


Figure 4.29 LC-UV chromatogram at 210 nm showing oxidative transformation of E3 by Fe_2O_3

The results obtained when E3 was added in Fe_2O_3 catalysts, showed no formation of transformation products. Figure 4.29, Shows E3 detected at high concentration in the reaction mixture. Also as can be seen in Figure 4.20, during the 20 days of reaction period no significant change occurred in the concentration of E3. The LC-MS showed the presence of E3 and no other product peaks were observed.

The results of this study suggest that Fe (III) – SM is the best catalysts among the studied catalysts for oxidative transformation of E3 by 1 mM $NaNO_2$ + Fe (III) - SM. MnO_2 performed equally well for transformation of E3, but quinone type and 16α-hydroxyestrone intermediate products were formed which are highly toxic and related to cancer. No transformation of E3 was observed on Fe_2O_3 and NM up to 20 days.

Wang et al., demonstrated use of $FeCl_3$ and $NaNO_2$ as photocatalysts for oxidative transformation of E3.[190] In their study 99 % transformation of E3 under similar

conditions took 13 days. Sun light was used as a source for photoinitiation of the catalyst. The concentration of $NaNO_2$ used in our study is higher than the concentration used by Wang et al., but in our studies the presence of light for initiation of catalyst was not required. Hence the catalysts used by Wang et al., will be limited by the need to have a light source for initiation of the reaction. Fonseca et al., studied the transformation of estrogens in water by solar radiation. The concentration of hormones used in the study was 0.1 g/L. They found that the transformation of E3 took 120 days by solar radiation. The study demonstrated that the transformation of hormones can occur by natural solar radiation. The factors such as temperature, dissolved organic matter, biological activity and oxygen content will determine the rate of transformation in the environment.[215] Shappell et al., carried out the transformation of E3 using Fe-TAML/H_2O_2 catalysts. The concentration of hormone used was 80 μM. Complete transformation of E3 occurred within 15 min with a half-life of 5 min. However Fe-TAML in absence of H_2O_2 did not showed the transformation of E3.[118] Xu et al., studied the transformation of E3 by MnO_2 in water. The concentration of E3 used was 4 μM. In this study 100 % transformation of E3 occurred after 225 min at pH 4. The estrogenicity assay showed complete removal of estrogenic activity after treatment with MnO_2.[116] The studies mentioned above either show slow transformation of E3 or use catalyst that can increase the cost of the transformation process if employed for treatments in WWTPs. In the study by Xu et al., complete removal of estrogenic activity is shown, but our results contradict the statement. Formation of toxic transformation product 16α-hydroxyestrone was observed when the transformation of E3 was carried out using MnO_2.

4.4.7 Abiotic transformation of EE2 by Fe (III) - SM, NM, MnO_2 and Fe_2O_3

Abiotic transformation of EE2 was studied using various catalytic systems such Fe (III) - SM + 1 mM $NaNO_2$, Fe (III) - SM, NM + 1 mM $NaNO_2$, NM, MnO_2 and Fe_2O_3.

Complete transformation of EE2 was obtained with Fe (III) – SM + 1 mM $NaNO_2$ within 4 h. While with Fe (III)-SM 90 % transformation of EE2 occurred within 48 h. With MnO_2, 60 % EE2 was transformed after 48 h. The Fe_2O_3 and NM + 1 mM $NaNO_2$ and NM showed the least transformation of EE2, less than 15 % (Figure 4.30).

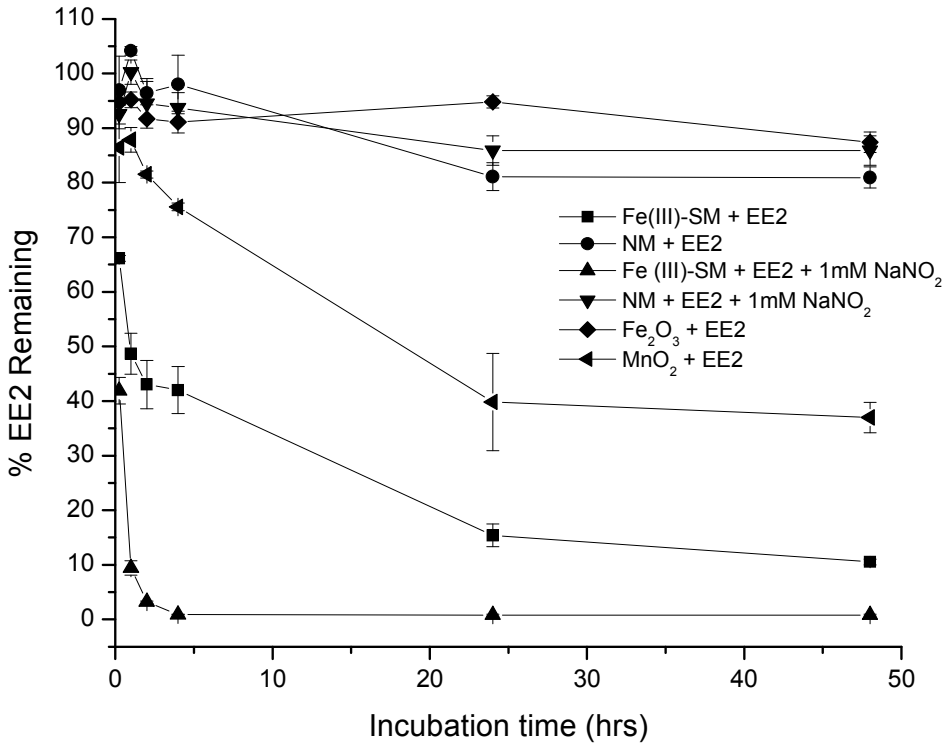


Figure 4.30 Oxidative transformation of EE2 using various catalysts monitored by LC-UV at 210 nm

Table 4.3 Half-life values and reaction rate constants for transformation of EE2 using various catalysts

Treatment	Reaction rate constant K $(mmol/g)^{-1}$ h	$t_{1/2}$ (hours)
Fe (III) – SM + EE2	108.78	1.03
Fe (III) – SM + 1 mM $NaNO_2$ + EE2	616.60	0.18
MnO_2 + EE2	4.17	27.0

Reaction rate constants and half-life value for transformation of EE2 using various catalysts is given in Table 4.3. Fastest reaction rate was observed with Fe (III) – SM + 1 mM $NaNO_2$ + EE2 with a half –life value of 0.18 h. The slowest transformation of EE2 occurred with MnO_2 with a rate constant of 4.17 and a half-life value of 27 h.

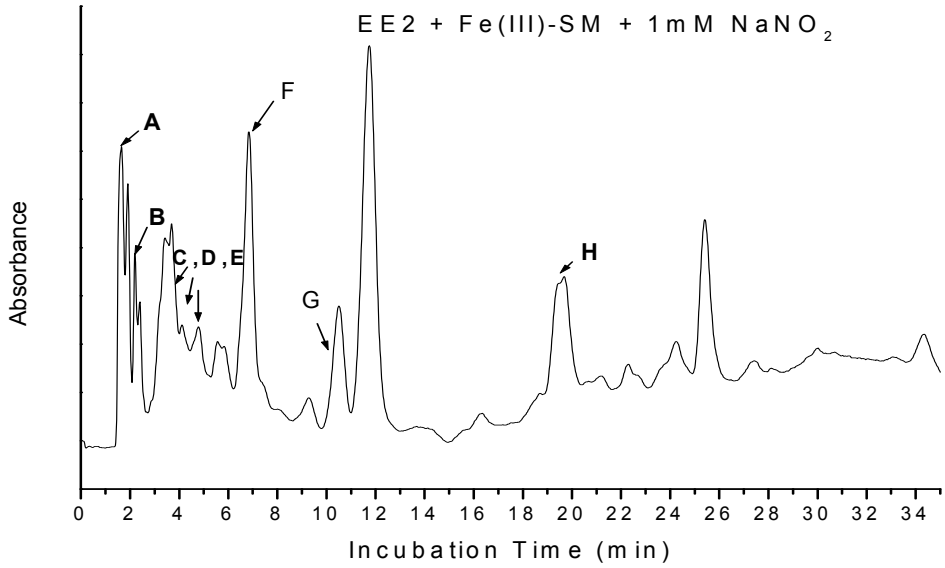


Figure 4.31 LC-UV chromatogram at 210 nm showing transformation products formed when EE2 was subjected to oxidative transformation by Fe (III) – SM + 1 mM $NaNO_2$.

Many transformation product peaks were observed (Figure 4.31) when transformation of EE2 was studied using Fe (III) – SM + 1 mM $NaNO_2$. The identification of these peaks using LC-MS revealed presence of several nitration products. Peak A shown in Figure 4.32 at retention time 1.52 min was identified as dinitro-EE2 and EE2 dimer or nitro-EE2 and nitro-EE2 dimer having a m/z of 679 $[M-H]^-$ (Figure 4.32 b). Peak B at retention time 1.76 min was 2, 4-dinitro EE2 having a m/z of 385 $[M-H]^-$ (Figure 4.32 a). Peaks C, D and E at retention time 3.65, 4.11, 4.63 min were identified as conformers of 2, 4-dinitro- hydroxy EE2, having m/z of 401 $[M-H]^-$ (Figure 4.32 c). While peaks F and G at retention time 6.02 and 9.73 min were identified as 2-nitro EE2 and 4-nitro EE2 having m/z 340 $[M-H]^-$ (Figure 4.32 d).

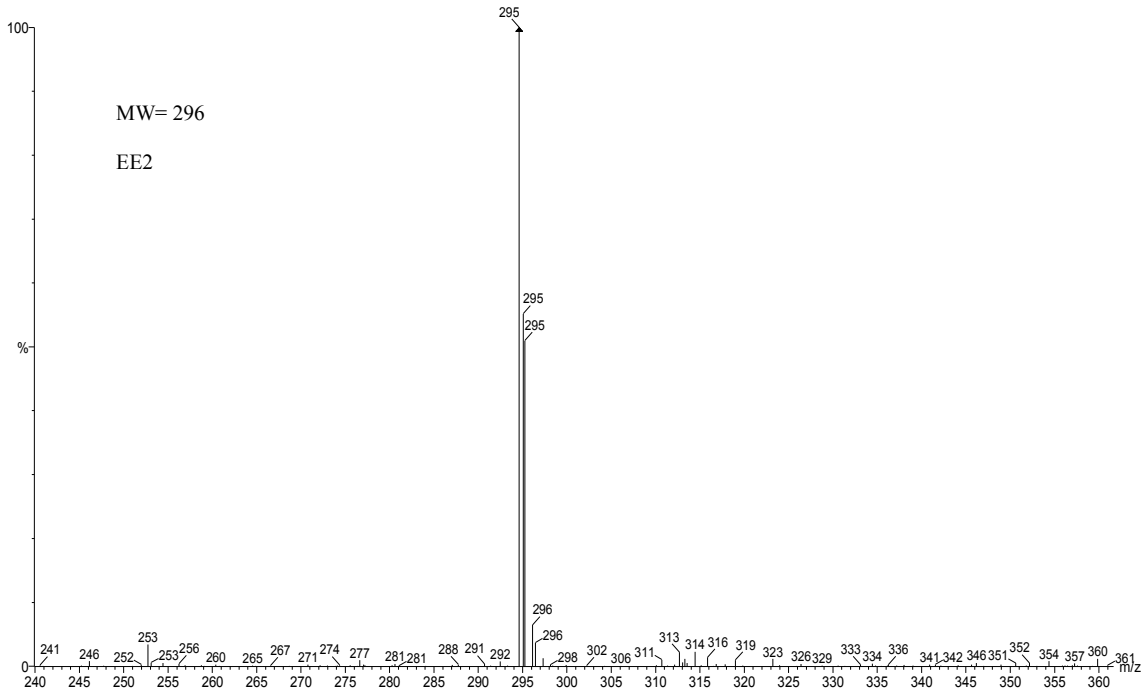
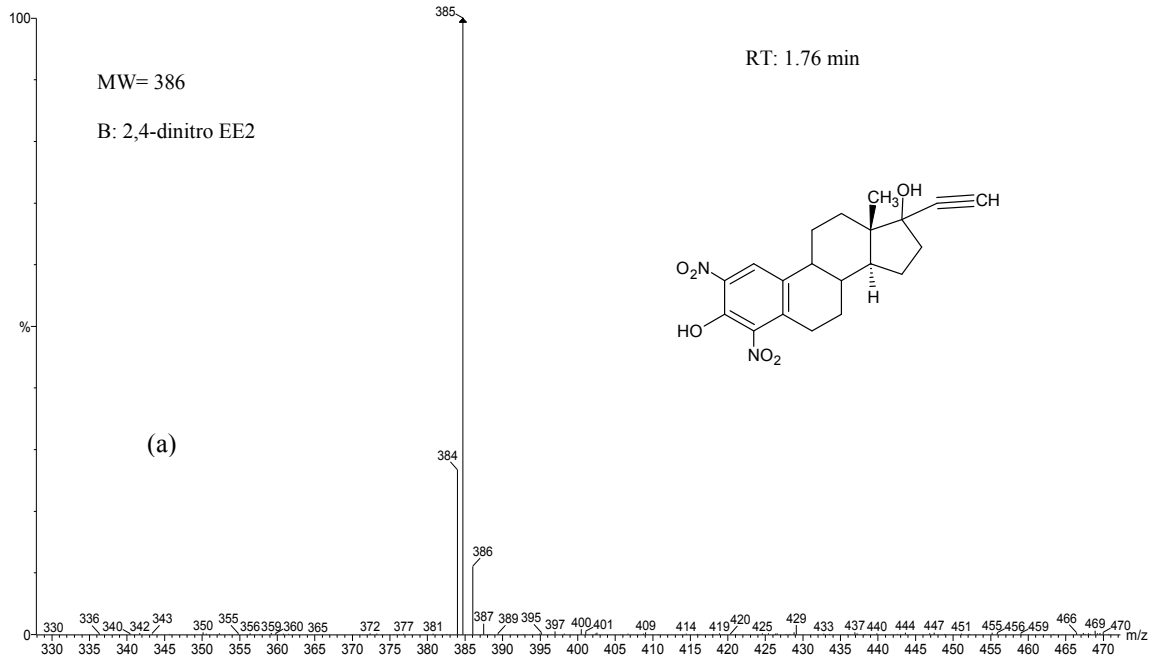


Figure 4.32 Mass spectrums showing the transformation products of EE2 identified. Reaction conditions: Fe (III)-SM + 1 mM $NaNO_2$ (a, b, c, d).



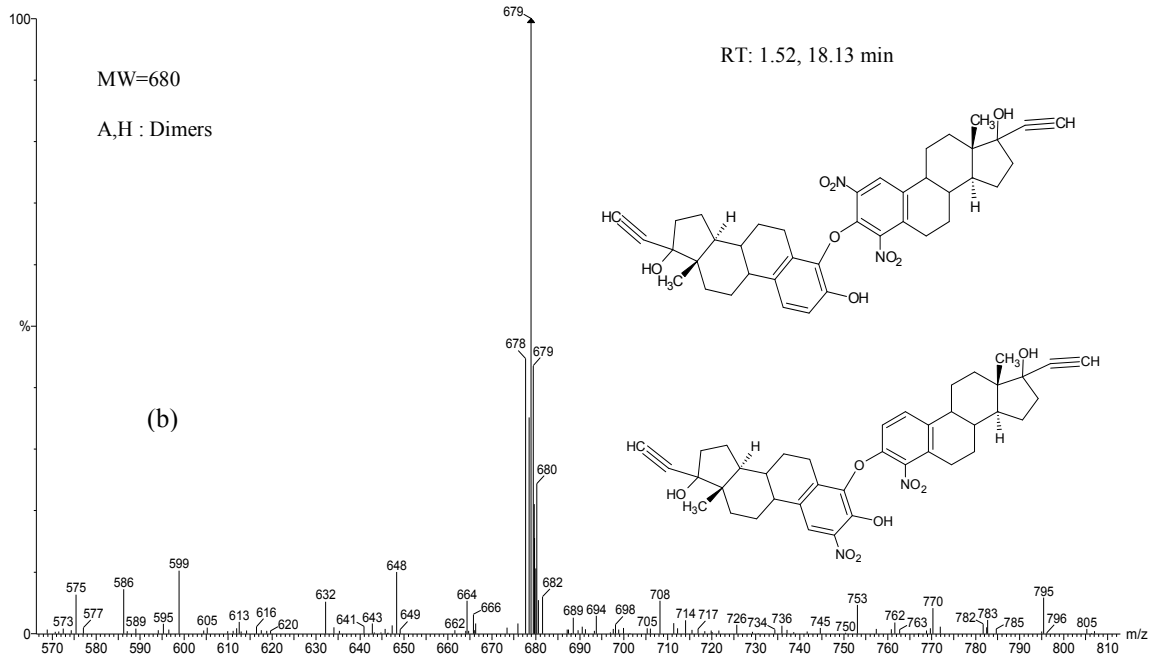


Figure 4.32 (continued)

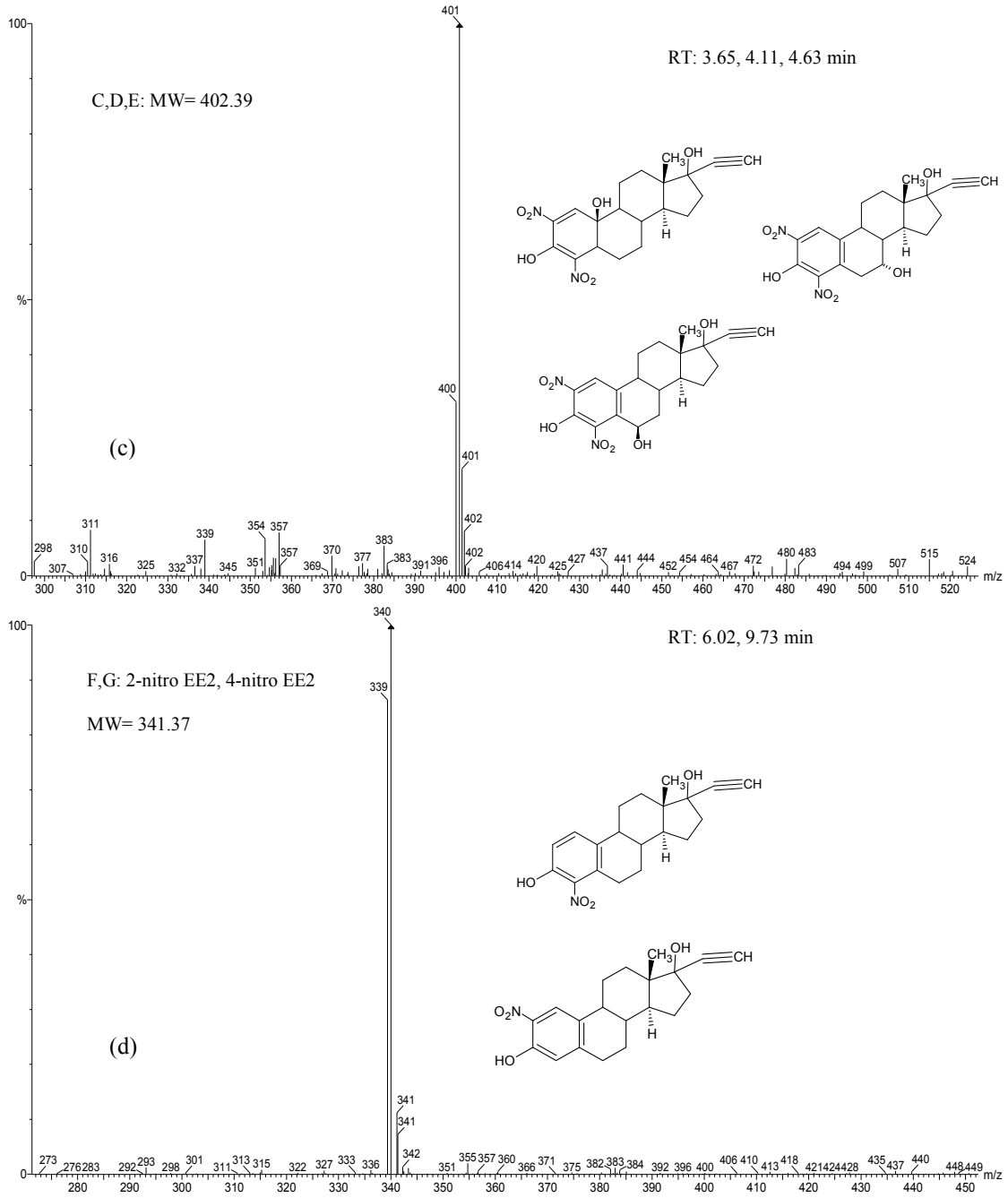


Figure 4.32 (continued)

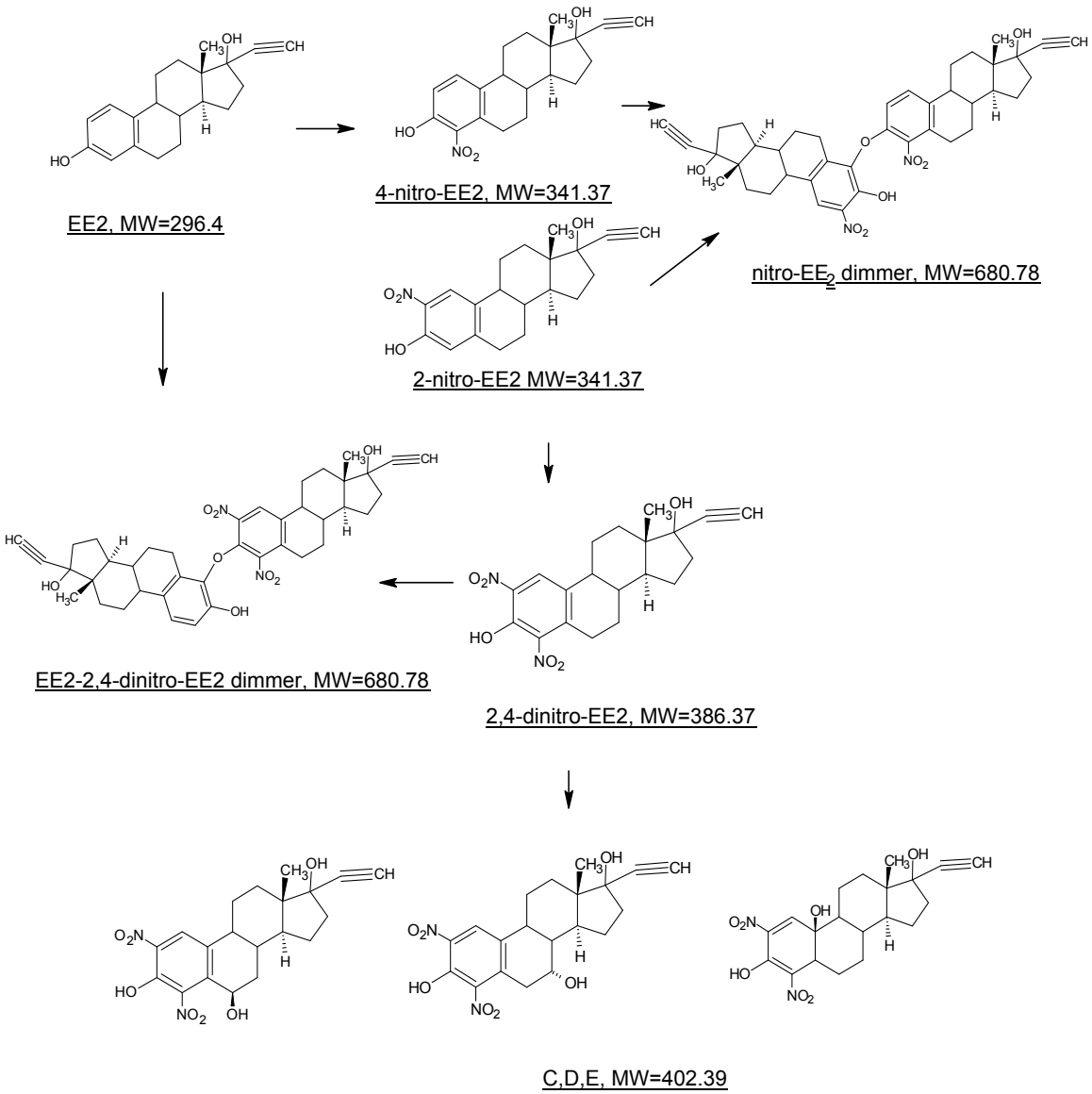


Figure 4.33 Proposed pathway for oxidative transformation of EE2 by Fe (III)-SM + 1 mM $NaNO_2$.

Based on the products detected in this study using LC-MS and proposed pathway for the chlorination of E2 by Hu et al.,[144] a pathway for the transformation of EE2 is suggested in Figure 4.33. As shown in the Figure 4.33 nitration products are the primary transformation products produced. These nitration products are further oxidize to produce two different types of dimers and hydroxylation of 2,4-dinitro EE2 results in introduction

of the –OH group, various possibilities are shown in Figure 4.33. As observed by Liyanapatirana et al and Gu et al., dimers were also produced.[202, 205] The mechanism for formation of dimer involves single electron transfer, which leads to the formation of radical cation. In this case nitroEE2 radical cation is formed and reacts with other nitroEE2 molecule to produce nitroEE2 dimer. Wang et al., demonstrated use of $FeCl_3$ and $NaNO_2$ as photocatalysts for oxidative transformation of EE2.[190] In their study 79.6 % EE2 was transformed after 13 days. Sun light was used as a source for photoinitiation of the catalyst. The concentration of $NaNO_2$ used in present study is higher than the concentration used by Wang et al., but in our studies presence of the light for initiation of catalysts is not required. Fe (III) – SM + 1 mM $NaNO_2$ was capable of achieving almost 100 % transformation of EE2 within 4 h. Shappell et al., reported complete transformation of EE2 using Fe-TAML/H_2O_2 at 365 nm.[118] However no transformation products have been reported. But estrogenicity assay showed removal of estrogenic activity after treatment with Fe-TAML/ H_2O_2. In our studies nitroEE2 derivatives are reported as the transformation products of EE2. Gaulke et al., conducted estrogenicity test for nitroEE2 derivatives and reported reduced estrogenicity.[192] When developing method for remediation of target organic compounds it is necessary to keep in mind the toxicity of the transformation products produced.

When EE2 was subjected to transformation using Fe (III) – SM fewer transformation product peaks appeared (Figure 4.34), compared to Fe (III)-SM + 1 mM $NaNO_2$. The peaks were identified using LC-MS, not many product peaks were detected. The only products peaks that were identified at retention time 10.48, 11.61, 13.01, 19.24 min (Figure 4.34) corresponded to EE2 dimers at m/z 589 $[M-H]^-$. The mechanism of

formation of dimer is similar to that reported for E2 transformation by Fe (III) – SM as shown in Figure 4.19.

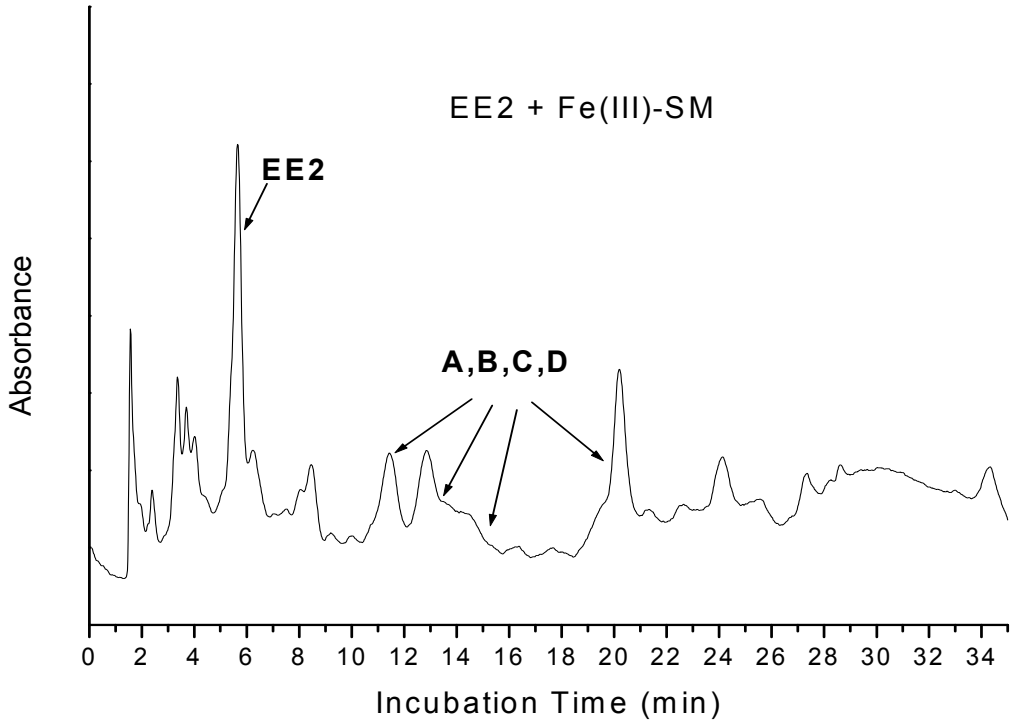


Figure 4.34 LC-UV chromatogram at 210 nm showing transformation products of EE2 formed by Fe (III)-SM.

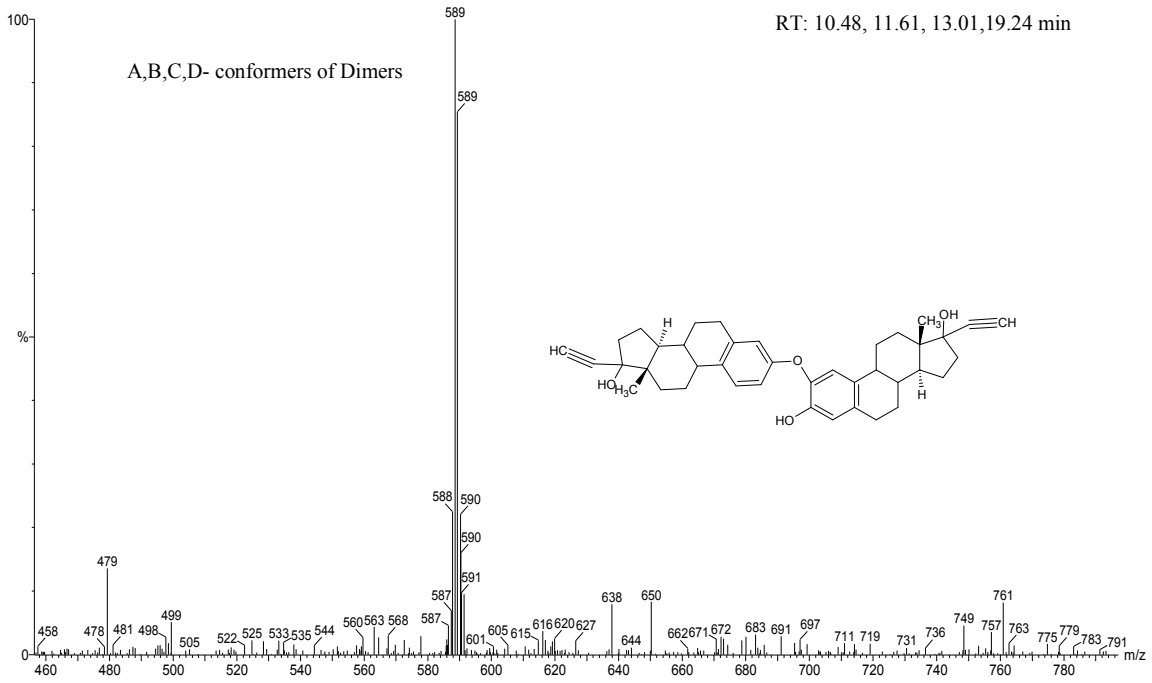


Figure 4.35 Mass spectrum of transformation products detected with LC-MS when EE2 was subjected to transformation using Fe (III) - SM.

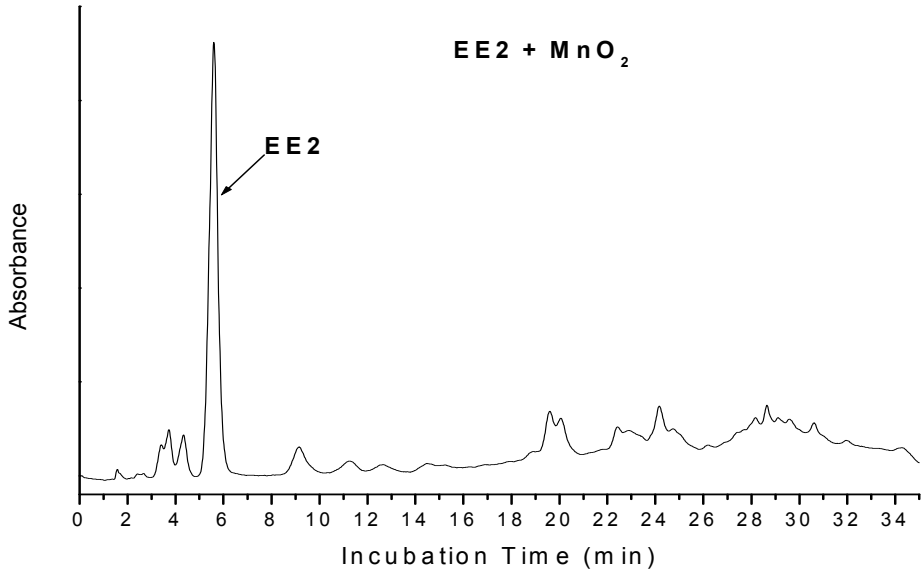


Figure 4.36 LC-MS chromatogram at 210 nm showing EE2 transformation using MnO_2.

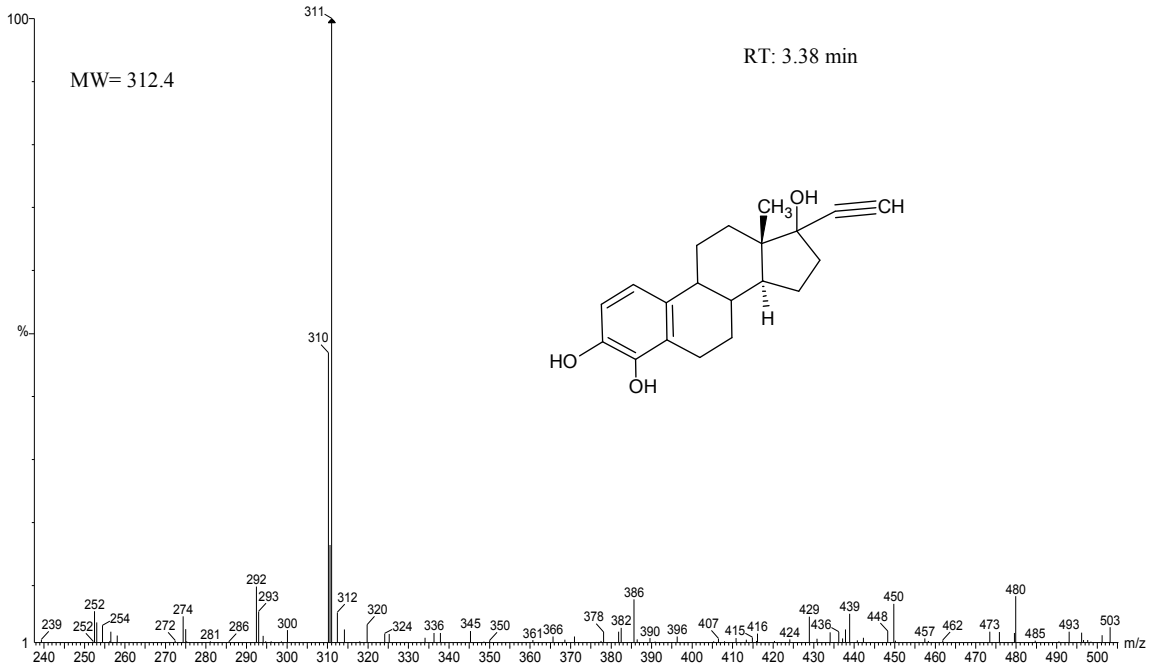


Figure 4.37 Mass spectrum of transformation product of EE2 produced by MnO_2

The transformation of EE2 in MnO_2 reaction mixture was slower compared to other catalysts studied, after 48 h, 60 % EE2 has transformed. As shown in LC-UV chromatogram (Figure 4.36) small product peaks are observed. But when LC-MS was used for identification of the peaks no peaks were detected, it may be due to low concentration of products formed or less sensitivity of the instrument towards the products formed. Only one peak at retention time of 3.38 min was detected at an m/z of 311 $[M-H]^-$, the structure of the identified compound is shown in Figure 4.37. Only one peak was observed at this m/z ratio, no dimers were detected. In the study by Xu et al., complete oxidation of EE2 was achieved using MnO_2 in less than 250 min.[116] The observed difference can be due to the pH employed. They used pH 4 for studying transformation of EE2 by MnO_2. While in this study experiments were carried in water without pH adjustment (~ 6). Studying the transformation of organic compounds at pH other that the pH of water does not represents the real environmental conditions.

Therefore the transformation rates observed in present study would be close representatives of EE2 transformation in the environment by MnO_2.

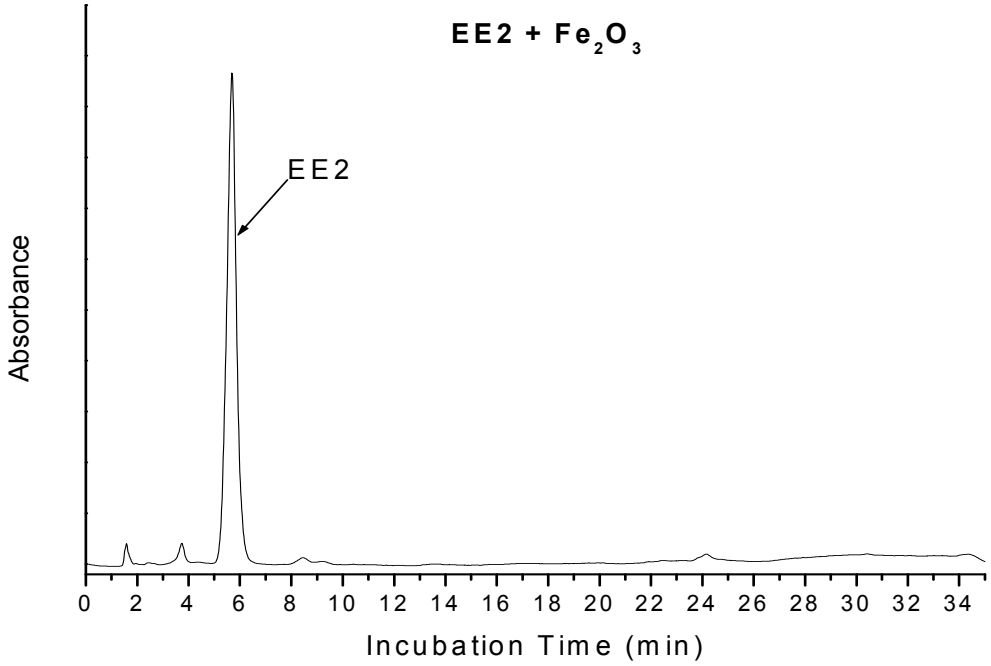


Figure 4.38 LC-UV chromatogram at 210 nm showing transformation of EE2 by Fe_2O_3

Fe_2O_3 was the least efficient catalyst of all catalyst used in this study, more than 90 % EE2 remained in reaction mixture after 48 h of exposure time. As can be seen in LC-UV chromatogram (Figure 4.38) no product peaks were detected. LC-MS analysis also confirmed that no transformation products were formed.

Same was true with NM, no transformation of EE2 was observed. According to the results of this study complete transformation of EE2 can be achieved within 48 h using 1 mM $NaNO_2$ – SM and Fe (III) - SM.

Fonseca et al., showed that the transformation of EE2 by solar radiation took more than 126 days.[215] In study by Moriyama et al., chlorination was used for transformation of EE2. Six transformation products were detected. Two transformation products were identified as 4-chloroethynylestradiol (4-ClEE2) and 2, 4-dichloroethynylestradiol (2, 4-diClEE2). The transformation was fast and occurred within 5 min. Although the products did not further transform after 60 min of chlorination.[36] The estrogenicity assay revealed that estrogenic activity of 4-ClEE2 was comparable to the parent EE2 and 2, 4-diClEE2 showed 10 times less estrogenic activity than EE2.

4.4.8 Abiotic transformation of estrone (E1) when exposed to Fe (III) – SM, NM, MnO_2, Fe_2O_3 with UV irradiation at 365 nm

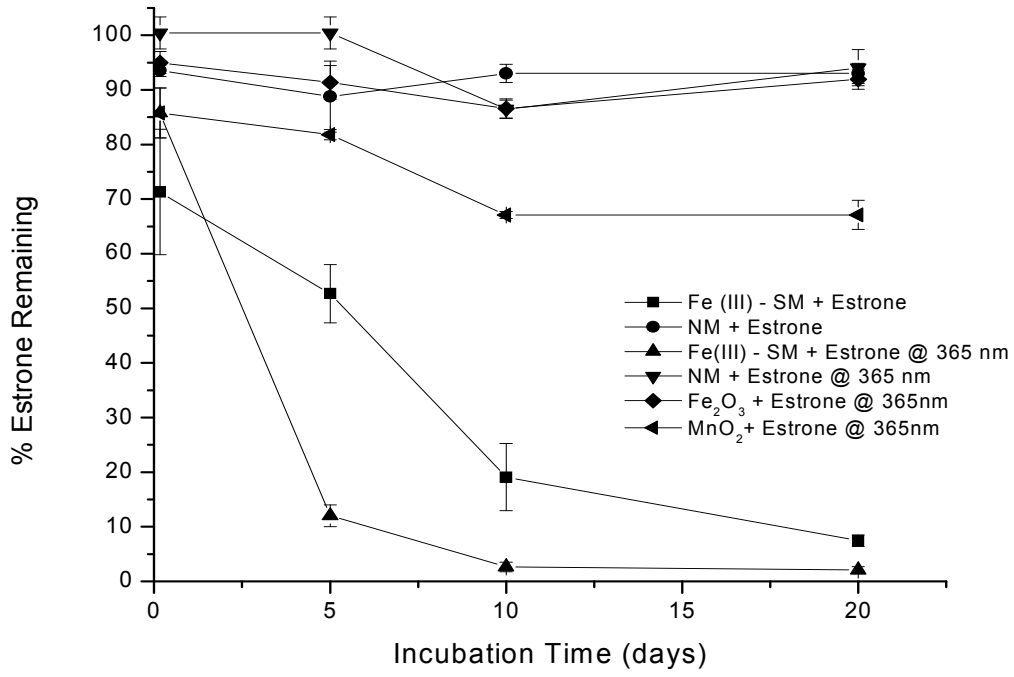


Figure 4.39 Graph showing oxidative transformation of estrone using different catalysts

Table 4.4 Half-life values and reaction rate constants for transformation of E1 using various catalysts

Treatment	Reaction rate constant K $(mmol/g)^{-1}$ days	$t_{1/2}$ (days)
Fe (III) – SM + E1	15.95	7.14
Fe (III) – SM + E1 + UV	115.25	0.97
MnO_2 + E1 + UV	3.69	30.00

When transformation of estrone was studied it was found that estrone showed slow transformation rate. In an attempt to accelerate the transformation, UV light at 365 nm was employed. The results in this study show that after 4 h, 70 % of the compound remained in the treatments using Fe (III) – SM + UV and 85 % remained using treatment Fe (III) – SM with no irradiation. UV light accelerated the transformation process to some extent (Figure 4.40). Half-life values of 7.14 and 0.97 days were observed for E1 transformation by Fe (III) – SM and Fe (III) – SM + UV, respectively (Table 4.4).While NM did not show any transformation of E1 (Figure 4.39). MnO_2 did not prove to be efficient for the transformation of E1. After 20 days only 30% E1 was transformed using MnO_2. Fe_2O_3 was the least efficient catalysts for transformation of E1 more than 90% still remained in the reaction mixture.

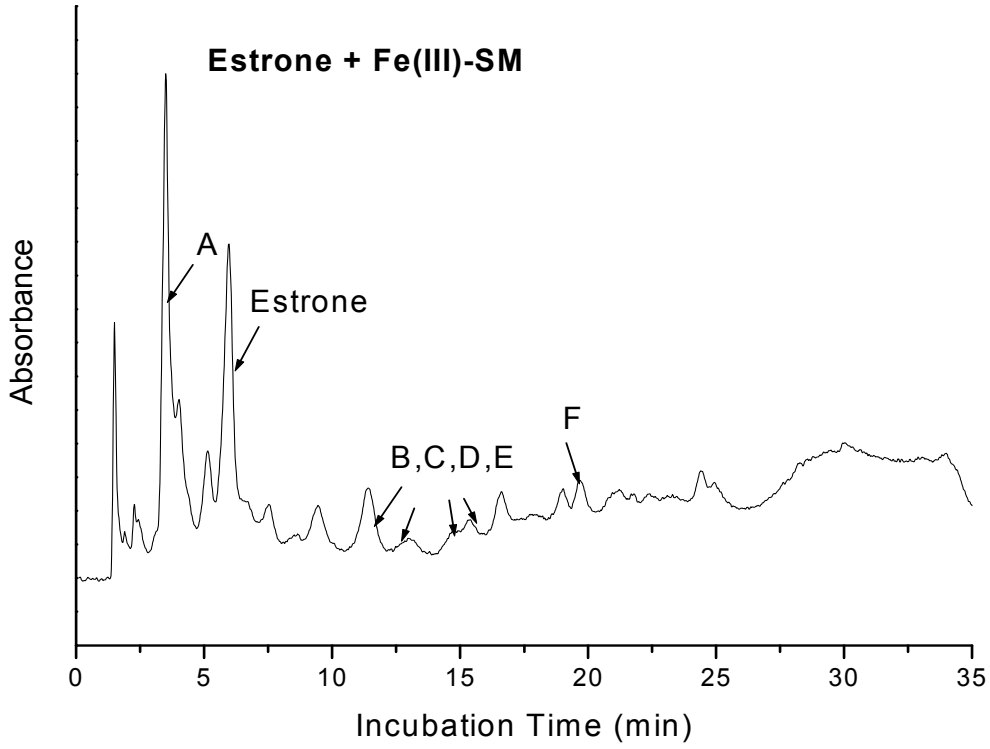


Figure 4.40 LC-UV chromatogram at 210 nm showing the transformation of E1 by Fe (III) - SM without UV irradiation

A peak was observed at m/z 299 $[M-H]^-$ in Fe (III)-SM at same retention time of 3.57 min (peak A, see Figure 4.41). Five peaks at m/z 537 $[M-H]^-$ (Figure 4.41) were observed at retention time 11.49, 12.64, 13.38, 14.6 and 20.12 as shown in Figure 4.40. These peaks corresponds to E1 dimers, five peaks represent five different conformers of E1 dimers. No other products were identified when Fe (III)-SM was used for oxidative transformation of E1. After 20 days less than 10% of E1 remained in the reaction mixture.

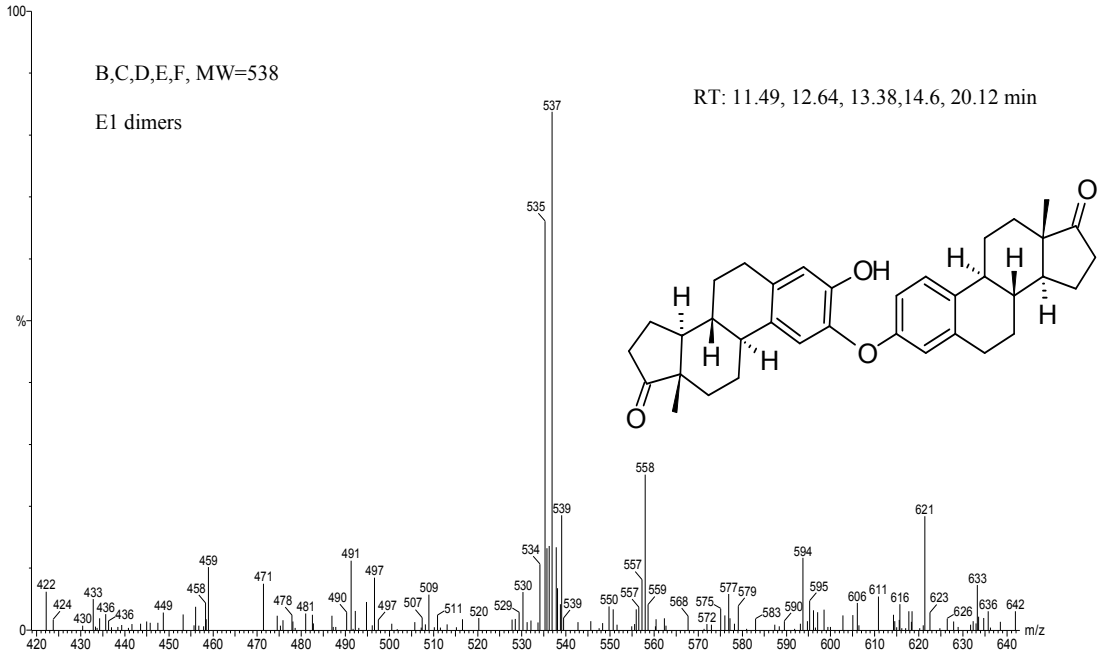


Figure 4.41 Mass spectrum of the oxidative transformation products of E1 by Fe (III) - SM

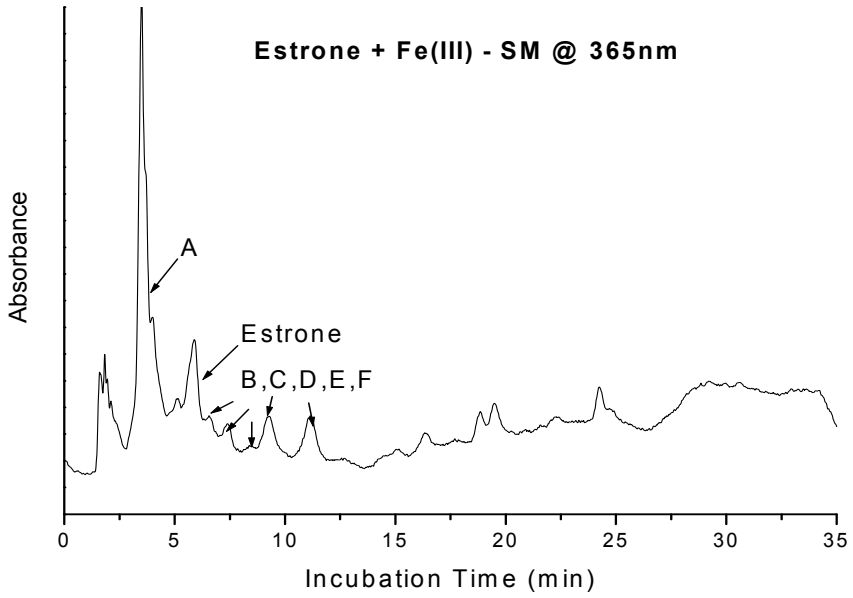


Figure 4.42 LC-UV chromatogram at 210 nm showing transformation of E1 by Fe (III) – SM + UV irradiation at 365 nm

The transformation of E1 took 20 days, which was longer compared to other hormones tested earlier. Many peaks appeared when E1 was subjected to transformation with Fe (III) – SM along with UV irradiation (Figure 4.42). When LC-MS was employed for identification of the peaks, peak A was identified as a transformation product with a m/z of 299 $[M-H]^-$ (Figure 4.43 a), no structure was assigned to this m/z. Peaks B, C, D, E and F were identified as dimers with m/z of 568 $[M-H]^-$ (Figure 4.43 b), but no structure assignment was possible due to lack of information in literature regarding the possible transformation products obtained. There is a lack of mass spectrum database for the transformation products obtained for hormones; hence GC-MS would also not provide accurate identity of the products. NMR can provide information on identity of the compounds but isolation of formed products from a complex mixture will be a challenging task.

Addition of UV irradiation did accelerate the transformation rate to some extent. However towards the end of the incubation period the transformation rate became same for E1 transformation on Fe (III)-SM with and without UV irradiation.

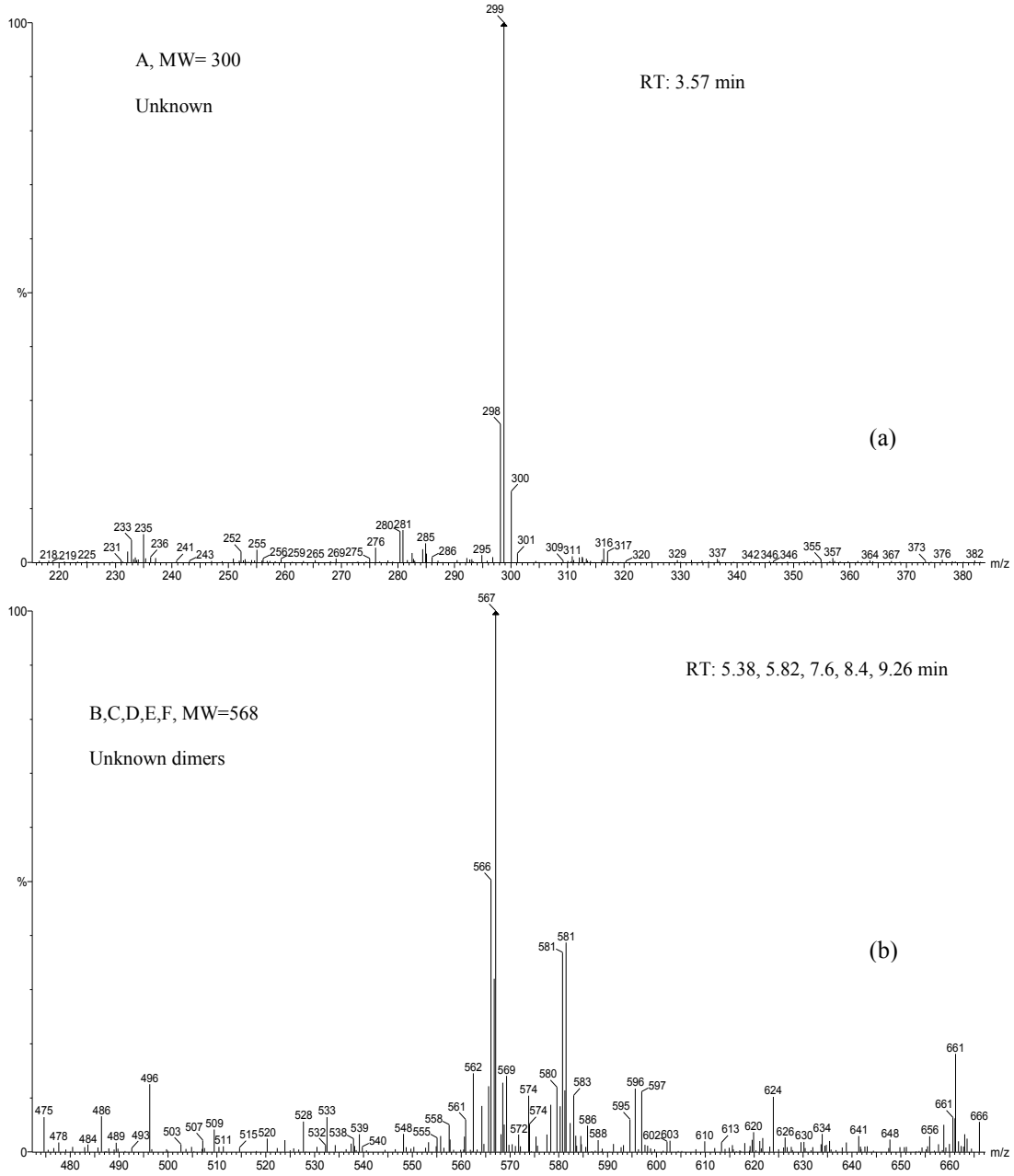


Figure 4.43 Mass spectrum of unidentified transformation products detected during oxidative transformation of E1 by Fe (III)-SM with UV irradiation.

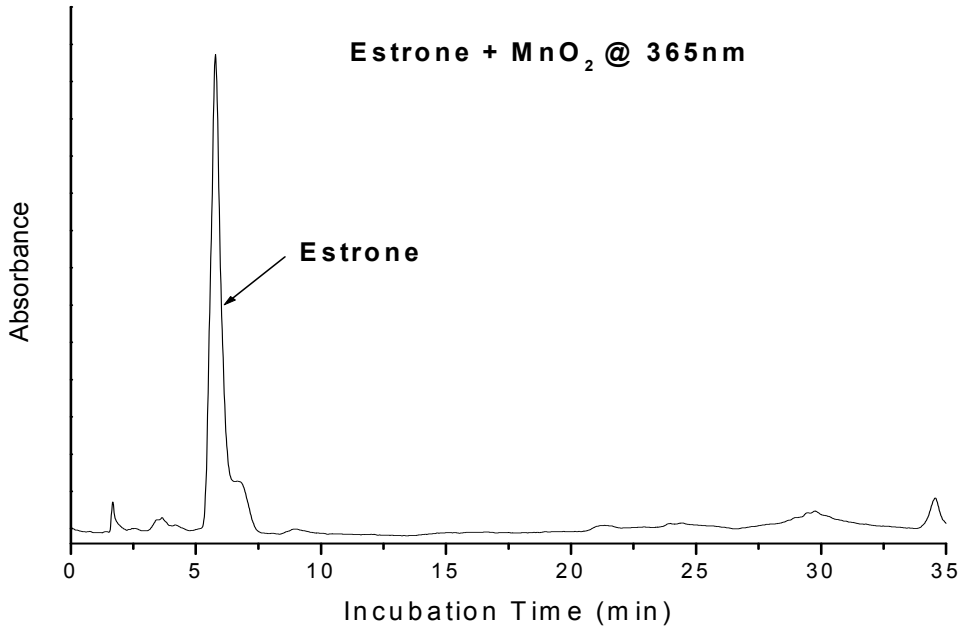


Figure 4.44 LC-UV chromatogram at 210 nm obtained for reaction mixture containing MnO_2 and E1.

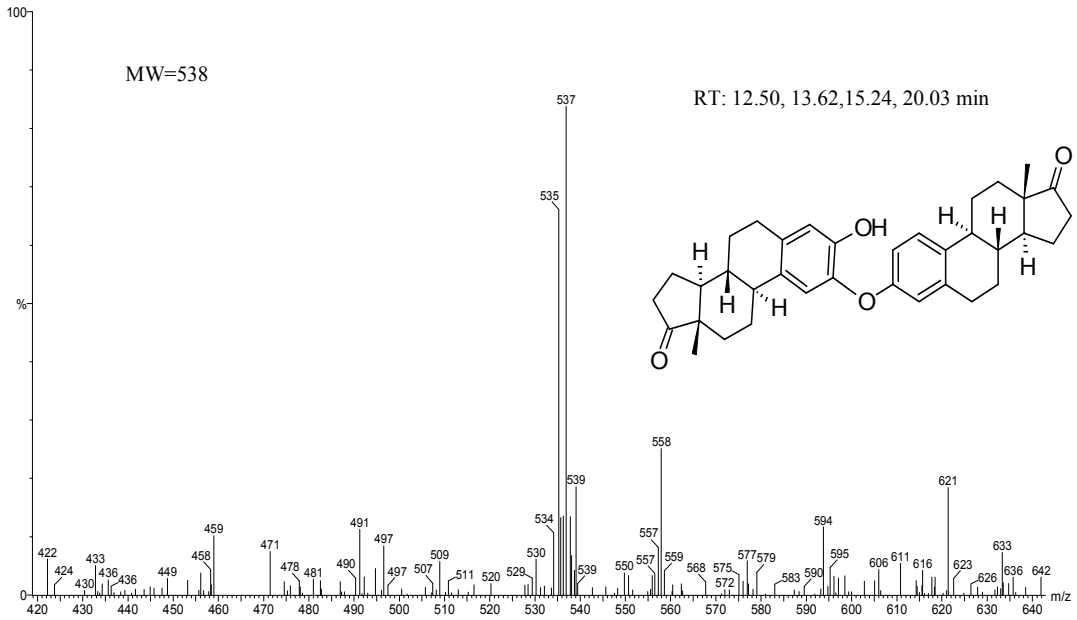


Figure 4.45 Mass spectrum of the oxidative transformation products of E1 by MnO_2 with UV irradiation at 365 nm.

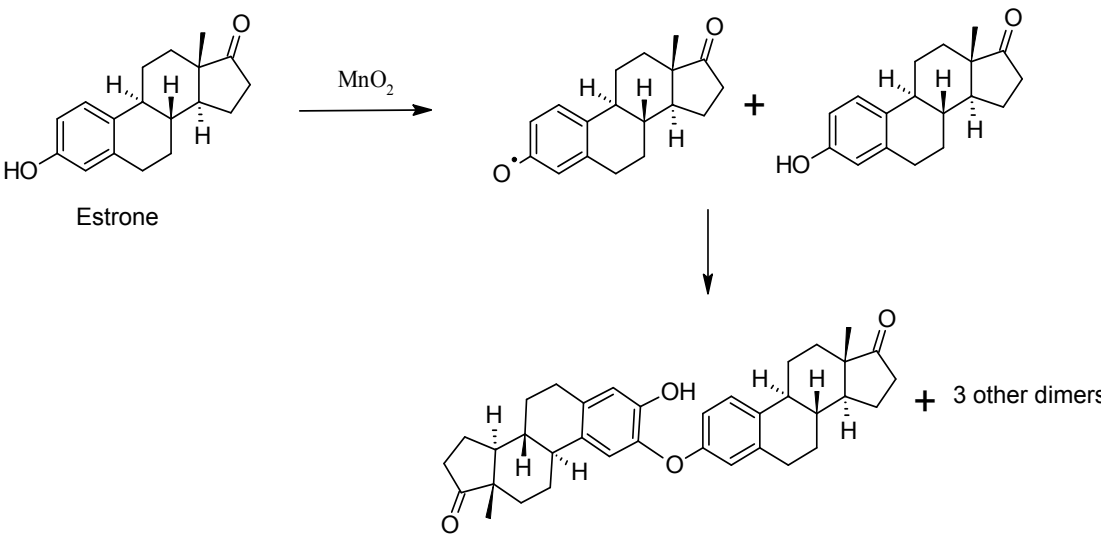


Figure 4.46 Proposed mechanism for E1 dimer production by MnO_2 at 365 nm.

When transformation of E1 was carried out by MnO_2 along with UV irradiation at 365 nm, four peaks at m/z 537 $[M-H]^-$ (Figure 4.45) were observed at retention time of 12.50, 13.62, 15.24 and 20.03. These peaks corresponded to E1 dimers; four peaks represent four different conformers of E1 dimers. The concentration of peaks formed was very small, as shown in LC-UV chromatogram no dimer peaks can be seen (Figure 4.44). The half-life of E1 subjected to transformation by MnO_2 was calculated to be 30 days (Table 4.4). No other products were identified when MnO_2 was used for oxidative transformation of E1. MnO_2 did not show efficient removal of E1 when subjected to transformation. After 20 days less than 30 % of E1 was transformed in the reaction mixture. Zhang et al., studied transformation of TCS and chlorophene by MnO_2. They also detected TCS and chlorophene dimers upon transformation by MnO_2.[216] Based on the pathway proposed by Zhang et al., and products detected in this study using LC-MS for transformation of E1 by MnO_2 a reaction mechanism is proposed in Figure 4.46. According to the proposed pathway the hydroxyl of E1 is oxidized into a free radical. This free radical further reacts with another molecule of E1 to produce a dimer of E1.

Different conformers are formed, since the C-1, C-2, C-3 and C-4 can involve in dimer formation.

As shown in Figure 4.47 one peak corresponding to E1 was observed in LC-UV chromatogram when E1 transformation was carried out with Fe_2O_3 and UV irradiation at 365 nm. No products were detected with LC-MS. Fe_2O_3 and NM showed the least potential as catalyst for transformation of E1. After 20 days more than 90 % E1 stayed in reaction mixture.

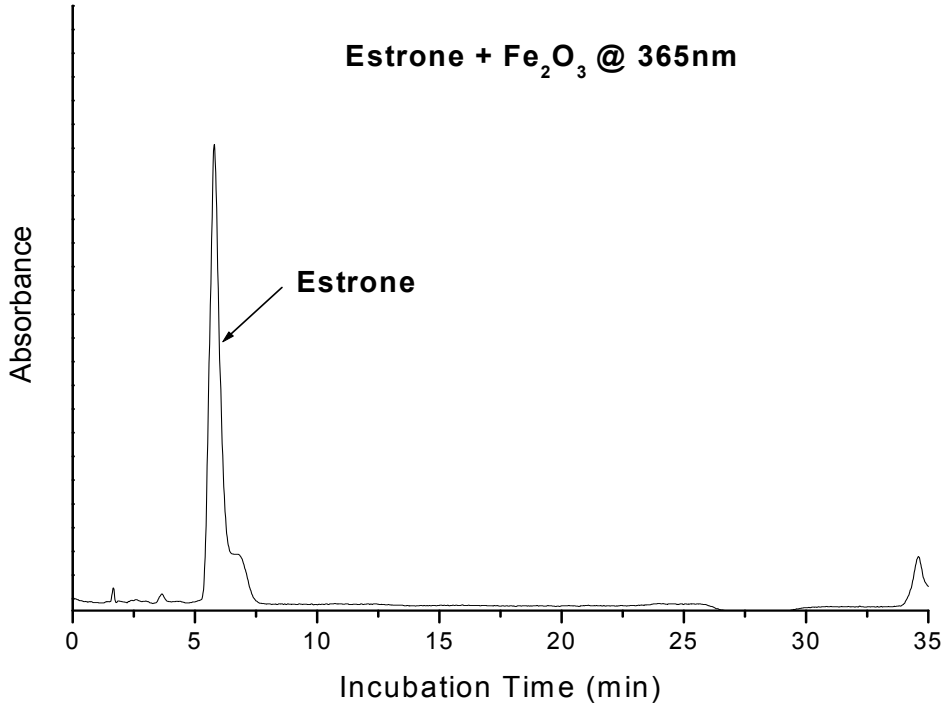


Figure 4.47 LC-UV chromatogram showing oxidative transformation of E1 by Fe_2O_3

The results of this study suggest that estrone takes longer to transform when compared with E2, E3 and EE2. UV irradiation did not accelerate the transformation of E1 to large extent. Fe (III)-SM was the best catalyst among all other catalysts studied for

transformation of E1. MnO_2 is a strong oxidizer, but proved to be inefficient in fast removal of E1. Fe_2O_3 and NM showed the least potential as catalysts for the transformation of E1 in the aqueous environment.

Atkinson et al., studied the transformation of E1 by photolysis using UVB (λ = 280 – 320 nm) lamp. After 6 h of irradiation 500 ng/L initial concentration of E1 was reduced to 122 ng/L.[211] In another study by Chowdhary et al., the transformation of E1 was carried out in the aqueous solution at pH 6.0 by solar irradiation (290-700 nm). The half life of E1 transformation varied from 40 – 123 min depending on the intensity of light and E1 concentration used.[189] Oxidative transformation of E1 when carried out by Fe-TAML/H_2O_2 at 365 nm took 60 min for ~ 95 % E1 transformation.[118] The treatments used in the studies mentioned above are not practical if employed in WWTPs. UV radiation and the amount of dose required for the transformation of hormones can prove to be costly treatments. Also in surface water, lakes and ground water penetration of solar radiation depends on the depth of the water and dissolved organic matter present in the water. While the catalyst developed in the study has been shown to work without UV radiation and montmorillonite mineral is easily available at low cost.

4.4.9 Abiotic transformation of testosterone by Fe (III) - SM, NM, MnO_2, Fe_2O_3 with UV irradiation at 365 nm

When the transformation of testosterone was studied it was found that testosterone was the hardest compound to get transformed. It is due to fact that testosterone does not possess the reactive -OH group at C-3 position of the benzene ring, which is usually present in other hormones. The results in this study show that after 4 h, 70 % of the compound remained in the treatments using Fe (III)-SM and Fe (III)-SM + UV at 365 nm. No significant difference was observed between the two treatments,

which suggest that UV light did not accelerate the transformation of testosterone by Fe (III)-SM. While NM did not show any transformation of testosterone see Figure 4.48.

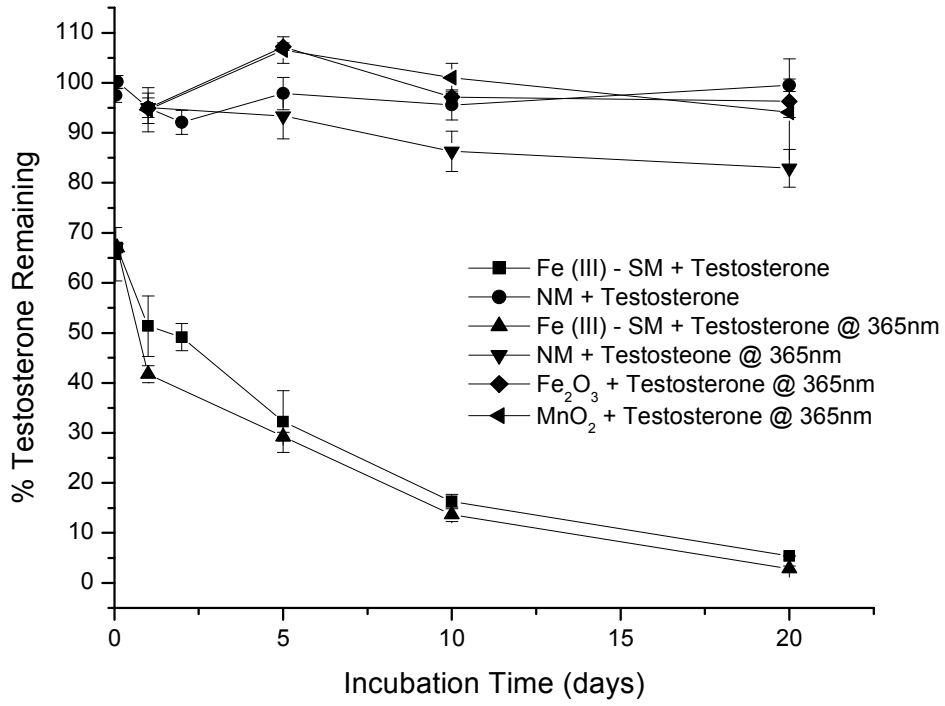


Figure 4.48 Graph showing transformation of testosterone during a period of 20 days.

Table 4.5 Half-life values and reaction rate constants for transformation of testosterone using various catalysts

Treatment	Reaction rate constant K $(mmol/g)^{-1}$ days	$t_{1/2}$ (days)
Fe (III) – SM + Testosterone	39.07	2.85
Fe (III) – SM + Testosterone + UV	176.67	0.66

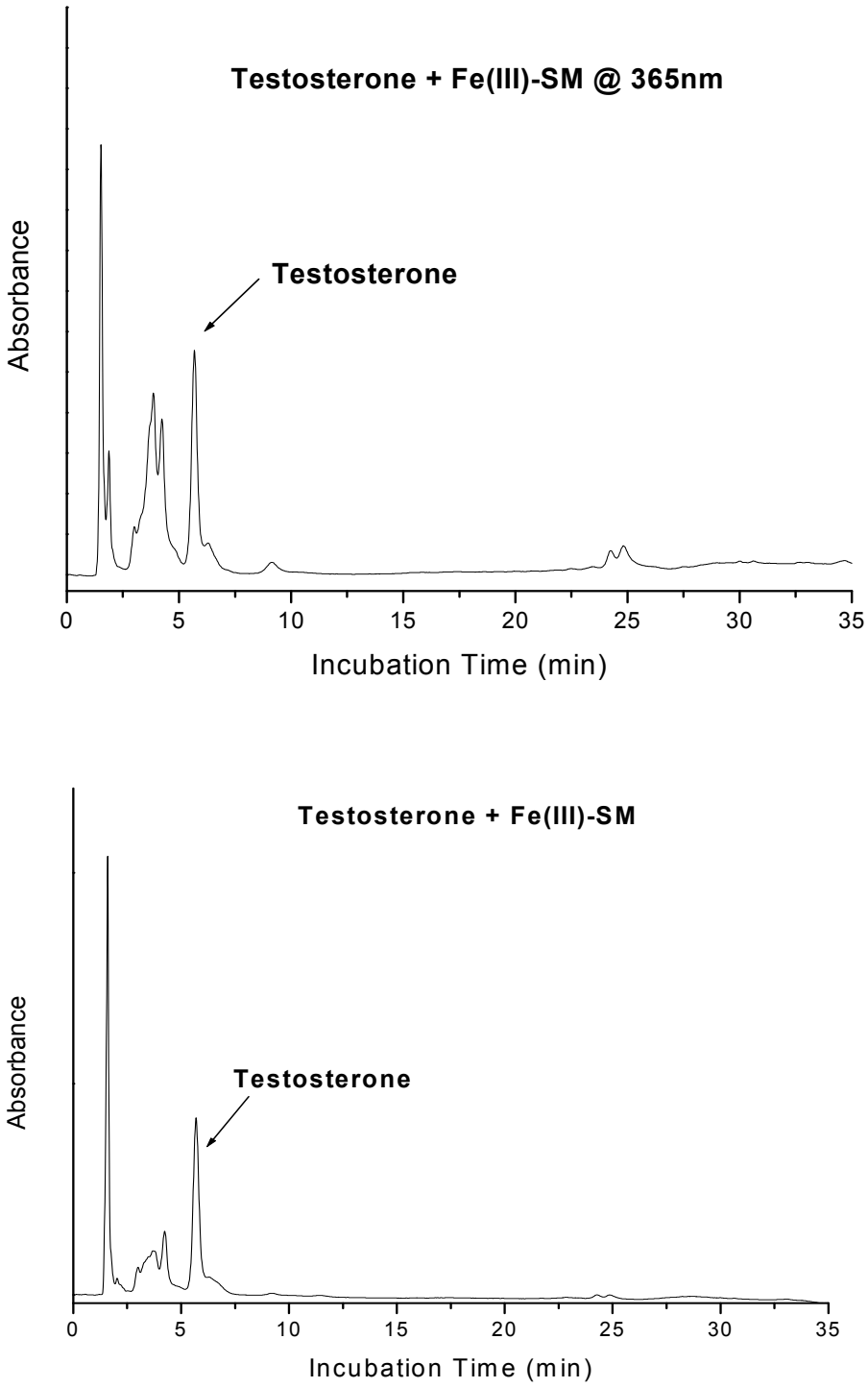


Figure 4.49 LC-UV chromatograms obtained at 240 nm for testosterone by Fe(III)-SM with and without UV irradiation at 365 nm

The transformation rates observed for both treatments with and without UV light by Fe (III)-SM were almost same (Table 4.5). After 20 days the concentration of testosterone had gone down approximately to 0 in both treatments. Half-life values of 2.85 and 0.66 days were calculated for testosterone transformation by Fe (III) – SM and Fe (III) – SM + UV, respectively. Not many product peaks were observed (Figure 4.49), and due to lack of time no attempts were made to identify product peaks using LC-MS.

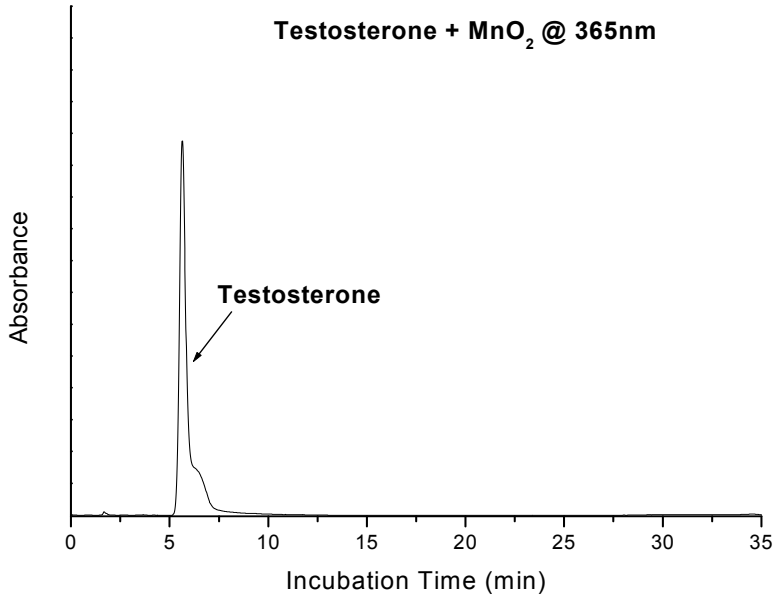


Figure 4.50 LC-UV chromatogram of transformation of testosterone by MnO_2 with UV irradiation at 365 nm

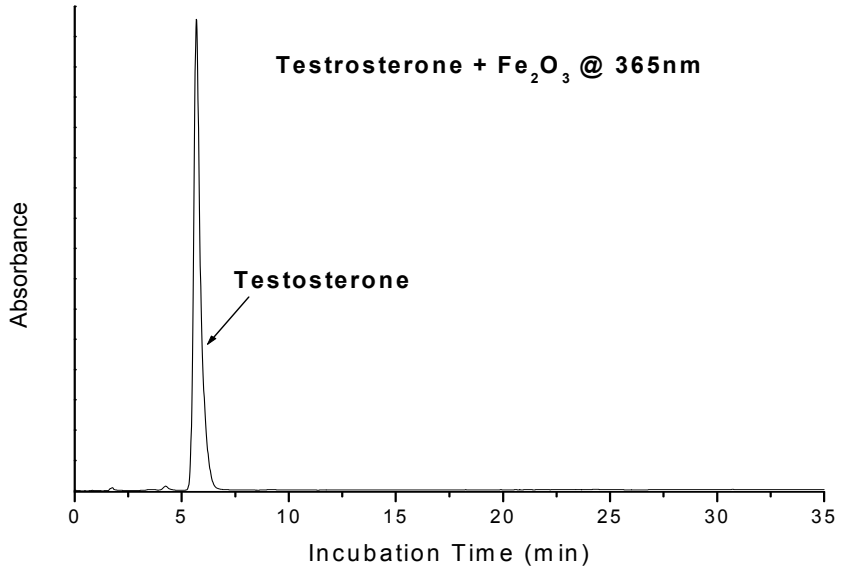


Figure 4.51 LC-UV chromatogram at 240 nm showing testosterone after being subjected to transformation by Fe_2O_3

The Figure 4.50, shows that no transformation of testosterone took place when MnO_2 was used for heterogeneous catalysis. As shown in Figure 4.49, during the period of 20 days concentration of testosterone stayed same in the treatment.

Similar results were obtained when testosterone was added in Fe_2O_3 catalytic system, no transformation was observed. Figure 4.51, shows testosterone detected at high concentration in the reaction mixture. Also as can be seen in Figure 4.48, during the 20 days of reaction period no significant change occurred in the concentration of testosterone.

There is only one report in literature about the transformation of testosterone using heterogeneous catalytic system. Shappell et al., studied transformation of testosterone using Fe-TAML/H_2O_2 in the aqueous environment. They tested three concentrations 83, 300 and 3000 nM of Fe-TAML catalysts for transformation of

testosterone. The lower concentration of Fe-TAML was not able to transform testosterone, while highest concentration achieved almost 70 % transformation of testosterone after 60 mins. No transformation products were reported.[118] This study is a stepping stone towards studying the transformation of testosterone in the aqueous environment using various catalysts. This study shows that Fe (III)-SM is ideal for the transformation of testosterone. UV irradiation is not essential for the transformation to occur using Fe (III)-SM. Complete transformation is achieved within 20 days. Even though catalysts used in study by Shappell et al., achieved fast transformation of testosterone. High concentration of catalysts was required along with H_2O_2 and UV, which makes this transformation process expensive. Other catalysts MnO_2 and Fe_2O_3 which are widely employed for the transformation studies did not transform testosterone within 20 days. However results of this study suggest that testosterone will be resistant to the transformation process in the environment. As seen in this study, UV light will not be an efficient means for elimination of testosterone from the environment. Strong catalysts as employed in the study by Shappell et al., or used in this study are required to achieve transformation of testosterone.

4.5 Conclusions

In this study clay-catalyzed oxidation was used for the transformation of estrogenic hormones. The Fe (III)-saturated montmorillonite proved to be efficient in removal of E2, E3, EE2, E1 and testosterone from the aqueous samples. Our studies were done in the room light (i.e., bright during day and dark at night) which simulates day and night pattern in the real environment In the true environment, UV rays from the sunlight can expedite the transformation process, even though our studies revealed that UV light is

not required for the transformation using Fe(III)-saturated montmorillonite. When $NaNO_2$ was used to accelerate the transformation process, nitro derivatives of E2, E3 and EE2 were produced. The nitro derivatives are not proved yet to possess toxicity comparable to that of the parent estrogenic hormone itself. But more work needs to be done to test this hypothesis. The developed catalyst was able to transform E2 within 4 h, hence this catalyst can be considered for fast remediation of E2 in the dark as well as in the sunlight. E3 was quickly transformed when 1mM $NaNO_2$ was used along with Fe (III)-saturated montmorillonite. Nitration products were produced in abundance. Complete transformation occurred within 1 day. While transformation of E3 was slower in other treatments and 90% E3 was transformed after 20 days using MnO_2 and Fe (III)-saturated montmorillonite by itself. Toxic compounds such as quinone derivative of E3 and 16α-hydroxyestrone were produced as the transformation products of E3 by MnO_2. EE2 showed complete transformation in treatment containing 1mM $NaNO_2$ with Fe (III)-saturated montmorillonite within 4hrs. While 90 % transformation was observed with Fe (III)-saturated montmorillonite within 48 h and 70 % transformation was observed with MnO_2.

Our results showed that the transformation of E1 and testosterone was the slowest of all. It took 20 days for the complete transformation of E1 and testosterone using Fe (III)-saturated montmorillonite. The data from this study clearly shows the enhanced transformation of hormones on Fe (III)-saturated montmorillonite even when estrogens were present in such high concentrations as used in this studies. Studies have shown the effect of concentration of hormones on the transformation rates. According to these studies, lower concentration of hormones can lead to faster transformation.[147, 148] If the catalyst used in this study can work efficiently with high concentrations, it should be able

to handle environmentally relevant concentrations easily. When Fe (III)-saturated montmorillonite is used as catalyst, transformation can take place without any source of initiation such as the UV or sunlight. The order of general ease of the transformation of the studied hormones was as follows E2>EE2>E3>E1> testosterone. Feng et al., reported similar transformation order using photo-Fenton process for the transformation of estrogenic hormones. According to their studies, following transformation order is observed for hormones E2>EE2>E1.[152] The transformation efficiency is dependent on the structure of the hormones. E2, EE2 and E3 possess reactive –OH group at C-3 position. E1 also possess reactive –OH group but is a transformation product of E2, as determined by other studies. Therefore further transformation of E1 can be difficult. Testosterone lacks the –OH group at C-3 positions and the electron rich benzene ring which makes transformation of testosterone difficult. Further studies are required to identify the transformation products produced during the oxidative transformation of hormones and predict pathways for oxidative transformation.

CHAPTER V

CONCLUSIONS

The presence of pharmaceuticals and personal care products in the environment is well documented. Conventional water treatment plants are inefficient in removal of these compounds from the wastewater and water. The water from wastewater treatment plants is released into our aquatic environment. The biosolids generated from wastewater treatment plants is commonly used as fertilizers for land applications, this is the main cause of spreading of PPCPs in the environment. Other sources of introduction of PPCPs in the environment include animal husbandry, direct release of raw sewage into water streams and others.

The aims of this study were: to investigate the fate of antibacterial compounds triclosan and triclocarban in biosolids and biosolids-applied soil, to develop better sample extraction and cleanup approach for TCS and TCC analysis, and to study mineral surface catalyzed oxidative transformation process for five estrogenic hormones 17β-estradiol, estriol, 17α-ethynylestradiol, estrone and testosterone.

The goals of this dissertation were achieved by three studies which addressed the objectives outlined in section 1.7

- Development of a rapid analytical method for analysis of TCS and TCC in biosolids and biosolids-applied soil using sonication extraction and LC-MS detection and quantification.

- Development of a SPE sorbent based on molecularly imprinted polymer for selective extraction of TCS and TCC from biosolids and biosolids-applied soil using LC-UV
- Oxidative transformation of estrogenic hormones in the presence of Fe (III)-saturated montmorillonite heterogeneous catalytic system.

5.1 Development of a rapid analytical method for analysis of TCS and TCC in biosolids and biosolids-applied soil using sonication extraction and LC-MS detection and quantification

A method using sonication extraction coupled with LC-ESI-MS detection was developed to simultaneously analyze of TCS and TCC in biosolids-applied soils and biosolids. There are few methods for simultaneous determination of TCS and TCC in biosolids-applied soil and biosolids. The method developed in this study was able to chromatographically resolve both TCS and TCC. No additional clean up of sample extract was required for the LC/MS analysis. The concentrations of TCS and TCC observed in our studies are higher than found in other studies. This difference can arise because of different WWTPs from which biosolids are collected. WWTPs located in areas with large populations can receive higher input of TCS and TCC leading to higher detection concentrations. Also the different treatment employed in WWTPs can result in different removal efficiencies of these compounds from wastewater influents, resulting in higher or lower concentrations of these compounds in biosolids.

Studying the fate of TCS and TCC in the environment is necessary because of the concerns of these compounds leaching in the ground water supplies and surface runoff during rainy weather. Another consequence is the bioaccumulation of these compounds followed by widespread practice of biosolids land application. Using the developed

method we found that TCS and TCC are almost ubiquitous in all the biosolids-applied soil and biosolids samples. High concentration of TCS and TCC in surface soil of biosolids-applied field suggests that both compounds tend to accumulate in soil surface. Triclosan and TCC were also detected in lower concentration in biosolids compost, which suggest that significant transformation of TCS and TCC can occur during composting process. The detected concentrations of TCC were much higher than that of TCS, which suggest that TCC might be more persistent in the environment when compared with TCS. Previous studies suggested that the half-life of TCS in aerobic soil is about 18 days, while it is 108 days for TCC, under anaerobic conditions the degradation slows down ever further [103]. Longer half-life of TCC can be the reason for the higher concentrations of TCC found in all the samples analyzed in our studies.

The results obtained in this study were comparable with other studies. The developed method is easy, sensitive, and fast and no cleanup is required before LC/ESI/MS analysis. The development of this method was central to this work, as available alternative methods were not efficient for analysis of TCS and TCC in biosolids-applied soil and biosolids. The use of LC-ESI-MS for analysis proves to be expensive and limits the number of samples analyzed. Therefore, the next study of this thesis focused on developing method for analysis of TCS and TCC using MISPE coupled with LC-UV

5.2 Development of a SPE sorbent based on molecularly imprinted polymer for selective extraction and cleanup of TCS and TCC from biosolids and biosolids-applied soil followed by analysis using LC-UV

A molecularly imprinted polymer (MIP) able to selectively bind TCS and TCC was prepared using non-covalent molecular imprinting methods. The prepared MIP was evaluated as a selective sorbent in solid phase extraction for sample cleanup before HPLC/UV analysis of TCS and TCC in soil and biosolids samples. The MIP was also compared with the commercially available C_{18} SPE sorbent. Presence of humic acid reduced the TCS and TCC recovery using both C-18 SPE and MISPE. MISPE clean-up and recovery were better than C-18 for various environmental sample. LC-UV and LC-MS gave similar concentrations of TCS and TCC when MISPE was used as a clean-up method. But large discrepancy was observed between concentrations obtained using LC-MS and LC-UV when C-18 SPE was employed as clean-up method. Single MISPE cartridge was reused 35 times. While C-18 SPE cartridges cannot to reuse. Comparing to the C_{18} SPE, using MISPE for sample cleanup may result in significant reduction of analytical cost because MISPE can be reduced and HPLC/UV instead of HPLC/MS can be used for instrumental analysis following sample cleanup with the MISPE.

The results of this study proved that the MIP developed was adequate to be used for the solid phase extraction of soil and biosolids samples for analysis of TCC and TCS using HPLC/UV. Intraday and inter-day repeatability of < 5%RSD was achieved using this method.

5.3 Oxidative transformation of estrogenic hormones in presence of Fe (III)-saturated montmorillonite heterogeneous catalytic system

A catalytic system for oxidative transformation of estrogenic hormones was tested. Fe (III)-saturated montmorillonite proved to be efficient in transformation of E2,

E3, EE2, E1 and testosterone in aqueous samples. The studies were done to simulate environmental conditions. Experiments were conducted at room temperature and analyzed on regular intervals. Our studies revealed that UV light is not required for transformation of hormones using Fe (III)-saturated montmorillonite. Complete transformation of E2, E3, EE2, E1 and testosterone were observed using Fe (III)-saturated montmorillonite. When $NaNO_2$ was used to accelerate the transformation process, nitro derivatives of E2, E3 and EE2 were produced. The transformation of E2 occurred within 4 h, hence this catalytic system can be considered for fast remediation of E2 in dark as well as in the sunlight. The transformation of E3 occurred within one day when 1 mM $NaNO_2$ was used along with Fe (III)-saturated montmorillonite. Nitration products were produced in abundance. While 90 % E3 was transformed after 20 days using MnO_2 and Fe (III)-saturated montmorillonite by itself. No transformation was observed with non-modified montmorillonite and Fe_2O_3. EE2 showed complete transformation in treatment containing 1 mM $NaNO_2$ with Fe (III)-saturated montmorillonite within 4h. While 90 % transformation was observed with Fe (III)-saturated montmorillonite within 48 h and 70 % transformation was observed with MnO_2.

Our results showed that the transformation of E1 and testosterone was the slowest of all. Complete transformation of E1 and testosterone using Fe (III)-saturated montmorillonite took 20 days. The data from this study clearly shows the enhanced transformation of hormones on Fe (III)-saturated montmorillonite even when estrogens were present in such high concentrations as used in this studies. Faster transformation at low concentration using Fe (III)-saturated montmorillonite is possible. When Fe (III)-saturated montmorillonite is used as a catalyst, transformation can take place without any source of initiation such as UV or sunlight. The ease of transformation of the studied

hormones using Fe (III)-saturated montmorillonite was as follows E2>EE2>E3>E1> testosterone.

The results in this study suggest that hormones can undergo oxidative transformation in the environment. Two hormones E1 and testosterone can be more persistent and would not undergo transformation easily. UV light in incapable of removing these two hormones. Hence bioaccumulation of E1 and testosterone can be observed.

5.4 Further work

More studies are required for detection of TCS and TCC in biosolids and biosolids-applied soil to understand the TCS and TCC distribution. Numerous studies have been done to understand the occurrence, fate and transformation of TCS in aqueous environment. But there is lack of information on occurrence fate and transformation of TCC. There is a much higher need to direct attention towards the toxicity, transformation and bioaccumulation of TCC. Further studies are required to identify the transformation products produced during the oxidative transformation of hormones. GC-MS and NMR structures can provide information on the produced oxidation products, which can help predict the transformation pathways for hormones. Transformation studies using lower concentrations of hormones are required, but expensive instruments will be required for this studies. Also the toxicity of the produced products needs to be determined. Use of Fe (III)-saturated montmorillonite for possible advanced wastewater treatment should be studied in pilot scale.

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