FATE AND TRANSPORT OF AN ESTROGEN CONJUGATE 17ß-ESTRADIOL-17-SULFATE IN SOIL-WATER SYSTEMS

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment
for the Degree of
DOCTOR OF PHILOSOPHY

Major Department:
Soil Science

August 2013

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

The hypothesis of this study was that a sulfate conjugated estrogen, i.e. 17β-estradiol-17-sulfate (E2-17S), could be a precursor to free estrogens detected in the environment. The objectives of were to investigate the fate and transport processes of E2-17S in various soil-water systems.

Radiolabeled E2-17S was synthesized using a series of chemical for the subsequent soil batch experiments. The batch experiment results showed that E2-17S dissipated more quickly from the aqueous phase of the topsoil compared to the subsoil, demonstrating that soil organic carbon played a significant role. The aqueous dissipation of E2-17S was attributed to sorption to the soil surface and transformation to form multiple metabolites. The non-linear sorption isotherms indicated limited sorption of E2-17S, and the concentration-dependent log K_{OC} values were 2.20 and 2.45 for the sterile topsoil and subsoil, respectively. The total radioactive residue measured in the irreversible sites was greater than the reversible sites, demonstrating that irreversible sorption was the predominant sorption process.

The observed multiple metabolites suggested that E2-17S underwent complex transformation pathways. For the aqueous phase speciation, mono- and di-hydroxy-E2-17S were consistently detected under all soil conditions, which indicated that hydroxylation was the major transformation process. Also, the hydroxyl metabolites were found at higher concentrations in the topsoil than the subsoil. In the reversibly sorbed phase, free estrogens (i.e. 17β-estradiol and estrone) were detected at relatively low levels (≤ 2% of applied dose) for all soils, demonstrating that deconjugation/hydrolysis and subsequent oxidation did occur. Furthermore, both hydroxylation and hydrolysis of E2-17S took place under the non-sterile and sterile conditions.

Although deconjugation was not a major pathway, E2-17S could be a precursor of free estrogens in the environment.

A comprehensive one-site fully kinetic model was applied to simulate the overall governing processes in the soil-water systems and to describe the distribution of multiple metabolites in the aqueous, reversibly sorbed, and irreversibly sorbed phases.. The model gave rise to a satisfactory fit for all experimental data obtained from the batch studies, and the 36 estimated parameters were derived at relatively high confidence.

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to my advisor Dr. Frank Casey, for his guidance, encouragement, patience, and support during my graduate study. Under his direction, I have learned how to perform scientific research from research questions to published results.

I would like to acknowledge my committee member Dr. Heldur Hakk for his guidance and mentoring on all my laboratory work in the Biosciences Research Laboratory of USDA-ARS.

I also want to thank the other members of my advisory committee, Dr. Tom DeSutter, Dr. Eakalak Khan, and Dr. Peter Oduor, for their comments and advice on my research and manuscripts.

I sincerely thank Mrs. Colleen Pfaff (Biosciences Research Laboratory, USDA-ARS, Fargo, ND) and Mr. Nathan Derby (Department of Soil Science, North Dakota State University, Fargo, ND) for their technical support and contribution to this research. I appreciated Dr. Jane Schuh (Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo, ND) who provided great help for the soil gamma-irradiation procedure. I want to thank the postdoctoral fellow Dr. Suman Shrestha in the Department of Soil Science for his help on the radioactive material synthesis and modeling paper. I would like to thank the Department of Soil Science for providing a pleasant work environment.

This project is supported by Agriculture and Food Research Initiative Competitive Grant No. 2010-65102-20400 from the USDA National Institute of Food and Agriculture. I was fortunate to be awarded by the North Dakota Water Resources Research Institute fellowship to pursue my Ph.D.

I am grateful to my father, brother, sister-in-law, and nephew for their love, understanding, and encouragement.

DEDICATION

To my late mother Mrs. Meijun Song, for who she had been, what she had done, and all the love she had given to me.

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GENERAL INTRODUCTION

Naturally occurring estrogenic hormones are considered as emerging contaminants due to their adverse effects to aquatic wildlife by disrupting endocrine systems of organisms. Human and animal wastes are the major sources of estrogenic hormones in the environment. Manure from animal feeding operations (AFOs) is land-applied as a soil amendment, which would allow estrogenic hormones to enter into adjacent water systems constantly. Among the steroidal estrogens, 17β-estradiol (E2) is of the highest potency and thus the greatest concern. There have been wide-spread environmental detections of E2 at concentrations above its lowest observable adverse effect levels (LOAEL). Despite the relatively high and frequent detections of E2 in the environment, laboratory studies indicate that E2 has high soil sorption affinity and readily degrades. The discrepancies between the field and laboratory observations suggest that other pathways may facilitate the transport and persistence of E2 in the environment.

Estrogens are released by animals and humans as glucuronide or sulfate conjugated forms to facilitate estrogen excretion in urine by increasing water solubility. These conjugated estrogens can act as precursors to free estrogens in the environment. Several studies have reported that conjugated estrogens can be hydrolyzed to release free estrogens in various media. The research hyphothesis is that conjugated estrogens can increase the total estrogen levels and facilitate the transport of estrogens in the environment. Therefore, this dissertation research was performed to investigate the fate and transport processes of a sulfate conjugate, 17β-estradiol-17-sulfate (E2-17S), in agricultural soils, with the hypothesis that it can be potentially converted to free estrogens in various soil-water systems.

Organization of the Dissertation

The dissertation consists of six parts that includes a general introduction, two published and two manuscripts to be published in peer-reviewed journals, and a general conclusion. The general introduction includes a literature review of natural steroidal estrogens and their conjugates in the environment, discussing the adverse effects, sources, and environmental fate of the estrogenic compounds. The first paper introduces a method of in-house synthesis of radiolabeled glucuronide and sulfate conjugated E2. The second paper presents the persistence and transformation pathways of E2-17S in non-sterile agricultural soils. The third paper discusses the sorption and metabolism of E2-17S in sterile soils. The fourth paper applied a mathematical model to simulate the experimental data to describe the coupled sorption and transformation of E2-17S under various soil conditions. The dissertation concludes with the general conclusions to summarize the entire research. References are listed at the end of each paper in which they are cited; and references for the general introduction and conclusion are listed together at the end of the dissertation.

Literature Review

Adverse effects to aquatic wildlife

Endocrine disrupting chemicals (EDCs) can cause adverse effects to aquatic wildlife at very low concentrations (parts-per-trillion) and are becoming an increasing concern to water quality regulatory and environmental science studies. Some of the most potent EDCs are naturally occurring estrogenic hormones released by humans and animals, including E2, and its less active metabolite estrone (E1) and estriol (E3) (Khanal et al., 2006). Among the steroidal hormones, E1 and E2 are of primary concern because they exert their adverse effects at lower concentrations

compared to other steroidal hormones and EDCs. Moreover, E2 and E1 can be frequently detected in the environment at concentrations above their lowest observable effect level (LOEL $\approx$ 10 ng L^{-1}) (Shore and Shemesh, 2003; Khanal et al., 2006).

The adverse effects caused by estrogen exposure to aquatic wildlife have been studied since the mid-1990s. Vitellogenin is a protein normally produced only in female fish; however, male fish can have very high plasma vitellogenin concentrations after exposure to estrogenic chemicals in sewage effluents (Purdom et al., 1994). An *in vivo* study of estrogenic responses in male rainbow trout (*Oncorhynchus mykiss*) and roach (*Rutilus rutilus*) was conducted by Routledge et al. (1998). After exposed to E2 or E1 for 21 d, the elevated vitellogenin concentrations in male fish indicated that environmentally relevant concentrations of such estrogens were sufficient to induce vitellogenin production (Routledge et al., 1998). Panter et al. (2000) investigated effects of intermittent exposure to estrogenic substances, where male fathead minnows (*Pimephales promelas*) were exposed to E2 at 30, 60, and 120 ng L^{-1} continuously, or 120 ng L^{-1} intermittently for 21 or 42 d. They found that plasma vitellogenin levels from intermittent exposure were equal to those in response to continuous exposure to the same concentration. Irwin et al. (2001) measured E2 levels ranging from 0.05 to 1.8 ng L^{-1} in farm ponds near livestock pastures. They also found that vitellogenin production in male painted turtles (*Chrysemys picta*) could not be induced after exposure to 9.45 ng L^{-1} of E2 for 28 d in the laboratory (Irwin et al., 2001). So far few studies have examined the relationship between manure-borne estrogens from livestock and their adverse effects to aquatic wildlife (Hanselman et al., 2003).

Sources

Human and livestock wastes are the major sources of natural steroidal estrogens. Steroidal estrogens detected in the environment are attributed to discharges from sewage treatment plants (STPs), land application of municipal sewage sludge, and soil amendment using animal manure (Lai et al., 2000).

Human

Women excrete about 5 μg day^{-1} of E1 and E2, and the amount of estrogens excreted from pregnant women can be 1000 times higher depending on the stage of pregnancy (e.g. late gestation daily production is 26 mg of E2 and 37 mg of E1) (Shore and Shemesh, 2003). Daily estrogen excretion was estimated to be 1.6 μg of E2, 3.9 μg of E1, and 1.5 μg of E3 in male urine, and 3.5 μg of E2, 8 μg of E1 and 4.8 μg of E3 in female urine (Johnson et al., 2000). The amount of E1 and E2 in human urine is in the order of 4.4 kg per year per million inhabitants, which accounts for 50% of the total observed estrogens in the influents to STPs (Johnson et al., 2000).

Estrogens derived from humans are mainly released into sewage systems and collected by STPs, and the discharge from STPs is considered an important source of estrogenic chemicals to the environment. Estrogenic chemicals in the effluents of STPs can be discharged into rivers at sufficient levels to induce disruption to reproduction systems of male fish (Jobling et al., 1998). Therefore, measuring the estrogen levels in STP effluents is critical to improve the removal efficiency of such chemicals. Ternes et al. (1999) found that E2 was at a median level of 6 ng L^{-1} in Canadian STPs, and that E1 was the only estrogen detected in the studied rivers and streams of Germany at concentrations ranging from 0.7 to 1.6 ng L^{-1}. Baronti et al. (2000) measured the

average concentrations of E3, E2, E1, and ethinyl estradiol (EE2) in the influents of six Italian STPs at 80, 12, 52, and 3 ng L^{-1}, respectively. Additionally, estrogens were detected in three Dutch STPs from lower than limit of detection (LOC) to 48 ng L^{-1} for E2, 11 to 140 ng L^{-1} for E1, and <0.2 to 8.8 ng L^{-1} for EE2 (Johnson et al., 2000).

Animals

Animal feeding operations (AFOs) are considered another major source of steroidal hormones, accounting for 90% of the total estrogen load to the environment of the United States (Maier et al., 2000). Lange et al. (2002) reported that the total estrogens released by farm animals in the European Union and the United States were 33 and 49 metric tons per year, respectively (Table 1), and in the United States, cattle (*Bos taurus*), pigs (*Sus scrofa*), and poultry (*Gallus domesticus*) contributed 45, 0.8, and 2.7 Mg of estrogens per year. Animals release estrogenic hormones to the environment in urine and feces of all species, sexes, and classes; however, different estrogens are associated with different livestock species (Hanselman et al., 2003). Cattle excrete estrogens 17α-estradiol (E2α), E2, and E1 as free and conjugated metabolites (Ivie et al., 1986; Hoffmann et al., 1997). However, swine or poultry rarely excrete E2α, but E2, E1, and E3 plus their conjugates in the excreta (Moore et al., 1982). Furthermore, different species produce estrogens by different routes, many studies demonstrated that cattle excrete estrogens mostly in feces (58%); however, swine and poultry excrete estrogens mostly in urine (96% and 69%, respectively) (Ivie et al., 1986; Palme et al., 1996). Also, urinary estrogens are major in conjugated forms, whereas fecal estrogens are excreted as unconjugated free steroids (Palme et al., 1996).

Table 1. Estimated yearly steroid hormone excretion by farm animals in the European Union and the United Sates in 2000 (Lange et al., 2002).

Species	European Union			USA		
	Million heads	Estrogens	Androgens	Million heads	Estrogens	Androgens
		metric tons			metric tons	
Cattle	82	26	4.6	98	45	1.9
Pigs	122	3	1.0	59	0.83	0.35
Sheep	112	1.3		7.7	0.092	
Chickens	1002	2.8	1.6	1816	2.7	2.1
Total	1318	33	7.1	1981	49	4.4

Manure land application is widely used as an economic way of disposing animal manure and recycling nutrients. Thus, manure applied to agricultural land can be a potential source of estrogenic compounds (Khanal et al., 2006). In AFOs, animal manure is generally collected and temporarily stored in tanks, piles, or lagoons, where estrogens are either present in the aqueous phase or sorbed to the solid phase of the storage systems. It is reported that E2 concentrations in dairy, swine, and poultry manure ranged from below detectable limits (BDL) to 239 ± 30 μg kg^{-1}, BDL to 1215 ± 275 μg kg^{-1}, and 33 ± 13 to 904 μg kg^{-1}, respectively (Hanselman et al., 2003). The total free estrogen levels (E1, E2, E3, and E2α) were measured in various lagoon samples by Hutchins et al. (2007), which were 1000−21000 ng L^{-1} in swine lagoons, 1800−4000 ng L^{-1} in poultry lagoons, 370−550 ng L^{-1} in dairy lagoons, and 22−24 ng L^{-1} in beef lagoons, respectively.

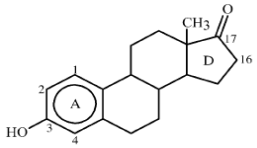
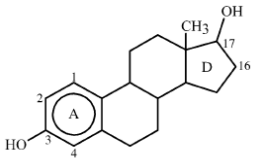
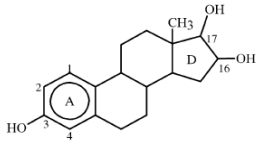
Fate and transport of estrogens

Properties of estrogens

Natural occurring steroidal estrogens (E1, E2, and E3) have the common steroid structure that are composed of four rings: a phenol, two cyclohexanes, and a cyclopentane (Khanal et al.,

2006). Steroidal estrogens have relatively low aqueous solubilities, and they are non-volatile and hydrophobic as indicated by the low vapor pressure and high log K_{OW} values (Lai et al., 2000) (Table 2). Estrogens are expected to be readily sorbed to soils and sediments and have relatively low aqueous mobility and persistence in the environment (Ingerslev and Halling-Sørensen, 2003).

Table 2. Structure and properties of natural steroidal estrogen hormones.

Estrogen	Chemical Structure	MW[a] (g mole^{-1})	Sw[b] (mg L^{-1})	log K_{OW}[c]	VP[d] (kPa)	E2 Equivalent	Reference
E1		270.37	0.8−12.4	3.1−3.4	3×10^{-8}	0.1−0.2	(Ternes et al., 1999)
E2		272.38	5.4−13.3	3.8−4.0	3×10^{-8}	1	(Lai et al., 2000)
E3		288.38	3.2−13.3	2.6−2.8	9×10^{-13}	0.02	(Lai et al., 2000)

[a]molecular weight; [b]solubility in water; [c]octanol-water partitioning coefficient; [d]vapor pressure.

Sorption

A series of laboratory-based experiments was conducted by Lai et al. (2000) to determine the partitioning of natural and synthetic estrogens between water and sediments. Synthetic estrogens, EE2 and mestranol, with higher K_{ow} values, were found to have greater sorption coefficients and more rapidly removed from the aqueous phase compared to natural estrogens, E2, E1, and E3. The authors also reported that sorption of estrogen is correlated to soil organic carbon content (OC), particle size distribution, salinity, and competition of binding sites on the sediments (Lai et

al., 2000). Colucci et al. (2001) studied sorption and transformation of radiolabeled E2 and E1 in agricultural soils. After 3 day incubation in loam, sandy loam, and silt loam soil, the non-extractable radioactivity was 90.7, 70.3, and 56.0% respectively, indicating a rapid removal from the aqueous phase. A further study (Colucci and Topp, 2002) demonstrated that E2 as low as part-per-trillion levels could also be expected to rapidly dissipate in agricultural soils through soil binding and formation of non-extractable residues.

Freundlich sorption coefficients of E2 were reported to range from 86 to 6670 L Kg^{-1} as determined by batch equilibrium studies with four types of soil (Casey et al., 2003). The sorption affinity of E2 was highly correlated to silt content and soil OC, and may also be associated with surface area and/or cation exchange capacity (Casey et al., 2003). Another study reported that Freundlich sorption coefficients of E2 were 3.56 and 83.2 L Kg^{-1} in two soils (Lee et al., 2003). Moreover, equilibrium sorption of E2 and E1 in soils was achieved between 5 and 24 h following linear sorption isotherms, and the OC normalized sorption coefficients (log K_{OC}) were 2.49 for E2 and 2.99 for E1 (Casey et al., 2005). The formation of soil-bound residues may significantly reduce the environmental risks of estrogens to water systems nearby agricultural soils treated with municipal biosolids or livestock manure (Colucci et al., 2001).

Degradation

Mineralization and degradation of estrogens have been widely studied under various conditions. Laboratory mineralization of estrogens and testosterone were investigated using biosolids from wastewater treatment systems by Layton et al. (2000). They found that 70 to 80% of E2 was mineralized to CO_2 within 24 h in biosolids with first-order rate constant of 0.0042 min^{-1}. Jacobsen et al. (2005) investigated persistence of E2 in soils receiving swine manure and municipal biosolids. 17β-Estradiol was found rapidly converted to E1 within a few days in

manured and unmanured soils, and the negligible mineralization rates of E2 in sterile soils indicated that it was a soil microorganism dependent process (Jacobsen et al., 2005).

In addition to mineralization, degradation to less active metabolites is also a major metabolism pathway for E2. The degradation half-lives of E2 in aerobic soil and sediment slurries ranged from 0.8 to 9.7 d, and the primary product was found to be E1 (Lee et al., 2003). Biodegradation of E1 and E2 in grassland soils amended with cattle and sheep manure resulted in half-lives of 5 to 25 d (Lucas and Jones, 2006), where the degradation rates of the estrogens in manure amended soils were more rapid compared to unamended soils, demonstrating that animal manure could effectively remove estrogens in soils (Lucas and Jones, 2006). Although most literature reports degradation of estrogens to be a biotic process, abotic transformation of E2 can also occur. Colucci et al. (2001) observed that E2 was readily removed from the aqueous phase of agricultural soils, and that the occurrence of E2 degradation in autoclaved soils demonstrated an abiotic process. Sheng et al. (2009) also found that E1 could be produced from E2 via abiotic oxidation with naturally occurring MnO_2 playing a role.

Environmental detections

The aforementioned laboratory-based studies have found that estrogenic hormones are of relatively low mobility due to high sorption affinity and rapid degradation rates. As a result, estrogens can hardly be expected to enter freshwater systems at levels that are sufficient to impact water quality and threaten aquatic wildlife. The environmental risk of estrogenic hormones is considered to be low and overestimated. However, many field studies have provided evidence that estrogens are sufficiently mobile and persistent to impact surface and ground water quality.

Steroidal hormones can enter fresh water through surface runoff when animal manure is applied to agricultural land. Nichols et al. (1997) measured E2 at 133 and 102 μg kg^{-1} in normal and aluminum treated poultry litter, and detected a maximum concentration of E2 in surface runoff at 1280 ng L^{-1} after litter application. A further study (Finlay-Moore et al., 2000) reported that the background E2 concentrations in surface runoff from ungrazed pasture were 50 to 150 ng L^{-1}; however, after poultry litter was applied, E2 concentrations increased to 20 to 2530 ng L^{-1} in surface runoff, and E2 levels in soils rose from 55 to 675 ng kg^{-1} after litter application.

In northwest Arkansas, E2 concentrations were detected ranging from 6 to 66 ng L^{-1} in five groundwater springs that were affected by nearby AFOs (Peterson et al., 2000). According to a nationwide reconnaissance (Kolpin et al., 2002) of the occurrence of organic contaminants in 139 streams of 30 states, the median concentration of E2 and E1 was 160 and 27 ng L^{-1}, respectively; and the frequency of detection was 10.6 and 7.1% for E2 and E1, respectively. In groundwater impacted by a residential septic system in Cape Cod, MA, the predominant estrogens detected were E1 (≤ 120 ng L^{-1}) and E2 (≤ 29 ng L^{-1}) (Swartz et al., 2006). Matthiessen et al. (2006) determined estrogenic activity in water samples from streams running through livestock farms, where E1 and E2 were almost ubiquitous in the streams with E2 equivalents ranging from 0.04 to 3.6 ng L^{-1} across all sites. More recently, estrogen levels (E1, E2α, E2, and E3) in three headwater streams were monitored monthly for a year within an AFO in upstate New York (Zhao et al., 2010), and estrogen concentrations in the streams were lower than 1 ng L^{-1} and increased in spring due to snow melting and precipitation. The low concentrations were likely due to degradation during the long residence time (~8 months) of manure storage, where 99.8% of the excreted estrogens were degraded (Zhao et al., 2010).

Estrogen conjugates

Steroidal estrogens are released by humans and animals primarily as sulfate or glucuronide conjugates, which allows them to be easily excreted in urine or bile because of the increased water solubility (Johnson and Sumpter, 2001). Conjugated estrogens have a sulfate and/or glucuronide moiety attached at the C-3 and/or C-17 position of the parent compound (Hanselman et al., 2003). Conjugated estrogens have much greater aqueous solubilities and mobility than free estrogens due to the polar glucuronide or sulfate functional groups (Hanselman et al., 2003). Glucuronide conjugation of steroidal estrogens in humans and animals is catalyzed by uridine 5'-diphospho-glucuronosyltransferase (UGT) enzymes (Kiang et al., 2005), and sulfate conjugation is catalyzed by sulfotransferases utilizing 3'-phosphoadenosine-5'-phosphosulfate (PAPs) as the sulfur donor (Gomes et al., 2009).

Conjugated estrogens have been consistently detected at relatively high levels in human and animal wastes. Baronti et al. (2000) monitored natural and synthetic estrogens in the influents and effluents of six Roman STPs for five months. The inlet concentrations of E3, E2, E1, and EE2 averaged 80, 12, 52, and 3 ng L^{-1} in the six STPs, respectively. Based on the daily excretion of estrogens, Baronti et al. (2000) suggested that deconjugation occurred preferentially in the sewers and increased the free estrogen levels. D'Ascenzo et al. (2003) found that free estrogens were never detected in pregnant female urine except E3, and that 106, 14, and 32 μg of conjugated E3, E2, and E1 were daily excreted in women, respectively. Estrogen sulfates were less abundant than estrogen glucuronides in female urine and accounted for approximately 20% of the total conjugated estrogens released from women. Moreover, sulfate conjugates were found more recalcitrant to deconjugation in activated sludge than glucoronides, with half-lives of more

than 2.5 d compared to 10 h for glucuronide conjugates (D'Ascenzo et al., 2003). Hutchins et al. (2007) reported that conjugates accounted for at least a third of the total estrogen load in different types of AFO lagoons, where the detected estrogen conjugates included estrone-3-sulfate (E1-3S; 2−91 ng L^{-1}), 17β-estradiol-3-sulfate (E2-3S; 8−44 ng L^{-1}), 17α-estradiol-3-sulfate (E2α-3S; 141−182 ng L^{-1}), and E2-17S (72−84 ng L^{-1}). The fact that all the estrogen conjugates found in the lagoon samples were sulfate forms suggested that sulfate conjugates were more persistent than glucuronides.

After being released to the environment, sulfate and glucuronide estrogen conjugates are biological inactive; therefore not a concern unless they deconjugate to yield the active parent estrogen (Ingerslev and Halling-Sørensen, 2003). Deconjugation is a common enzymatic hydrolysis process in the environment, which is governed by bacterial enzymes β-glucuronidase or sulfatase for glucuronide and sulfate conjugates, respectively (Khanal et al., 2006). Due to the persistence and recalcitrance of estrogen sulfate conjugates and their potentials to release free estrogens, it is important to understand the environmental fate of these conjugates.

Laboratory microcosm studies were conducted to determine aerobic degradation of E1-3S in three pasture soils at three temperatures (Scherr et al., 2008). The results showed that E1-3S was degraded rapidly without a lag phase in all soils forming E1 as a primary metabolite. The dissipation times for 50% and 90% (DT_{50} and DT_{90}) of E1-3S ranged from several hours to several days, and the degradation rates were temperature dependent (Scherr et al., 2008). A further study (Scherr et al., 2009) reported that the degradation of E2-3S followed first-order kinetics with half-lives ranging from 0.424 to 7.69 h. Two primary metabolites, E1-3S and E2, and one secondary metabolite, E1, were formed during the incubation, and furthermore, soil arylsulphatase activity played a major role in the degradation and metabolite formation of E2-3S

(Scherr et al., 2009). Additionally, Gomes et al. (2009) investigated the fate of multiple sulfate and glucuronide conjugates using batch studies in activated sludge. The first order deconjugation rates of glucuronides were less than 0.5 h, while 74 to 94% of sulfate conjugates still remained at 8 h, suggesting that glucuronide conjugates dissipated more quickly than sulfate conjugates. The stability of the conjugates can also be affected by conjugation positioning: D-ring glucuronides are more resistant than A-ring glucuronides to deconjugation (Gomes et al., 2009).

Objectives of the Study

The hypothesis of this study was that the manure-borne estrogen conjugate, E2-17S, can act as a precursor to free estrogens in agricultural soils and potentially increase the estrogen load and transport in the environment. To test this hypothesis, the persistence, sorption, and transformation of E2-17S were investigated in various soil-water systems. The effects of soil OC content, soil sterility, and initial concentrations of the applied conjugate were determined. The specific objectives were to (1) synthesize a radiolabeled E2-17S and perform characterization of the synthesized materials; (2) conduct laboratory soil batch studies to determine the persistence and transformation pathways of E2-17S under different soil conditions; (3) illustrate the effects of OC and initial concentrations on the dissipation and persistence of E2-17S in soils; (4) determine the role of soil microorganisms in the metabolism of E2-17S using non-sterile versus sterile soils; (5) develop sorption isotherms to estimate the sorption coefficients of E2-17S; and (6) build a mathematical model to simultaneously fit the sorption and transformation processes underlying in the soil-water systems.

PAPER 1. SYNTHESIS AND CHARACTERIZATION OF RADIOLABELED 17ß-ESTRADIOL CONJUGATES

Abstract

The use of radioactive tracers for environmental fate and transport studies of emerging contaminants, especially for those that are labile, offers convenience in tracking study compounds and their metabolites, and in calculating mass balances. The aim of this study was to synthesize radiolabeled glucuronide and sulfate conjugates of 17β-estradiol (E2). The conjugates [4-^{14}C]17β-estradiol-3-glucuronide (E2-3G) and [4-^{14}C]17β-estradiol-17-sulfate (E2-17S) were synthesized utilizing immobilized enzyme and chemical syntheses, respectively. Microsomal proteins from the liver of a phenobarbital induced pig (*Sus scrofa domestica*) were harvested and used to glucuronidate [^{14}C]E2. Synthesis of [^{14}C]E2-17S consisted of a three-step chemical process – introducing a blocking group at the C-3 position of [^{14}C]E2, sulfation at C-17 position, and subsequent deblocking to yield the desired synthetic product. Successful syntheses of [^{14}C]E2-3G and [^{14}C]E2-17S were achieved as verified by liquid chromatography, radiochemical analyses, quadrupole-time-of-flight (QTOF) mass spectrometry, and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Radiochemical yields of 84 and 44 percent were achieved for [^{14}C]E2-3G and [^{14}C]E2-17S, respectively. Synthetic products were purified using high performance liquid chromatography (HPLC) and radiochemical purities of 98% or greater were obtained.

Introduction

Medical research has used radiolabeled estrogenic compounds to study breast and uterine cancers (Mull et al., 2002), estrogenic receptors (Cummins, 1993), and as imaging agents in

breast tumors (Dence et al., 1996). More recently though, the radiolabeled hormonal compounds (e.g. [^{14}C]17β-estradiol (E2), [^{14}C]estrone (E1), and [^{14}C]testosterone (Casey et al., 2003; Casey et al., 2004; Fan et al., 2006; Sangsupan et al., 2006; Fan et al., 2007) and 6,7-^{3}H-estradiol (Sangsupan et al., 2006) have been used to study the fate and transport of steroids in the environment. Exposures to exogenous reproductive hormones have been associated with adverse effects in certain aquatic (Desbrow et al., 1998; Larsson et al., 1999; Teles et al., 2005) and terrestrial (Preziosi, 1998; Lintelmann et al., 2003; Park et al., 2009) species. Human waste treatment and animal feeding operations (AFOs) are sources of E2 and E1 to the environment. Estradiol is the most potent of these natural estrogens (Payne and Talalay, 1985; Palme et al., 1996; Matsui et al., 2000; Legler et al., 2002).

Laboratory studies suggest estrogens should have little to no mobility and should not persist in the environment because they bind rapidly and strongly to soil and degrade within hours (Holthaus et al., 2002; Casey et al., 2003; Fan et al., 2007). Field studies, however, have indicated that estrogens are present in the environment at frequencies and concentrations that imply they are moderately mobile and persistent (Kolpin et al., 2002; Schuh, 2008). Estrogen conjugates, which have different water solubilities, sorption coefficients, and degradation rates relative to their "free" estrogen counterparts, may offer insights into why steroidal estrogens are frequently detected in the environment (Kolpin et al., 2002). Swine (*Sus scrofa domesticus*), poultry (G*allus domesticus*), and cattle (B*os taurus*) excrete 96%, 69%, and 42%, respectively, of the urinary estrogens as conjugates (Hanselman et al., 2003). In fact, appreciable amounts of E2 in conjugated forms have been measured in swine manure slurry (liquid urine and feces) from AFO manure storage lagoons (Hutchins et al., 2007). Conjugates form a major portion of total environmental estrogen loading from AFOs and might play a significant function in the

detections of “free” steroidal estrogens in the environment. Although estrogen conjugates are biologically inactive, they can potentially be cleaved by microbial enzymes to form the more potent parent compound (Khanal et al., 2006).

Conjugation reactions are a common vertebrate mechanism in which hormones, drugs, toxicants, and non-nutritive organic molecules are eliminated (Amdur et al., 2001). During conjugation a charged, polar moiety is attached to a hydrophobic compound (e.g. estrogen), which increases its water solubility and excretion in urine or bile. Estrogens are typically conjugated with glucuronic or sulfuric acid at the C-3 and/or the C-17 positions (Khanal et al., 2006) (Fig. 1 and 2). Glucuronidation of estrogen is catalyzed by uridine 5'-diphospho-glucuronosyltransferase (UGT) enzymes in the endoplasmic reticulum and sulfation is catalyzed by cytosolic sulfotransferases (SULTs) (Nishiyama et al., 2002).

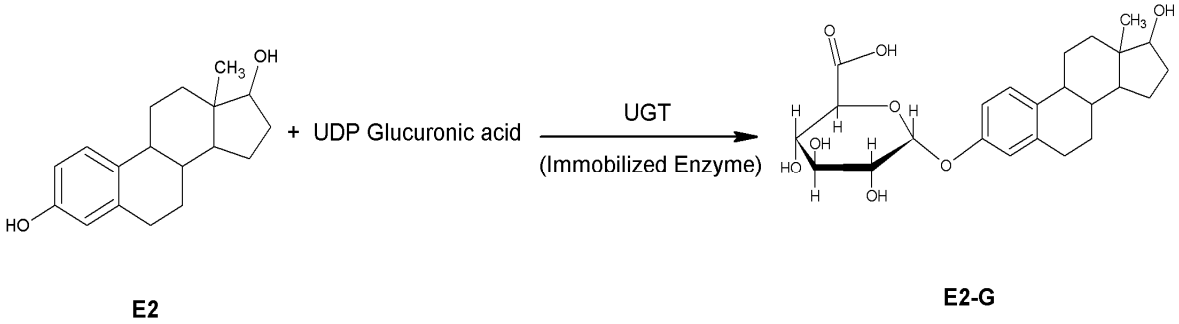


Figure 1. Glucuronidation of the hydroxyl group at C-3 of [4-^{14}C]17β-estradiol by uridine 5’-diphospho-glucuronosyltransferase (UGT).

The environmental fate of estradiol conjugates has not been extensively studied, possibly because radiolabeled conjugates are not commercially available. The availability of radiolabeled conjugated hormones would enable studies to be conducted that would improve the understanding of the fate and transport of these labile compounds in the environment. The

objective of this paper is to provide a method to synthesize radiolabeled [4-^{14}C]17β-estradiol-3-glucuronide (E2-3G) and [4-^{14}C]17β-estradiol-17-sulfate (E2-17S).

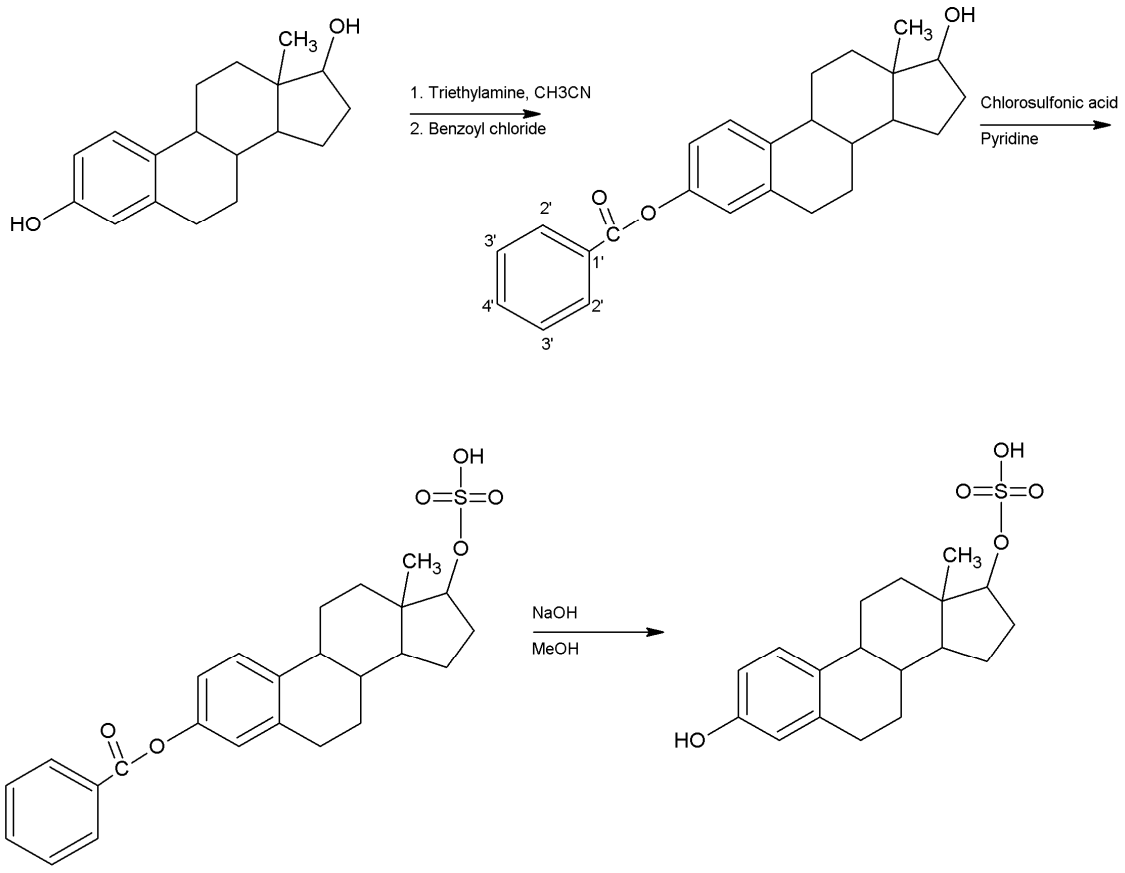


Figure 2. Chemical synthesis of [4-^{14}C]17β-estradiol-17-sulfate conjugate from [4-^{14}C]17β-estradiol.

Experiment

Materials

[^{14}C]17β-Estradiol (55 mCi/mmole) was purchased from American Radiolabeled Chemicals (St Louis, MO). Unlabeled E2, UDP glucuronic acid, magnesium chloride, ethanol, potassium phosphate monobasic, potassium phosphate dibasic, potassium hydroxide, hydrochloric acid, ethyl acetate, pyridine, sodium hydroxide, chlorosulfonic acid and acetic acid were obtained

from Sigma-Aldrich. Triethylamine (Fluka); benzoyl chloride (Bayer); trisodium phosphate (Mallinkrodt, Paris, KY) were obtained from other sources. Acetonitrile (ACN) was obtained from EMD Chemicals (Gibbstown, NJ). Scintillation fluid EcoLite™ was obtained from MP Biomedicals (Santa Ana, CA). SPE cartridges Bond Elut™ C18 (6 g, 20 mL) and Sep-Pak® Vac C18 were obtained from Varian (Harbor City, CA) and Waters (Milford, MA), respectively.

Uridine 5'-diphospho-glucuronosyltransferase (UGT)

A castrated, cross-bred hog weighing 24.4 Kg was used as the source of the UGT enzymes, following USDA Animal Care and Use Committee guidelines. The hog was intramuscularly (2 d) then intraperitoneally dosed (2 d) with approximately 20 mg Kg^{-1} phenobarbital for four consecutive days, after which, the hog was euthanized. The liver was homogenized and microsomes were isolated via differential centrifugation. Proteins were solubilized and immobilized onto Sepharose beads (Pallante et al., 1986) and were stored in a 1:1 suspension with 0.1 M Tris buffer (pH 7.4) at 4 °C until use.

Liquid scintillation counting

Radioactivity was quantitated with a Packard 1900 CA scintillation analyzer (Downers Grove, IL), and samples were dissolved in EcoLite™ scintillation cocktail.

High-performance liquid chromatography

Analytical HPLC for E2-3G was performed using a Waters 600E System Controller and pump (Milford, MA), equipped with a Jasco FP 920 fluorescence detector (Jasco, Easton, MD) with the following conditions: Phenomenex-C18, 4.6 × 250 mm, 5 μm; A: 10% ACN in 50 mM ammonium acetate (pH 4.5), B: 90% ACN in 50 mM ammonium acetate (pH 4.5); gradient: 20 to 100% B, 29 min, 100% B, 3 min hold, 1.0 ml/min, excitation and emission wavelengths of

280 and 312 nm, respectively. Prep-HPLC was performed on Jones Chromatography-C18, 10 × 250 mm, 5 ìm; A: 5% ACN in 50 mM ammonium acetate (pH 4.5), B: 90% ACN in water; isocratic 85% solvent A, 15% solvent B; 4.7 mL/min.

For E2-17S, analytical HPLC was performed on a Gilson System 45NC Gradient Analytical instrument (Gilson Medical Electronics, Middleton, WI) equipped with a variable wavelength UV detector with the following conditions: Radial-Pak-C18, 8 × 100 mm (Waters Associates, Milford, MA); A: 10:90 methanol/water, B: 90:10 methanol/water; gradient: 20% B to 100% B, 28 min., 4 min hold; 1.0 ml/min 220 nm. HPLC for 17β-estradiol-3-benzoate (E2-3B) was conducted using following conditions: Radial-Pak-C18, 8 × 100 mm; A: 10:90 methanol/water, B: 90:10 methanol/water; gradient: 20% B to 100% B, 30 min, 15 min hold; 1.0 ml/min, UV 220 nm.

Mass spectral analysis

Negative ion LC/MS was performed with a Waters Alliance 2695 HPLC (Symmetry-C18, 2.1 × 100 mm; A: 40% ACN in water, B: 60% ACN in water; gradient: 40 to 100% B, 10 min, 5 min hold, 0.2 mL/min), and a Waters Micromass QTOF (API-US in a ES- mode, MassLynx software, FWHM: 6500, source temperature 120 °C, desolvation temperature 350 °C, cone voltage 35 V, capillary voltage 2500 V, collision energy 5 eV for sulfate and 20 eV for glucuronide conjugates.

NMR spectra

A Bruker AM-400 spectrometer (Billerica, MA) operating at either 400.13 MHz or 100.61 MHz was used to record the ^{1}H- and ^{13}C-NMR spectra respectively. ^{1}H-NMR spectra were run in fully coupled mode with 128 scans and an acquisition time of 3.9713 s. ^{13}C-NMR spectra were

run in CPD mode, with 64K scans obtained with an acquisition time of 1.307 s. The chemical shifts for the NMR spectra for E2 were ^{13}C NMR (MeOH-d_4) δ: 155.84, 138.8, 132.32, 127.22, 116.05, 113.72, 82.49, 51.26, 45.34, 44.35, 40.5, 38.00, 30.72, 30.68, 28.83, 27.53, 24.03, 11.71; ^{1}H NMR (MeOH-d_4) δ (aromatic A-ring protons): 7.06 (d), 6.53 (d), 6.47 (s).

Synthesis of [4-^{14}C]17β-estradiol-3-glucuronide

5 mL of 0.1 M phosphate buffer (pH 7.4) was added to 20 mL of pre-rinsed microsomal proteins immobilized on Sepharose beads. Forty μL of 2.63 M magnesium chloride, 63 mg of UDP glucuronic acid (5 mM final concentration), and 164.7 μg of [^{14}C]E2 (0.60 μmole; 33 μCi; dissolved in 567 μL ethanol) and 6477 μg of unlabeled E2 (23.78 μmole, dissolved in 540 μL ethanol) were added to the reaction flask. The reaction flask was slowly stirred on a Roto-Vap (Büchi, Flawil, Switzerland) without vacuum at 37 °C for 24 h determined *a priori*. The aqueous fraction was collected by filtration. [4-^{14}C]17β-Estradiol-3-glucuronide was partially purified on a Bond Elut™ C18 SPE cartridge preconditioned with ACN and nanopure water by eluting with 20:80 ACN-water. The final radiochemical purity was 99% obtained after preparative HPLC. ^{13}C NMR (MeOH-d_4) δ: 176.52, 156.99, 135.66, 127.20, 117.96, 115.41, 102.65, 82.47, 77.71, 76.68, 74.74, 73.59, 51.26, 45.41, 44.32, 40.34, 38.97, 37.97, 30.69, 30.04, 28.40, 27.51, 24.00, 11.67. ^{1}H NMR (MeOH-d_4) δ (aromatic A-ring protons): 7.18 (d), 6.87 (d), 6.81 (s). LC/MS-QTOF: M-H = 447.21, m/z 271.17, 175.03, 113.02.

Synthesis of [4-^{14}C]17β-estradiol-17-sulfate

Synthesis of [^{14}C]E2-17S consisted of a three-step chemical process that involved introducing a blocking group at C-3 position of [^{14}C]E2, sulfation at C-17 position, and subsequent deblocking to yield the desired synthetic product (Fig. 2).

[4-^{14}C]17β-Estradiol-3-benzoate

Radiolabeled E2 (259.5 µg, 0.95 µmole, 47.7 µCi) was mixed with unlabeled E2 (11.43 mg, 42 µmole) in ethanol and the solvent was evaporated (Hooijerink et al., 2005). The residue was re-dissolved in 2 mL of acetonitrile, and 13 µL triethylamine and 11 µL benzoyl chloride (13.3 mg, 94.7 µmole) were added; the reaction mixture was stirred at room temperature for 2 h and subsequently dried under a stream of nitrogen. To the white residue, 4 mL of 0.1 M trisodium phosphate solution was added and the mixture was sonicated for 30 min resulting in a light yellow suspension. The suspension was extracted with ethyl acetate (3 mL × 3), and the organic solvent was evaporated under a stream of nitrogen. The residue E2-3B was dissolved in ethyl acetate (3 mL) and water (1 mL) for further purification using HPLC. The yield of E2-3B was 59.3% and radiochemical purity was 98%. ^{13}C NMR (MeOH-d_4) δ: 165.94, 150.13, 139.49, 139.38, 134.86, 130.99, 130.99, 129.83, 129.83, 127.47, 122.63, 119.79, 82.45, 51.33, 45.55, 44.34, 40.14, 37.99, 30.69, 30.56, 28.27, 27.48, 24.04, 11.68. ^{1}H NMR (MeOH-d_4) δ (aromatic A-ring protons): 7.44 (d), 6.94 (d), 6.89 (d); δ (benzoate protons): 8.14 (d), 7.66 (dd), 7.54 (dd). LC/MS-QTOF: M-H = 375.21, m/z 361.21, 356.85, 334.82, 332.82.

[4-^{14}C]17β-Estradiol-3-benzoate-17-sulfate

Sulfur trioxide-pyridine complex was synthesized in-house (Itoh et al., 1999) by adding chlorosulfonic acid (138 µL, 2.07 µmoles) with stirring to dry pyridine (1.66 mL) at 0 ˚C. The solution was allowed to warm to room temperature, followed by dilution with dry pyridine (623 µL). 17β-Estradiol-3-benzoate was dissolved into 1.1 mL of pyridine, and the solution was heated to 50 °C, to which sulfur trioxide-pyridine complex, also heated to 50 °C, was added. The mixture was stirred for 30 min at 50 °C followed by solvent evaporation under nitrogen, addition

of water (4 mL), and adjusting to pH 8 (1 M NaOH). The mixture was partially purified with a Sep-Pak® Vac C18 cartridge, and 17β-estradiol-3-benzoate-17-sulfate (E2-3B-17S) was eluted with methanol. ^{13}C NMR (MeOH-d_4) δ: 166.95, 150.11, 139.42, 139.27, 134.95, 130.85, 130.85, 129.89, 129.89, 127.52, 122.64, 119.84, 88.22, 50.78, 45.41, 44.23, 40.29, 37.98, 30.53, 29.22, 28.18, 27.47, 24.11, 12.24. ^{1}H NMR (MeOH-d_4) δ (aromatic A-ring protons): 7.30 (d), 6.90 (d), 6.86 (s); δ (benzoate protons): 8.13 (d), 7.66 (dd), 7.52 (dd). LC/MS-QTOF: M-H = 455.10, m/z 351.12.

[4-^{14}C]17β-Estradiol-17-sulfate

Hydrolysis (Kirdani, 1965) of E2-3B-17S was accomplished by adding 5% NaOH in methanol (5 mL), stirring for 1 h at room temperature, then neutralization with 10% acetic acid, and evaporation under nitrogen. After purification by HPLC, 21 µCi (18.9 µmoles; 7.1 mg; 98% pure) of E2-17S was obtained (overall yield: 44%). ^{13}C NMR (MeOH-d_4) δ: 155.89, 138.76, 132.54, 127.26, 116.04, 113.76, 88.19, 50.78, 45.3, 44.24, 40.34, 38.00, 30.71, 29.22, 28.48, 27.48, 24.10, 12.19. ^{1}H NMR (MeOH-d_4) δ (aromatic A-ring protons): 7.06 (d), 6.53 (d), 6.47 (s). LC/MS-QTOF: M-H = 351.07, m/z 96.96.

Results and Discussion

Synthesis of [4-^{14}C]17β-estradiol-3-glucuronide

A one-step enzymatic synthesis of E2-3G is described that permitted regioselective attachment of a glucuronide acid moiety to E2. Since the reaction occurred in a buffered solution, reaction progress (Scheme 1, as shown in Fig. 1) could be readily followed by reversed-phase HPLC. The radiolabeled parent peak (E2) at 27.57 min dropped steadily in intensity while the increase in peak intensity at 5.55 min occurred for the desired product E2-3G (Fig. 3). The

reaction was essentially complete by 24 h. C-18 SPE purification yielded a radiochemical purity of 95%; semipreparative HPLC improved radiochemical purity to 99%. LC/MS-QTOF analysis of E2-3G showed ions at m/z 447.21, 271.17, 175.03, and 113.02, representing the molecular ion of E2-3G and ions of E2, glucuronic acid, and a glucuronide fragment, respectively.

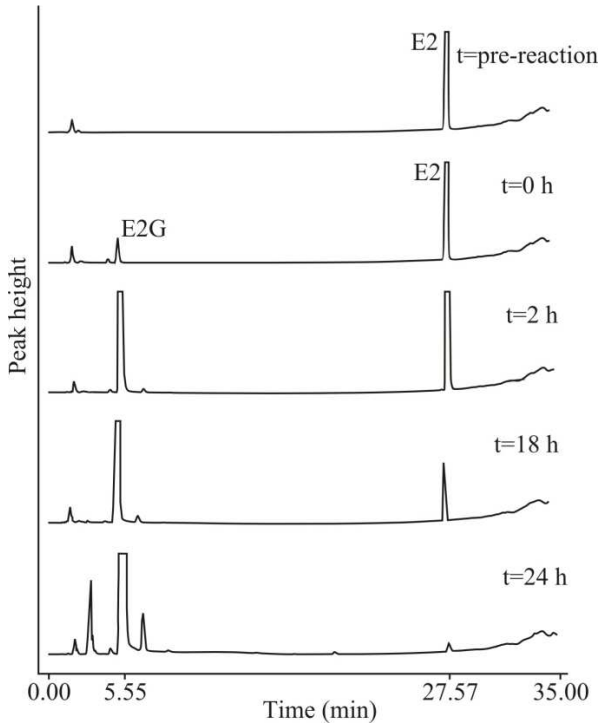


Figure 3. Progress of enzymatic synthesis of [4-^{14}C]17β-estradiol-3-glucuronide with time and the concurrent consumption of [4-^{14}C]17β-estradiol.

To determine the site of conjugation, ^{13}C-NMR spectra of E2 and E2-3G were compared to each other and with literature values of E2 (Dionne et al., 1997; Kashima et al., 2010) and bisphenol A glucuronide (Kurebayashi et al., 2003). Glucuronidation was indicated by the presence of an additional 6 carbons in the ^{13}C-NMR spectrum of E2-3G; and the site of conjugation was indicated by the downfield shift of C-3 from 132.32 to 135.66 ppm in the spectrum of E2-3G. Chemical shifts in the ^{1}H-NMR spectrum of E2-3G were also consistent with glucuronidation at C-3. For example, protons ortho and meta to C-3 were shifted downfield

6.53 to 6.87, 7.06 to 7.18, and 6.47 to 6.81 ppm) for E2 and E2-3G, respectively. In addition, as one of the most diagnostic components of a sugar conjugated spectrum, an anomeric singlet at 4.30 ppm also confirms the formation of E2-3G. Chemical shift assignments for the C-17 remained invariant for E2 and E2-3G.

Diglucuronide conjugation was theoretically possible due to two hydroxyl groups in E2, one a phenolic in the A-ring, and the other an aliphatic on the D-ring. However, only one site of conjugation was expected because enzyme-catalyzed reactions are usually regio- and stereospecific (Alonen et al., 2009). UGT enzymes are divided into two distinct subfamilies, UGT1 and UGT2 (King et al., 2000; Kiang et al., 2005). Phenobarbital treatment of hepatoma cell lines is known to induce hepatic bilirubin UGTs (Brierley et al., 1996), which show a strong selectivity for phenolics (Lepine et al., 2004).

Product yield of E2-3G was 84%, and was attributed to the induction of UGT's by phenobarbital (Watanabe and Yoshizawa, 1982). The same microsomal proteins also were active at glucuronidating hydroxylated polybrominated diphenyl ether metabolites, triclosan, and ractopamine hydrochloride.

Synthesis of [4-^{14}C]17β-estradiol-17-sulfate

The synthesis of E2-17S, presented in Scheme 2 (as shown in Fig. 2), was initiated by blocking the more reactive C-3 hydroxyl in E2, which was accomplished with a 59.3% product yield to form the intermediate E2-3B. The negative ion LC/MS analysis of E2-3B resulted in a molecular ion at 375.21, a methyl loss fragment at 361.21, and a water loss at 356.85. Losses of propanyl and propenyl groups were consistent with fragments at m/z 332.82 and 334.82, respectively. ^{1}H-NMR analysis of E2 and E2-3B indicated shifts in protons ortho- and meta- to

C-3 occurred in E2-3B relative to E2 (6.53 to 6.94; 7.06 to 7.44; 6.47 to 6.89 ppm). Benzoate protons were present at 8.14, 7.54, and 7.66 ppm of E2-3B. The ^{13}C-NMR spectrum confirmed that the blocking had occurred at C-3 because carbons ortho- and meta- to C-3 of E2-3B were shifted downfield relative to their chemical shift position in E2 (113.72 to 119.79; 132.32 to 134.86; 116.05 to 122.63 ppm).

The formation of E2-3B-17S was confirmed by a molecular ion at 455.09 in the LC/MS spectrum, and was accompanied by a prominent fragment at m/z 351.12, which was consistent with a benzoate fragment loss. Sulfation at C-17 was suggested by significant downfield chemical shifts for the C-17 proton (3.67 to 4.31 ppm) and carbon (82.45 to 88.22 ppm) in the ^{1}H and ^{13}C NMR spectra of E2-3B and E2-3B-17S, respectively. A radiochemical purity of 95% was achieved and was considered satisfactory for the next step.

The purification of the final product (E2-17S) yielded 21 µCi (18.9 µmoles; 7.1 mg) of 98% radiochemical purity. The formation of E2-17S was confirmed by a molecular ion at 351.07 and the sulfate moiety ion at m/z 96.96 in the LC/MS spectrum of E2-17S. ^{13}C NMR analyses of E2-17S indicated a significant upfield chemical shift of C-3 relative to E2-3B-17S (132.54 from 134.95 ppm), as well as for carbons ortho to C-3 (116.04 from 122.64; 113.76 from 119.84 ppm). Upfield shifts of the aromatic protons between E2-17S and E2-3B-17S were observed (6.53 from 6.90; 7.06 from 7.30; 6.47 from 6.86 ppm), but no chemical shift difference was observed for the C-17 proton. Collectively the physical data provide convincing evidence that sulfation of E2 had occurred at C-17. The overall yield of E2-17S was 44%, which possibly could have increased if the reaction conditions had been optimized; however optimization was not an immediate objective. The radiochemical yield was satisfactory for the immediate needs of this study.

Conclusions

Radiolabeled E2-3G and E2-17S were successfully synthesized using enzymatic and chemical approaches, respectively, which permitted their use for laboratory scale fate and transport experiments in soil-water systems. Though our objective was measuring and modeling the movement of endocrine disrupting compounds in the environment, these studies are but a small portion of the potential studies in which radiolabeled conjugates could be used. Since glucuronidation and sulfation are the major conjugation pathways in vertebrates for not only steroid hormones, but other xenobiotics (Dutton, 1980), we hypothesize that radiolabeled glucuronides and sulfates of other emerging contaminants can also be synthesized following the approaches provided in this paper, or with appropriate modifications of them.

Acknowledgements

The authors wish to thank Colleen Pfaff, Dee Anne Ellig, Barb Magelky, Mike Giddings, Grant Harrington, Jason Holthusen (USDA-ARS), and Nathan Derby (Soil Science Dept., NDSU) for their support in the laboratory work and Glenn Wittenberg (USDA-ARS) for his IT support. This research was based upon work supported by the National Science Foundation under Grant No. 0730492. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

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PAPER 2. DISSIPATION AND TRANSFORMATION OF 17ß-ESTRADIOL-17-SULFATE IN SOIL-WATER SYSTEMS

Abstract

In the environment, estrogen conjugates can be precursors to the endocrine-disrupting free estrogens, 17β-estradiol (E2) and estrone (E1). Compared to other estrogen conjugates, 17β-estradiol-17-sulfate (E2-17S) is detected at relatively high concentrations and frequencies in animal manure and surface runoff from fields receiving manure. To elucidate the lifecycle of manure-borne estrogens and their conjugates in the environment, the fate of radiolabelled E2-17S in agricultural soils was investigated using laboratory batch studies with soils of different organic carbon (OC) content (1.29% for topsoil versus 0.26% for subsoil). E2-17S was found relatively persistent in the aqueous phase throughout the duration of the 14 d experiment. The aqueous E2-17S persisted longer in the subsoil (half-lives (DT_{50}) = 64–173 h) than the topsoil (DT_{50} = 5–26 h), and the aqueous persistence of E2-17S depended on its initial concentration. The major transformation pathway was hydroxylation, yielding mono- and di-hydroxy-E2-17S (OH-E2-17S and diOH-E2-17S). Free estrogens, E2 and E1, were only observed in the sorbed phase of the soil at low concentrations (~1% of applied dose), which demonstrated that deconjugation and subsequent oxidation had occurred. Although deconjugation was not a major pathway, E2-17S could be a precursor to free estrogens in the environment.

Introduction

Naturally occurring estrogenic hormones are endocrine-disrupting compounds (EDCs). For example, the lowest observable adverse effect level (LOAEL) of 17β-estradiol (E2) is 10 ng L^{-1} for aquatic organisms (Routledge et al., 1998). In US streams, E2 is frequently detected

(frequency = 10.3%) at concentrations (9–160 ng L^{-1}) above the LOAEL (Kolpin et al., 2002). However, E2 degrades within hours and is found immobile in laboratory soil experiments (Lai et al., 2000; Casey et al., 2003; Lee et al., 2003). The discrepancies between field observations (high detection concentrations and frequencies) and laboratory experiments (highly degradable and immobile), indicate that there are other mechanisms that facilitate transport and persistence of estrogens in the environment. Conjugated estrogens may contribute to the mobility and persistence of free estrogens in the environment. Estrogens are excreted in the urine primarily as conjugates of sulfate or glucuronide, which are more water-soluble than their counterpart free estrogens (Andreolini et al., 1987; Guengerich, 1990). Furthermore, unlike free estrogens, conjugates are not biologically active (Desbrow et al., 1998), because they do not bind to estrogen receptors (Hobkirk, 1985). However, bacteria and enzymes can hydrolyze estrogen conjugates to yield the biologically active free estrogens, E2 or E1, in municipal and animal wastes (Hanselman et al., 2003; Khanal et al., 2006). Estrogen sulfate conjugates are more persistent, and are detected more frequently than glucuronides in municipal sewage systems (D'Ascenzo et al., 2003), wastewater (Gomes et al., 2005), and wastewater treatment plants (WWTPs) (Isobe et al., 2003; Schlusener and Bester, 2005).

Manure from animal feeding operations (AFOs) is land-applied as soil amendments, and can be a major source of steroid hormones and their conjugates to the environment. Livestock manure is estimated to contribute 90% of estrogens in the environment (Maier et al., 2000), and estrogen conjugates can comprise one-third of the total estrogen load from AFO manure (Hutchins et al., 2007). Moreover, the highest conjugate levels measured in various AFO lagoons were sulfated forms, where estrone-3-sulfate (E1-3S), 17β-estradiol-3-sulfate (E2-3S), 17α-estradiol-3-sulfate (E2α-3S), and 17β-estradiol-17-sulfate (E2-17S) were measured at

concentrations of 2−91 ng L^{-1}, 8−44 ng L^{-1}, 141−182 ng L^{-1}, and 72−84 ng L^{-1}, respectively (Hutchins et al., 2007). When poultry (*Gallus gallus*) manure was applied to an agricultural field, no glucuronide conjugates were detected in surface runoff; only sulfate conjugates were found, and E2-17S concentration (107 ng L^{-1}) was higher than any other sulfate conjugates (E2-3S, E2α-3S, and E1-3S) (Dutta et al., 2012). Additionally, runoff concentrations of E2-17S (0.3−3.9 ng L^{-1}) were higher than free E2 (0.5−1.9 ng L^{-1}) (Dutta et al., 2010).

Free estrogens are non-volatile and relatively hydrophobic compounds, and they are sorbed rapidly by soils and sediments (Casey et al., 2003; Lee et al., 2003). Degradation of free estrogens is reported to be rapid in soils, with half-lives of less than one day (Colucci et al., 2001). Considering that estrogen conjugates can act as precursors to free estrogens, it is imperative to understand the fate and transport of conjugates in the environment. Deconjugation potentials of estrogen sulfates have been studied in municipal sewage systems (D'Ascenzo et al., 2003; Gomes et al., 2009; Kumar et al., 2012); however, estrogen sulfates may behave differently in agricultural soils due to different microbial populations. The fate of E1-3S (Scherr et al., 2008) and E2-3S (Scherr et al., 2009) has been studied in pasture soils, and both sulfate conjugates were deconjugated to release free estrogens. A more recent study reported that a glucuronide conjugate, 17β-estradiol-3-glucuronide (E2-3G), was quickly transformed into free E2 and E1 in soil-water slurries, which can be a significant contributor to free estrogens to the environment (Shrestha et al., 2012b).

Compared to other conjugates, E2-17S is detected more frequently and at higher concentrations in AFO manure and in runoff from fields receiving manure. Swine manure is usually applied to agricultural land as slurry. A previous field study by Schuh et al. (2011a) reported that the manure application rates were 120 m^3 ha^{-1}, which supplied approximately 48

mm of water to the field. The surface runoff after manure slurry application can thus contain significant amounts of estrogen conjugates. To date, no studies have investigated the fate of E2-17S in soils, which is necessary to fully understand the behavior of manure-borne estrogens, the most significant source of steroid hormones to the environment. It is hypothesized in the present study that if E2-17S was applied to soils, then E2-17S could serve as a precursor to endocrine-disrupting, free estrogens in the environment. The objective of this study was to use batch experiments to determine the dissipation and transformation of E2-17S in the presence of agricultural soils.

Materials and Methods

Analytical methods

All experiments were conducted using radiolabelled [^{14}C]E2-17S (specific activity = 241.8 Bq μg^{-1}; radiochemical purity = 98%), which was synthesized in-house from [4-^{14}C]E2 (American Radiolabels, Inc., St. Louis, MO) (Shrestha et al., 2011). Also, all radiometric methods were based on those developed by Shrestha et al. (2012a).

Briefly, for metabolite fractionation and quantification, a combination of high performance liquid chromatography (HPLC) and liquid scintillation counting (LSC) was used. The HPLC (Beckman Coulter Inc., Fullerton, CA) was equipped with a C18 column (250 × 4.6 mm; Phenomenex; Torrance, CA), a System Gold 508 auto-sampler, a 126 solvent module pump, a 168 UV detector, and a Gilson FC 204 fraction collector (Middleton, WI). The HPLC solvents and gradient were identical to the previous study (Shrestha et al., 2012a). Aliquots of fraction-collected samples (1 mL) from the HPLC were transferred to 6 mL scintillation vials, to which 4 mL of Ecolite scintillation cocktail (MP Biomedicals, Santa Ana, CA) were added, and then

assayed for radioactivity for 10 min using LSC (1900 CA, Packard, Downers Grove, IL). Metabolites were identified and characterized by liquid chromatography with tandem mass spectroscopy (LC-MS/MS) in negative-ion mode (ESI-). The mass spectrometer used for the analysis was an Ultima API-US Quadrupole-Time of Flight mass spectrometer (Waters, Beverly, MA) equipped with an electrospray ionization source. The capillary voltage was 2.33 kV, cone voltage was 55 V, source and desolvation temperatures were 120 and 400 °C, respectively. The cone and desolvation gas flows were 0 and 500 L h^{-1}, respectively. The HPLC column was a Symmetry C18, 3.5 µm, 2.1 × 100 mm with a 2.1×10 mm guard column (Waters, Milford, MA). The initial mobile phase consisted of 60% 95:5 water:acetonitrile (solvent A) and 40% acetonitrile (solvent B). A linear gradient to 100% B was used from 0 to 10 min followed by a hold for 5 min at a flow rate of 0.2 mL min^{-1}.

To determine non-extractable, or irreversibly bound, radioactive residue in soil, combustion analysis was used. A mass of 0.1 g of extracted, air-dried soil (5 ×) was placed in fiber thimbles and combusted in a Packard Model 307 Oxidizer (Downers Grove, IL). Radiolabelled carbon dioxide was trapped with 8 mL of CarboSorb E (Waltham, MA) and analyzed by LSC after mixing with 12 mL of Permafluor cocktail (Waltham, MA).

Batch experiments

The soils used for the batch experiments were sampled from southeastern North Dakota, which is from the Hecla-Hamar Series, a loamy fine sand (sandy, mixed, frigid Oxyaquic Hapludolls). Previous laboratory (Fan et al., 2007a, b, 2008; Zitnick et al., 2011; Shrestha et al., 2012b) and field (Thompson et al., 2009; Schuh et al., 2011a; Schuh et al., 2011b) studies from this research group used the same soil type, which aided in interpreting observations and

discerning various fate and transport processes of steroid hormones in the environment. Soil samples were collected from two depths: topsoil (0−15 cm) (A horizon) and subsoil (46−61 cm) (C horizon). Swine manure slurry is applied to agricultural fields either by direct surface application or by injection beneath the upper 15 cm of soil (Schuh et al., 2011a). Consequently, the two soil depths can be potentially affected by manure slurry. The properties (Table 3) of the two soils were similar except for their organic carbon (OC) content. Before conducting the batch experiments, the soil samples were air-dried and passed through a 2 mm sieve.

Table 3. Selected properties of soil samples used in the batch experiments (Hecla-Hamar Series; loamy fine sand).

	Topsoil	Subsoil
Depth (cm)	0−15	46−61
Organic matter (OM) (%)	1.70	0.50
Organic carbon (OC) (%)	1.29	0.26
Inorganic carbon (IC) (%)	0.00	0.00
pH	7.0	7.4
Cation exchange capacity (CEC) ($cmol_c\ Kg^{-1}$)	9.3	9.8
Sand:silt:clay (%)	83:10:7	90:4:6
Mn ($\mu g\ g^{-1}$)	292	223

All batch experiments were conducted at room temperature (23 ± 1 °C), and followed methods from a previous study (Shrestha et al., 2012b). [^{14}C]E2-17S (in 10 µL MeOH) was added at four different concentrations: 0.6, 2.9, 8.9, and 30 mg L^{-1}, to triplicate 10 mL glass vials containing a soil-water mixture (1.6 g and 8 mL of 0.01 M $CaCl_2$ solution, respectively). Controls consisted of 0.01 M $CaCl_2$ and 0.6 mg L^{-1} of [^{14}C]E2-17S with no soil. The concentration ranges in this study were higher than concentrations normally found in the environment, which were used to ensure the adequate quantification and high resolution for the

parent compound and potential metabolites by LSC. Similar concentration ranges have been selected for a radiolabelled glucuronide estrogen, [^{14}C]E2-3G (Shrestha et al., 2012b). The batch vials were agitated by rotation from top to bottom (360° every 5 s) for 14 d (336 h). Aliquots of 100 and 150 µL were removed from the aqueous phase using a sterile syringe at 4, 8, 24, 48, 72, 168, and 336 h for LSC and HPLC analysis. The HPLC aliquot was passed through a 0.45 µm pore-size glass fiber filter, stored in a LC-MS/MS glass vial with formaldehyde (2.7% of final volume), and frozen until further analysis.

At each sample time, a single batch vial (hereafter called "stop vials") was removed from the low dose group (0.6 mg L^{-1}) and analyzed for parent compound and metabolites that partitioned to the sorbed phase. The removed "stop vials" were preserved with formaldehyde (2.7% of final volume) and then centrifuged. After centrifuging, the supernatant was transferred into new, clean batch vials, and both the liquid and soil samples were frozen until further analysis. To determine the radioactive residues bound to the soil, the soil was first extracted by water (5 mL × 3) and then by acetone (5 mL × 3) during 30 min of sonication. Aliquots (500 µL) from the water and acetone extracts were assayed for total radioactivity by LSC. The water extracts were then dried with a centrifugal rotary evaporator (Savant, Farmingdale, NY), and the acetone extracts were dried under a gentle stream of nitrogen. The extracts were reconstituted in methanol for HPLC analysis. Radioactivity that was extractable from the soil was considered reversibly sorbed; and non-extractable radioactivity was considered irreversibly sorbed, which was analyzed by soil combustion as described in section 2.1. Additionally, no gas phase analysis of the batch vial headspace was conducted because earlier studies showed that mineralization of free (Fan et al., 2007b) and glucuronide conjugated estrogen (Shrestha et al., 2012b) did not occur under these soil conditions.

First-order kinetics

Dissipation of E2-17S in the aqueous phase was described using the following first-order kinetic model (SigmaPlot® 2000 for Windows®; version 6.00 SPSS Inc.):

Parent compound: $\frac{C}{C_0} = e^{-kt}$ [1]

where C/C_0 is the relative concentration of [^{14}C]E2-17S, t (h) is time, and k (h^{-1}) is the dissipation rate constant of [^{14}C]E2-17S. Dissipation times for 50% (DT_{50}) and 90% (DT_{90}) of the parent compound were directly calculated using the first-order dissipation rate constants ($DT_{50} = \ln 2/k$ and $DT_{90} = \ln 10/k$).

Statistical analysis

Significance of main effects and interactions was determined using analysis of variance (ANOVA). Tukey's and Student's *t*-tests were used to determine whether there were significant differences between levels. For all statistical analysis, an α of 0.05 was used, and a probability of $p \leq 0.05$ was considered significant. The program JMP (version 9.0.2 SAS Institute Inc.) was used for all statistical analysis.

Results and Discussion

Aqueous phase observations

Parent compound dissipation

For the topsoil (Fig. 4A), aqueous concentrations of E2-17S reached steady-state between 48 h and 72 h for all initial concentrations. The aqueous E2-17S concentrations in the subsoil never reached steady-state, but continued to dissipate for the 14 d duration of the experiment (Fig. 4B). The first-order dissipation rate constants (k) of the topsoil were greater than the subsoil values

(Eq. 1; Table 4), reflecting faster aqueous dissipation of E2-17S, which can be attributed to greater sorption capacity and faster transformation. Soil with higher OC would result in higher sorption capacity of estrogens (Casey et al., 2003; Lee et al., 2003). Also, higher soil OC and microbial biomass would result in greater biotic metabolism of sulfate conjugates (Scherr et al., 2008, 2009). Although the microbial activity was not measured in the present soils, it is widely reported that microbial biomass and enzymatic activities decrease with increasing soil depth (Burns and Dick, 2002). Similar differences in aqueous dissipation between the subsoil and topsoil were also observed for E2-3G (Shrestha et al., 2012b). However, compared to E2-3G, E2-17S persisted longer in the aqueous phase, where the DT_{50} values for E2-17S (Table 4) were greater than values reported for E2-3G in the topsoil (DT_{50} = 1.5−3.3 h) and subsoil (DT_{50} = 41−116 h) (Shrestha et al., 2012b). Moreover, the DT_{50} values for E2-17S were greater than values reported for the free E2 (0.96 h to 12 h) in various soils (Colucci et al., 2001; Colucci and Topp, 2002), indicating the greater persistence of E2-17S.

Table 4. Parameter estimates with standard deviation for [^{14}C]17β-estradiol-17-sulfate using the first-order kinetic model under multiple initial concentrations.

Initial Concentration (mg L^{-1})	k (h^{-1})	r^2	DT_{50} (h)	DT_{90} (h)
		Topsoil		
30	0.026 (0.003)	0.980	26	88
8.9	0.030 (0.005)	0.937	23	77
2.9	0.033 (0.005)	0.955	21	71
0.6	0.143 (0.022)	0.967	4.9	16
		Subsoil		
30	0.004 (0.001)	0.869	173	576
8.9	0.006 (0.000)	0.989	108	360
2.9	0.007 (0.001)	0.991	99	329
0.6	0.011 (0.003)	0.827	64	213

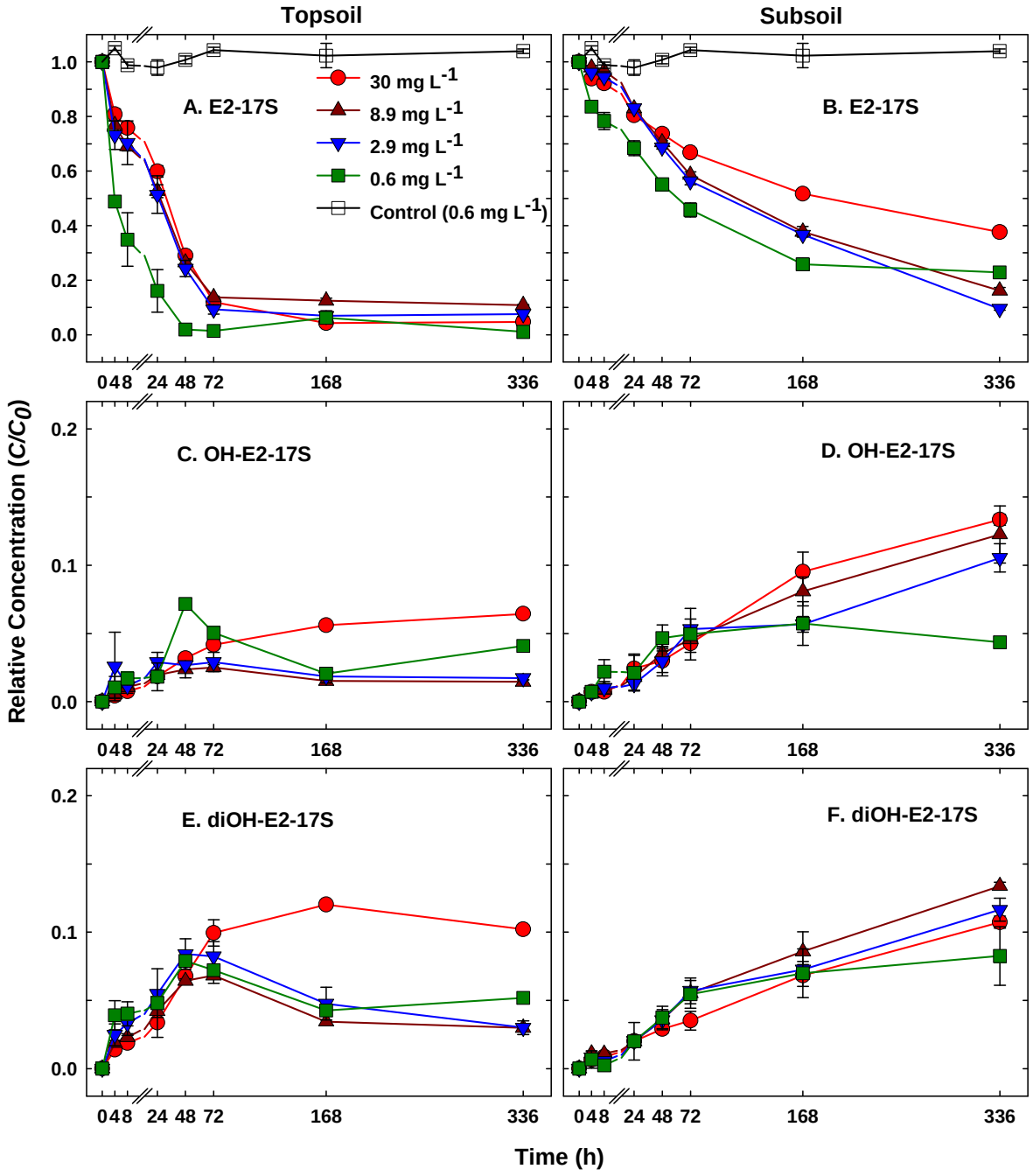

Figure 4. Aqueous concentration of [^{14}C]17β-estradiol-17-sulfate and its metabolites in topsoil and subsoil through time under different initial concentrations. The relative concentration represents the ratio between the measured concentration and the initial applied concentration of [^{14}C]17β-estradiol-17-sulfate in the present and other figures. Dotted symbols represent the average of three replicates of the measured data. Error bars represent one standard deviation.

Aqueous dissipation of E2-17S was greater for lower initial concentrations (Fig. 4A and 4B). Similar concentration-dependent dissipation in soils was observed for 17β-trenbolone acetate (Khan et al., 2008) and for E2-3G (Shrestha et al., 2012b), which is attributed to enzymatic saturability. In activated sludge, however, Chen and Hu (2009) reported that dissipation rates increased with increasing concentrations of E2, E2-3G, and E2-3S, which is attributed to the induction of greater microbial activities by the added substrate. These contrasting observations between the soil studies (current study, Khan et al., 2008; Shrestha et al., 2012b) and activated sludge study (Chen and Hu, 2009) may be caused by the greater biological activity, diversity, and capacity of the activated sludge compared to the soils.

Metabolite formation

Metabolites with higher polarity than E2-17S were detected in the aqueous phase, and were characterized as mono-hydroxy-E2-17S (OH-E2-17S) and di-hydroxy-E2-17S (diOH-E2-17S). The LC-MS/MS spectrum was consistent with the formation of OH-E2-17S ($[M-H]^-$ ion at m/z of 367.12 and fragments of 349.13 and 96.96) and diOH-E2-17S ($[M-H]^-$ ion at the m/z of 383.13 and fragments of 365.10 and 96.96); however, without standards, the position of the hydroxyl groups could not be determined definitively. The hydroxylated metabolites were already present at the first sample time of 4 h (Fig. 4C−4F), indicating that hydroxylation was a very rapid process.

The formation of hydroxylated E2-17S metabolites was likely caused by oxidation by soil enzymes (e.g. oxidases/hydroxylases). *In vitro* studies have demonstrated enzymatic processes that govern hydroxylation of E2, E2-17S, and other aromatic compounds. For example, hydroxylation of E2 and E2-17S at the C-2- or C-4- positions is catalyzed by microsomal cytochrome P450 enzymes harvested from rat (*Rattus norvegicus*) liver (Watanabe et al., 1991).

Also, during incubation with human placental microsomes in an NADPH-generating system, E2-17S is hydroxylated to 2- and 4-OH-E2-17S (Takanashi et al., 1993). Additionally, hydroxylation of E2-17S occurs when incubated with microsomes from female rat liver (Itoh et al., 2002). Outside of these *in vitro* studies, the present study appears to be unique in demonstrating hydroxylation of conjugated or free estrogens in soils. Soil microorganisms are reported to oxidize aromatic compounds to hydroxylated intermediates with mono- or di-oxygenases, which is then followed by ring-cleavage (Deveryshetty et al., 2007).

Sorbed phase observations

The total radioactive residue in the reversibly sorbed phase was relatively low for both soils (~15% of applied dose; Fig. 5). At the final sample time (336 h), the total radioactive residue in the irreversibly sorbed phase was greater than the reversibly sorbed phase for both the topsoil (irreversible ^{14}C = 70% of applied dose) and subsoil (irreversible ^{14}C = 45% of applied dose) (Fig. 5). Using the same soil type as the present study, Fan et al. (2007b) found that 73% of the applied radiolabelled E2 was irreversibly bound to soil (non-extractable fraction). The pH, CEC, and texture (Table 3) of two soils were similar, which indicated that the difference of radioactive residue levels in the sorbed phase likely resulted from the different OC content. Sorption of estrogens to soils and sediments is considered a hydrophobic interaction, with organic matter as the major sorption domain (Lee et al., 2003). Although the metabolites could not be characterized in the irreversible phase, irreversible sorption is due to interactions between the phenolic group of estrogenic compounds and humic acids and/or mineral surfaces to form hydrogen and covalent bonds (Yu et al., 2004).

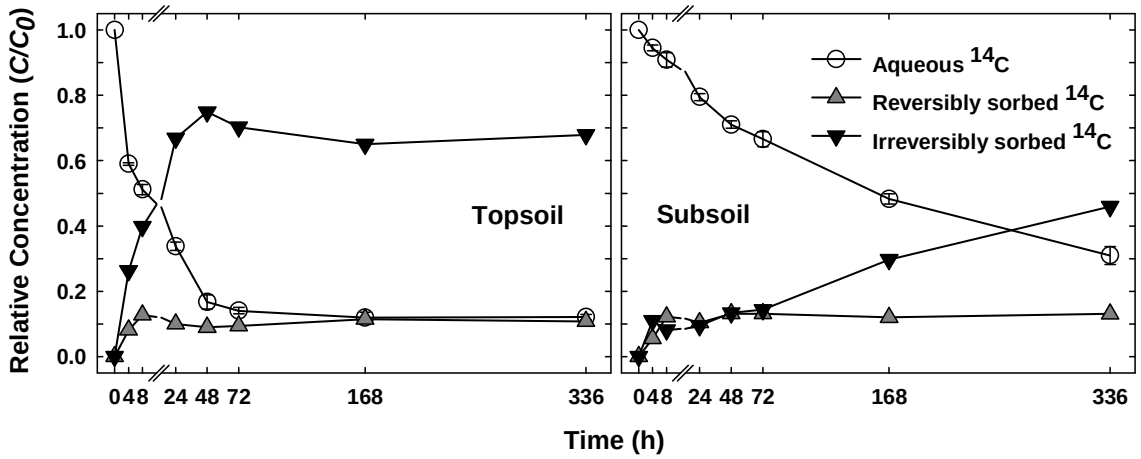

Figure 5. Distribution of radioactivity in the aqueous, reversibly sorbed, and irreversibly sorbed phase of topsoil and subsoil through time. The initial [^{14}C]17β-estradiol-17-sulfate concentration was 0.6 mg L^{-1}. Standard deviation error bars were available only for the aqueous data (n = 3), while the reversibly and irreversibly sorbed data were based on the "stop vials" (n = 1).

Compounds detected in the reversibly sorbed phase (extractable fraction) were diOH-E2-17S, OH-E2-17S, E2-17S, E2, estrone (E1), and an unknown metabolite (Fig. 6), in order of highest to lowest polarities based on the HPLC elution times. The most significant observation was the presence of free E2 and E1 in the sorbed phase, which were not detected in the aqueous phase. Although the measured concentrations of E2 and E1 were relatively low (~ 1% of applied dose; Fig. 6), these results demonstrated that E2-17S could be hydrolyzed to form free estrogens in agricultural soils. Additionally, an unknown metabolite that was more hydrophobic than E1 was consistently observed in the reversibly sorbed phase for both soils; however, it could not be characterized due to the low levels of recovery. This unidentified hydrophobic compound was present at lower concentrations in the topsoil compared to the subsoil throughout the entire experiment (0.7% vs. 1% of applied dose at 336 h; Fig. 6).

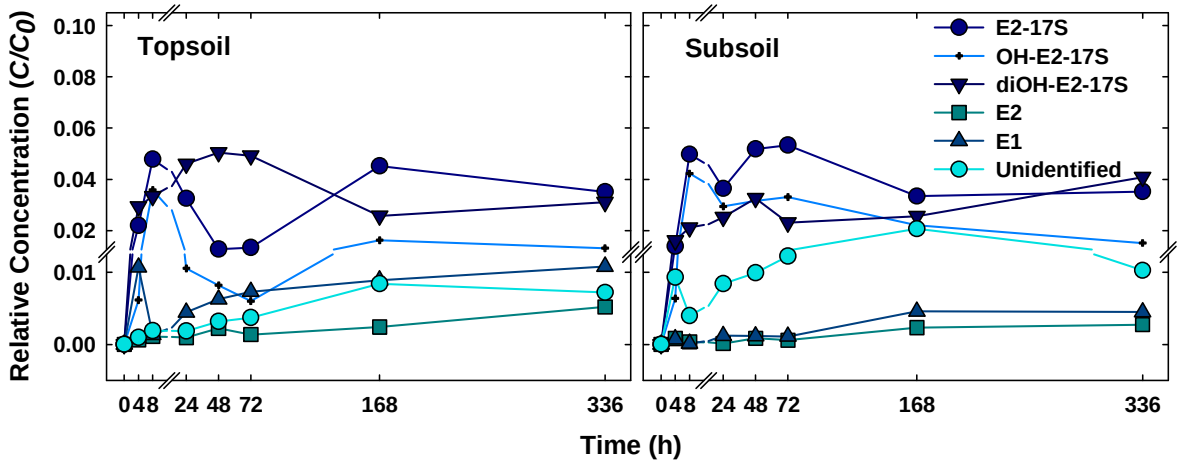

Figure 6. Reversibly sorbed concentration of [^{14}C]17β-estradiol-17-sulfate and its metabolites in topsoil and subsoil through time. Data were obtained from the "stop vials" with an initial [^{14}C]17β-estradiol-17-sulfate concentration of 0.6 mg L^{-1} (n = 1).

The observed E2 and E1 on the sorbed phase demonstrated that E2-17S was first hydrolyzed to form E2, which was subsequently oxidized to yield E1. Deconjugation of conjugate moieties is considered irreversible, and enzymes (i.e. sulfatases) are required to hydrolyze sulfate conjugates (Khanal et al., 2006). D'Ascenzo et al. (2003) found an acclimation period of 10 h was required to deconjugate E2-3S in domestic wastewater due to low inherent arylsulfatase activity. Also, arylsulfatase enzymes were found to be responsible for deconjugation of E2-3S in natural soils (Scherr et al., 2009). Arylsulfatases can distribute in the solid or aqueous phase of soils, which permits E1-3S deconjugation in both compartments (Scherr et al., 2008). In the present study, the deconjugated free estrogens, E1 and E2, were only observed on the sorbed phase, suggesting that sulfatase enzymes were only active on the solid phase. The oxidation of E2 into E1 was rapid, where E1 was detected without a lag period, occurring at the first sample time (4 h) (Fig. 6). As reported, oxidation of E2 is a fast process in soil with a half-life of 12 h (Colucci et al., 2001), and can occur on Mn-oxide reaction sites of soil surface (Sheng et al.,

2009). Because deconjugation of E2-17S appeared to be a sorbed-phase process, the proximity to surface Mn-oxide reaction sites may explain the observed immediate oxidation of E2 into E1. Additionally, the predominance of E1 compared to E2 (Fig. 6), was consistent with the observation that E1 is more stable than other naturally occurring estrogens in the environment (Hutchins et al., 2007).

Deconjugation/Hydrolysis

The present study observed that hydroxylation of E2-17S occurred approximately 10 times greater than deconjugation/hydrolysis. Similarly, deconjugation of other estrogen sulfates only occurred to a limited extent in sewage treatment systems and soils. For instance, an 8 h activated sludge and crude sewage batch study showed that aqueous concentrations of E1-3S and ethinylestradiol-3-sulfate remained between 87 and 94% of the applied dose, while free estrogens were only 3−7% of the applied dose (Gomes et al., 2009). Additionally, deconjugation of E1-3S and E2-3S was negligible in batch experiments with raw sewage and river water (Kumar et al., 2012). In a 10 d soil incubation study (sandy loam; OC = 1.1%; 25 °C), Scherr et al. (2009) found that the primary metabolite of E2-3S was E1-3S (55−68% of applied dose), indicating that oxidation was the predominant process. Deconjugation of E2-3S to yield E2 also occurred, but to a much lower extent compared to oxidation (2.7−3.5% of applied dose). Despite the different soil types and experimental designs, Scherr et al. (2009) and the current study both showed that deconjugation/hydrolysis was not the dominant metabolism pathway for E2-3S or E2-17S in agricultural soils.

In contrast to sulfate conjugates, the primary transformation pathway for the glucuronide conjugate, E2-3G, in the similar soil-water systems was deconjugation, which produced

maximum aqueous concentrations of E2 (18% of applied dose) within 24 h (Shrestha et al., 2012b). The different metabolism pathways of E2-17S and E2-3G in soils are consistent with observations in sewage systems that glucuronide conjugates were more susceptible to deconjugation than sulfate conjugates (D'Ascenzo et al., 2003; Gomes et al., 2009). In addition to difference in conjugate moieties (sulfate vs. glucuronide), the site of conjugation may also influence the metabolism pathways. Glucuronide conjugates with the moiety located on the D-ring are more resistant to hydrolysis than A-ring glucuronides (Gomes et al., 2009). The effect of conjugation sites would also explain the more rapid degradation of E2-3S (half-life of 1.5 h) compared to no degradation of 17β-estradiol-3,17-sulfate in batch studies of WWTP activated sludge (Okayasu et al., 2005). The recalcitrance of E2-17S to deconjugation in the present study can result from the relatively stable sulfate moiety compared to glucuronide, as well as the conjugation site, i.e. C-17.

Conclusions

The objective of this study was to investigate the fate of E2-17S in natural agricultural soils, under the context of understanding the fate and transport of manure-borne estrogens. The results showed that soil OC content significantly influenced the aqueous dissipation of E2-17S. Additionally, hydroxylation was found to be the primary transformation pathway of E2-17S. Deconjugation/hydrolysis of E2-17S did occur, but it was a minor transformation pathway compared to hydroxylation, with only low concentrations of free estrogens (E2 and E1) being released on the reversibly sorbed phase. Although, E2-17S was more persistent than the glucuronide conjugate (E2-3G), it has a lower potential of releasing free estrogens to the environment. Nonetheless, due to the large amounts of manure-borne estrogen conjugates arising from AFOs, even 1% of free estrogens deconjugated from E2-17S may result in environmental

levels higher than the LOAEL. Therefore, the relatively stable estrogen conjugate, E2-17S, cannot be excluded as a precursor of free estrogens in the environment.

Acknowledgements

The authors greatly thank Mrs. Colleen Pfaff (Biosciences Research Laboratory, USDA-ARS, Fargo, ND) and Mr. Nathan Derby (Soil Science, North Dakota State University, Fargo, ND) for their contributions on collecting data. This project is supported by Agriculture and Food Research Initiative Competitive Grant No. 2010-65102-20400 from the USDA National Institute of Food and Agriculture. The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the USDA, the Agricultural Research Service, or the Food Safety and Inspection Service of any product or service to the exclusion of others that may be suitable. USDA is an equal opportunity provider and employer.

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PAPER 3. SORPTION AND METABOLISM OF 17ß-ESTRADIOL-17-SULFATE IN STERILIZED SOIL-WATER SYSTEMS

Abstract

Significant amounts of manure-borne estrogens are found in their conjugated forms, which can release endocrine-disrupting free estrogens in the environment via deconjugation and become a threat to the aquatic wildlife. To identify the abiotic processes governing the fate of a sulfate conjugated estrogen, 17β-estradiol-17sulfate (E2-17S), soil batch experiments were conducted to investigate the dissipation, sorption, and metabolism of radiolabeled E2-17S under sterilized conditions. The aqueous dissipation half-lives (DT_{50}) for E2-17S ranged from 2.5 to 9.3 h for the topsoil of high organic carbon (OC) content, but E2-17S remained at ~80% of applied dose in the low OC subsoil for the 14 d duration. The non-linear sorption isotherms indicated limited sorption of E2-17S, and the concentration-dependent log K_{OC} values were 2.20 and 2.45 for the topsoil and subsoil, respectively. Additionally, two types of hydroxylated E2-17S were found as major metabolites in the aqueous phase, which represented 9−25% and 6−7% of applied dose for the topsoil and subsoil at 14 d, respectively. Free estrogens (i.e. 17β-estradiol (E2) and estrone (E1)) were only detected from the sorbed phase. These results demonstrated that E2-17S underwent complex abiotic metabolism pathways; and the sorption and hydroxylation processes governed the aqueous dissipation of E2-17S predominantly.

Introduction

In the United States, animal feeding operations (AFOs) produce about 453 million Mg yr^{-1} of manure (Kellogg et al., 2000), which can be land-applied as a valuable source of fertilizer and organic matter. However, manure-borne estrogenic hormones can pose a potential risk to aquatic

wildlife (Burkholder et al., 2007; Thorne, 2007). It is estimated that AFOs contribute 90% of total estrogens in the environment (Maier et al., 2000). Cattle (*Bos taurus*), swine (*Sus scrofa*), and poultry (*Gallus gallus*) produce 45, 0.8, and 2.7 Mg yr^{-1} of total estrogens in the United States, respectively (Lange et al., 2002). Despite steroidal estrogens possessing relatively low water solubilities and high sorption affinities to soil and sediment, these compounds are frequently detected in surface and subsurface water systems (Shore et al., 1993; Nichols et al., 1997; Peterson et al., 2000; Kolpin et al., 2002; Kolodziej et al., 2004). For example, the most potent free estrogen 17β-estradiol (E2) was detected at a frequency of 37% and at concentrations up to 1910 ng L^{-1} throughout a 2-m soil profile in an agricultural field where liquid swine manure was applied (Schuh et al., 2011a).

Swine, poultry, and cattle excrete approximately 96, 69, and 42% of estrogens as conjugates, respectively (Hanselman et al., 2003). In dairy waste lagoons, 57% of the total estrogens were detected as conjugates, while in poultry lagoons, nearly all (95%) estrogens were conjugates (Hutchins et al., 2007). Conjugated estrogens are not estrogenic because they cannot bind to estrogen receptors; however, they may hydrolyze (i.e. deconjugate) to form endocrine-disrupting free estrogens (Khanal et al., 2006). Compared to the free estrogens, conjugated estrogens have greater aqueous solubility, which potentially enhances their mobility in the environment. The role that estrogen conjugates play in contributing free estrogens to the environment is not well understood, especially under the context of manure management.

A key research question about estrogen conjugates is the uncertainty of whether and how much deconjugation occurs in the environment. Most existing studies are limited to the field-monitoring of conjugate levels in influent and effluent discharges of sewage systems, or in manure storage systems and surface runoff to indirectly assess the behavior of estrogen

conjugates. Comprehensive studies on fate and transport of estrogen conjugates are not widely published. Little information is available on the fundamental processes governing the fate of estrogen conjugates in agricultural soils. The few soil microcosm studies (Scherr et al., 2008, 2009a) in the literature indicated that the sulfate estrogen conjugates, estrone-3-sulfate (E1-3S) and 17β-estradiol-3-sulfate (E2-3S), could hydrolyze and serve as precursors to free estrogens, and that the degradation rates were correlated to soil biological activity. Compared to E1-3S and E2-3S, 17β-Estradiol-17-sulfate (E2-17S) was detected at higher concentrations in swine lagoons (i.e. 87 ng L^{-1}) (Hutchins et al., 2007) and surface runoff (i.e. 107 ng L^{-1}) after poultry litter application (Dutta et al., 2012). Moreover, in swine lagoons, E2-17S was found to be more stable and persistent than glucuronide conjugates (Hutchins et al., 2007). To determine the fate and transport of E2-17S in natural agricultural soils, Bai et al. (2013) found that hydroxylation was the primary metabolism process of E2-17S, and that deconjugation occurred to a lower extent than hydroxylation.

Since the previous study (Bai et al., 2013) was conducted using biologically active soils, it was unclear whether the governing processes were biotic or abiotic. One of the objectives of the present study was thus to identify the abiotic processes involved in the fate and transformation of E2-17S using batch experiments under sterilized conditions. Additionally, little is known about the sorption capacities of estrogen conjugates to soil so far. The interactions of estrogen conjugates with soils would impact their entry into aquatic systems and subsequent fate. Therefore, another objective of this study was to evaluate the physicochemical sorption affinity of E2-17S to soil.

Materials and Methods

Analytical methods

The radiolabeled [^{14}C]E2-17S used in the experiments was synthesized (Shrestha et al., 2011) from [4-^{14}C]E2 (American Radiolabels, Inc.; St. Louis, MO) and possessed a specific activity of 241.8 Bq μg^{-1} and a radiochemical purity of 98%. All analytical methods followed the radiometric methods developed by Shrestha et al. (2012a) to measure the concentrations of E2-17S and its metabolites. Briefly, high performance liquid chromatography (HPLC; Beckman Coulter Inc., Fullerton, CA) was used to separate metabolites. To quantify metabolites, the fraction-collected HPLC effluent was measured for radioactivity by liquid scintillation counting (LSC; Packard 1900CA Downers Grove, IL). Liquid chromatography with tandem mass spectroscopy (LC-MS/MS; Waters, Beverly, MA) was used in negative-ion mode (ESI^-) to characterize metabolites. The analytical procedures and conditions for HPLC, LSC, and LC-MS/MS have been described previously (Shrestha et al., 2012a; Bai et al., 2013).

Soil batch experiments

The soils selected in this study were from a Hecla-Hamar Series, loamy fine sand (sandy, mixed, frigid Oxyaquic Hapludolls), and were used in previous laboratory (Fan et al., 2007b; Zitnick et al., 2011; Shrestha et al., 2012b) and field (Thompson et al., 2009; Schuh et al., 2011a; Schuh et al., 2011b) studies on the fate and transport of manure-borne hormones. The soil samples were collected from two depths, topsoil (0–15 cm) and subsoil (46–61 cm), which were similar in all properties except organic carbon (OC) content (Table 3). Because liquid swine manure is applied to the top 150 cm of the soil (Schuh et al., 2011a), these soil depths are affected by manure-borne estrogens and estrogen conjugates. The soil samples were air-dried

and then passed through a 2-mm sieve. To determine metal oxides (manganese oxides) in the soil samples, dissolved and reducible manganese was measured using hydroxylamine hydrochloride ($NH_2OH \cdot HCl$) with procedures as developed by Chao et al. (1972).

To conduct soil batch experiments, 1.6 g soil and 8 mL of 0.01 M $CaCl_2$ solution were added into 10 mL glass vials in triplicate, and then sealed and irradiated for 14 h using a 7.6 kGy gamma source (M38-4 Gammator, Radiation Machinery Corp., Parsippany, NJ). Gamma-irradiation dose of 1 kGy can kill all fungi and bacteria in per gram soil (Jackson et al., 1967); thus the irradiation dose of 7.6 kGy was sufficient to sterilize the soil samples. The [^{14}C]E2-17S solvated in 10 μL methanol was injected into the gamma-irradiated soil-water slurries using a sterile syringe at different initial concentrations, i.e. 3.2, 7.4, and 18 mg L^{-1}. Controls containing only $CaCl_2$ solution but no soil were also sterilized before adding with 0.5 mg L^{-1} of [^{14}C]E2-17S. Additionally, a separate set of batch vials was prepared and gamma-irradiated in order to perform destructive solid phase analysis, which was labeled as "stop vials" with 0.5 mg L^{-1} of [^{14}C]E2-17S applied. The [^{14}C]E2-17S concentrations in these studies were higher than conjugate concentrations normally detected in the environment; however, they were necessary to ensure reliable analytical detection and quantification of the radiolabeled compounds (Shrestha et al., 2012a; Bai et al., 2013).

The batch vials were agitated by rotation from top to bottom (360° every 5 s) for 14 d (336 h) at room temperature (23 ± 1 °C). At 4, 8, 24, 48, 72, 168, and 336 h, the batch vials were centrifuged (300 × g) in a centrifugal rotary evaporator (Savant, Farmington, NY). Aliquots of 100 and 150 μL were sampled from the aqueous phase using a sterile syringe, respectively. The 100 μL aliquots were analyzed for total radioactivity by LSC, and the 150 μL aliquots were preserved with formaldehyde (2.7% of final volume), filtered, and frozen at −20 °C until further

HPLC and LSC analyses. Additionally, one "stop vial" was removed at each sampling, preserved with formaldehyde, centrifuged, decanted, and then frozen until further analysis. The solid phase analysis was only performed at 336 h for the higher dose groups (3.2, 7.4, and 18 mg L^{-1}). For solid phase analysis, the preserved soil samples were extracted with water (5 mL × 3) and then acetone (5 mL × 3) by 30 min sonication. The water extracts were then evaporated with the centrifugal rotary evaporator, the acetone extracts were dried under nitrogen, and both extracts were resolvated in methanol for metabolite characterization. Soil non-extractable (irreversibly sorbed) radioactivity was determined by combustion (5 × 0.1 g) using a Packard Model 307 Oxidizer (Downers Grove, IL) after air-drying.

Data handling

First-order kinetics

In order to estimate the dissipation rate constants and dissipation times of E2-17S in the aqueous phase, the measured data were fitted with a first-order kinetic model:

$$C/C_0 = e^{-kt} \quad [2]$$

where C/C_0 is the relative concentration of [^{14}C]E2-17S, t (h) is time, and k (h^{-1}) is the first-order dissipation rate constant of [^{14}C]E2-17S. The time required to dissipate 50% (DT_{50}) and 90% (DT_{90}) of [^{14}C]E2-17S from the aqueous phase was calculated using the first-order dissipation rate constant ($DT_{50} = \ln 2/k$; $DT_{90} = \ln 10/k$).

Sorption isotherm

Freundlich sorption isotherms were used to determine sorption coefficients for [^{14}C]E2-17S as follows:

$$S = K_f C^N \quad [3]$$

where S (mg Kg^{-1}) is the reversibly sorbed (extractable) concentration of the applied [^{14}C]E2-17S, C (mg L^{-1}) is the aqueous concentration of [^{14}C]E2-17S, K_f ($mg^{1-N}\ L^N\ Kg^{-1}$) is Freundlich sorption coefficient, and N is an unitless empirical constant. If $N < 1$, then the sorption is limited; and if $N > 1$, then the sorption is limitless. When sorption is non-linear, it is not useful to calculate or compare the corresponding linear sorption coefficients; furthermore, the Freundlich coefficients K_f are dependent on the N values, making comparisons of K_f values between soils unsuitable (Sarmah et al., 2008). Herein, a simplified method (Sarmah et al., 2008; Scherr et al., 2009b) was used to calculate the concentration-dependent effective partitioning coefficient K_d^{eff} (L Kg^{-1}) and the corresponding OC-normalized partitioning coefficient K_{OC} (L Kg^{-1}) for [^{14}C]E2-17S as follows,

$$K_d^{eff} = K_f C^{N-1} \quad [4]$$

$$K_{OC} = K_d^{eff} / f_{OC} \quad [5]$$

where f_{OC} is the fraction of soil OC content. In this case, K_{OC} is equivalent to a single-point OC-normalized partitioning coefficient, which was determined at the initial concentration of 0.5 mg L^{-1} of [^{14}C]E2-17S.

Statistical analysis

The models (Eq. [2]−[3]) were fitted using the least-squares regression with equal weighting across all data points (SigmaPlot®, version 6.00 SPSS Inc., Chicago, IL). The software, JMP (version 9.0.2 SAS Institute Inc., Cary, NC), was used for all statistical analysis. Significance of main effects and interactions were determined using analysis of variance (ANOVA). Tukey's and Student's *t*-tests were used to determine significance between levels. For all analysis, an α of 0.05 was used, and a probability of $p \leq 0.05$ was considered significant.

Results and Discussion

Dissipation from aqueous phase

The parent compound remained stable in the controls with no soil (Fig. 7a and 7b), and no metabolites were detected, demonstrating that neither sorption to the batch vials nor metabolism had occurred. For the sterile topsoil (Fig. 7a), E2-17S dissipated rapidly from the aqueous phase with less than 10% of the applied dose remaining after 48 h for all initial concentrations. For the sterile subsoil (Fig. 7b), the aqueous concentrations of E2-17S remained ~80% of the applied dose at the end of the batch experiments. The disparate behaviors of E2-17S in the topsoil and subsoil demonstrated the important role that OC played in the aqueous dissipation of E2-17S. Similar soil OC effects on the fate of estrogen sulfates have been reported by other studies. In soils with higher OC, estrogen sulfates have shorter dissipation half-lives due to the greater sorption and transformation (Scherr et al., 2008, 2009a; Bai et al., 2013).

First-order dissipation rate constants (k) (Table 5) were inversely correlated to the initial concentration of E2-17S for the topsoil. Compared to the current sterile soil (DT_{50} = 2.5−9.3 h) (Table 5), the concentration-dependent DT_{50} values for E2-17S were approximately 3−8 times greater for non-sterile soil (DT_{50} = 21−26 h) (Bai et al., 2013). The slower dissipation of E2-17S in non-sterile soil than sterile soil is likely due to less sorption, which can be caused by living soil microorganisms that clog soil pores and reduce sorption capacity and accessibility of organic compounds (Bellin and Rao, 1993).

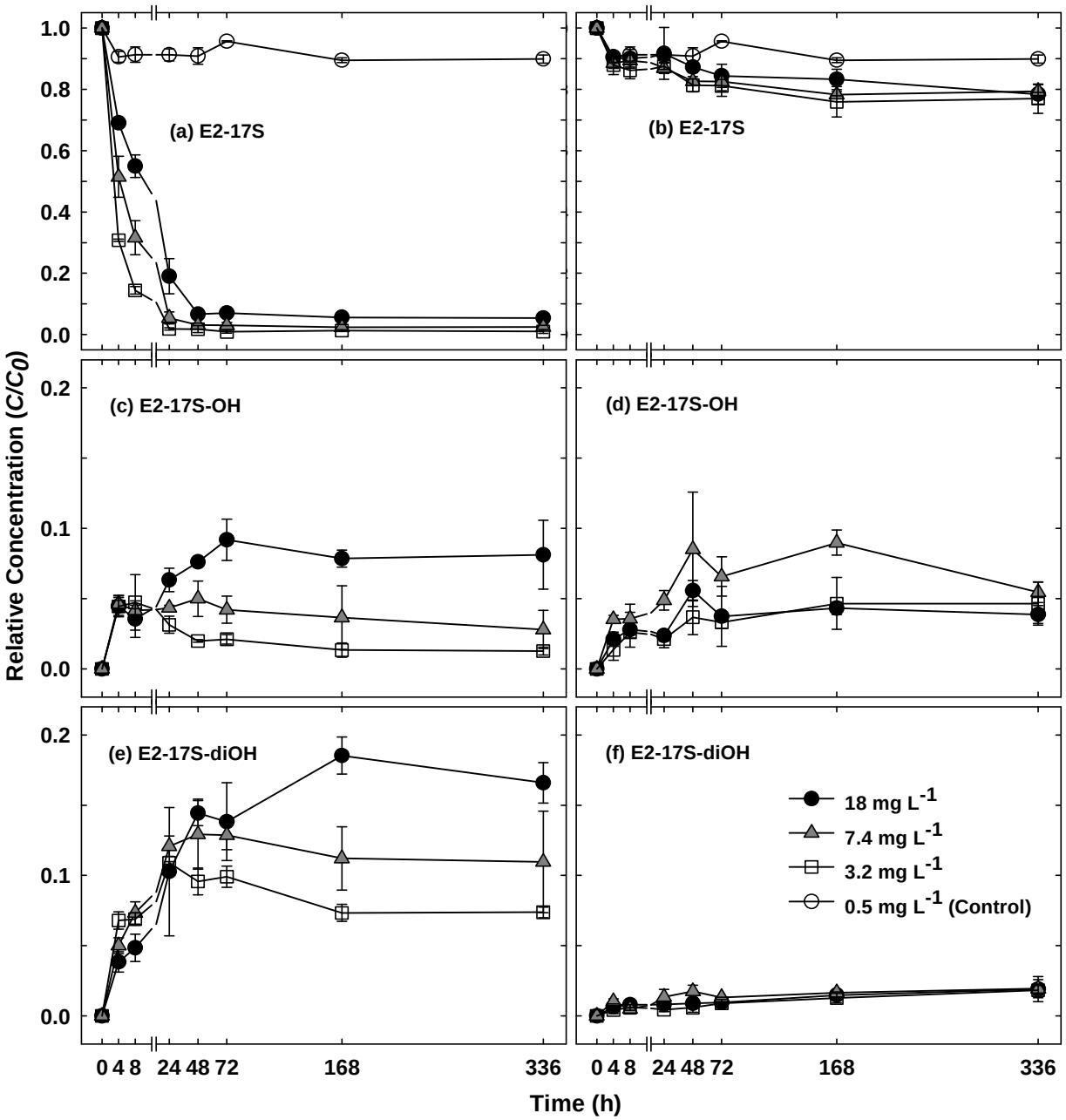

Figure 7. Aqueous concentration of [^{14}C]17β-estradiol-17-sulfate and its metabolites in topsoil (left) and subsoil (right) through time with multiple initial concentrations. The relative concentration represents the ratio of measured concentration to the initial concentration of [^{14}C]17β-estradiol-17-sulfate. Data represent average from three replicates. Error bars represent standard deviation. Controls represent batch vials with no soil.

Table 5. First-order parameter estimates with standard error (SE) for [^{14}C]17β-estradiol-17-sulfate in the topsoil with multiple initial concentrations.

Concentration [a] (mg L^{-1})	k (h^{-1} ± SE)	r^2	DT_{50} (h)	DT_{90} (h)
18	0.074 (0.009)	0.984	9.3	31
7.4	0.152 (0.010)	0.994	4.6	15
3.2	0.276 (0.014)	0.997	2.5	8.3

[a] Temporal aqueous concentrations of [^{14}C]17β-estradiol-17-sulfate could not be determined for the initial concentration of 0.5 mg L^{-1} due to the lower than detection limit levels.

Sorption isotherms

At 336 h, sorption equilibrium appeared to be reached for both soils as indicated by the steady-state aqueous concentrations of E2-17S (Fig. 7a and 7b). Also, the temporal distribution of radioactive residue on the sorbed phase showed equilibrium at 336 h (Fig. 8). Irreversible sorption (non-extractable fraction) was observed more dominant compared to reversible sorption (extractable fraction) for the topsoil. Irreversible sorption is associated with the naturally occurring organic matter of soil, i.e. humic substances (Fan et al., 2007b), thus the more abundant humic substances in the topsoil would have stronger irreversible sorption compared to the subsoil.

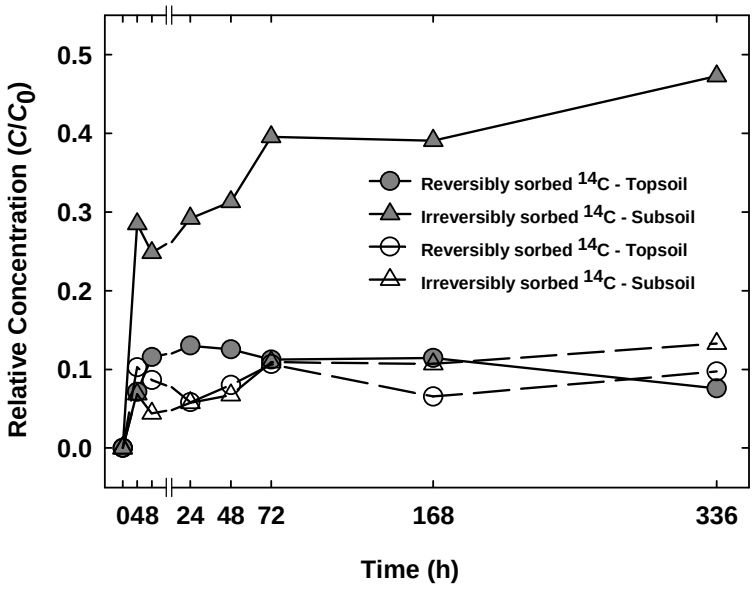

Figure 8. Radioactivity recovered from the sorbed (reversible and irreversible) phase of topsoil and subsoil through time with initial concentration of 0.5 mgL^{-1} for [^{14}C]17β-estradiol-17-sulfate (n = 1).

The total radioactive recoveries (Table 6) were acceptable except for the lowest initial concentration of E2-17S (0.5 mg L^{-1}). The relatively low radioactive recovery may result from incomplete combustion of the soil (Fan et al., 2007b). Sorption isotherms (Fig. 9) were constructed from directly measured concentrations of E2-17S at 336 h; where the aqueous concentrations ranged from 0.005 to 14.8 mg L^{-1}, and the sorbed concentrations were 0.125 to 4.01 mg Kg^{-1}. The Freundlich model provided an excellent fit for both soils as indicated by the high r^2 values, and the isotherms were non-linear as indicated by the *N* values (Table 7). The smaller than unity *N* values demonstrated limited sorption for E2-17S to both soils, which corresponded well with previous studies for other estrogen sulfates. The reported *N* values for E1-3S and E2-3S sorption to sterile activated sludge were 0.739 and 0.736, respectively (Chen and Hu, 2010). Sorption of E1-3S to agricultural soils resulted in *N* values ranging from 0.886 to

0.932 (Scherr et al., 2009b). The limited sorption to soil can be due to limited sorption sites within the soil organic matter domain (Yu et al., 2004).

Table 6. Summary of total radioactivity recovered at 336 h from the applied [^{14}C]17β-estradiol-17-sulfate in the topsoil and subsoil with multiple initial concentrations. (Average ± standard deviation (SD)).

Concentration (mg L^{-1})	Total ^{14}C recovery (% ± SD)	
	Topsoil	Subsoil
18	94 ± 6	97 ± 0
7.4	93 ± 2	99 ± 4
3.2	84 ± 5	99 ± 4
0.5	72 ± 9	73 ± 6

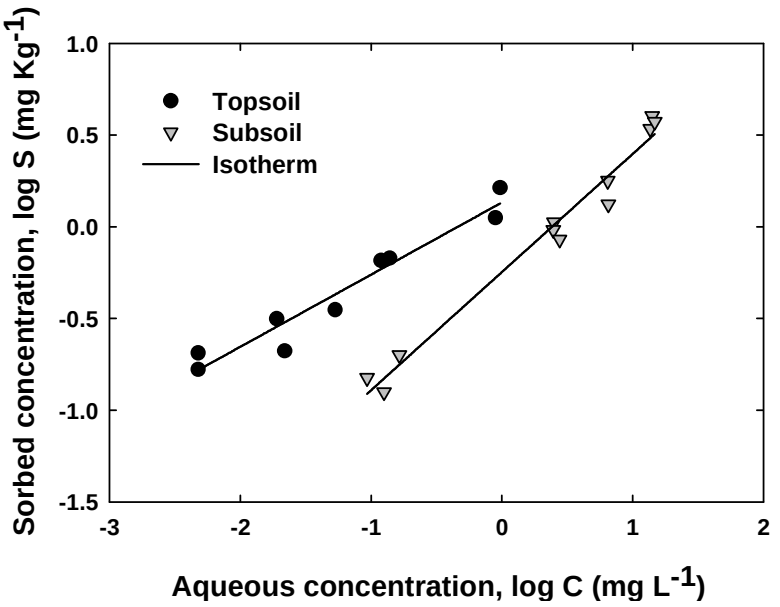

Figure 9. Freundlich sorption isotherms for [^{14}C]17β-estradiol-17-sulfate at contact time of 336 h for topsoil and subsoil.

The concentration-dependent log K_{OC} values (Table 7) of E2-17S for the topsoil and subsoil were comparable to the reported values for E1-3S, i.e. 1.73–2.08 (C = 0.25 mg L^{-1}) (Scherr et al., 2009b). Additionally, the free estrogen E2 had a concentration-dependent log K_{OC} value of 3.12

(C = 0.5 mg L^{-1}) (Sarmah et al., 2008). The nearly one order of magnitude smaller log K_{OC} values of E2-17S showed a reduced sorption affinity compared to its free counterpart E2. The octanol–water partitioning coefficient log K_{OW} value of E2-17S is 1.59 (calculated by the Windows-based software KOWWIN) (Tetko et al., 2005), which is slightly higher than E1-3S (0.95); while the log K_{OW} value for E2 is reported to be 3.94 (Lai et al., 2000). Considering the less hydrophobic nature of E2-17S, it is expected to have weaker hydrophobic interaction to soil organic matter compared to E2. However, the moderate sorption capacities of estrogen sulfates to soils observed in the present and previous study (Scherr et al., 2009b) suggested that other unknown interactions with soil organic matter and clay minerals, e.g. ligand binding, hydrogen, and covalent bonding might also occur.

Table 7. Freundlich isotherm parameters with standard error (SE) for [^{14}C]17β-estradiol-17-sulfate at contact time of 336 h for the topsoil and subsoil. The concentration-dependent equivalent partitioning coefficient K_d^{eff} (L Kg^{-1}) and log K_{OC} were determined at 0.5 mg L^{-1} of [^{14}C]17β-estradiol-17-sulfate.

	K_f ± SE ($mg^{1-N} L^N Kg^{-1}$)	N ± SE	r^2	K_d^{eff} (L Kg^{-1})	log K_{OC}
Topsoil	1.36 ± 0.055	0.394 ± 0.037	0.942	2.07	2.20
Subsoil	0.566 ± 0.029	0.644 ± 0.033	0.976	0.725	2.45

Metabolism pathways

Hydroxylation

Two types of hydroxylation products were found as major metabolites in the aqueous phase of both the sterile topsoil and subsoil (Fig. 7c−7f), which were characterized as OH-E2-17S and diOH-E2-17S by the LC-MS/MS analysis. The LC-MS/MS spectrum possessed an $[M-H]^-$ ion at m/z of 367.12 and fragments of 349.13 and 96.96 that consistent with OH-E2-17S; and an [M-

H]$^-$ ion at the m/z of 383.13 and fragments of 365.10 and 96.96 to confirm diOH-E2-17S. The hydroxyl positions could not be determined for the metabolites because no standards were available. Under the current sterilized conditions, abiotic hydroxylation of E2-17S catalyzed by metal oxides is likely to be a major mechanism. In soils, naturally occurring Mn and Fe oxides/hydroxides and smectite clays are known as potential oxidizing agents. Particularly, Mn oxides are believed to be among the strongest oxidizing agents that may be encountered in the absence of molecular oxygen (Laha and Luthy, 1990). Numerous studies have demonstrated that MnO_2 can oxidize phenols (Lin et al., 2009), aromatic amines (Li et al., 2003), and estrogens (de Rudder et al., 2004; Xu et al., 2008; Sheng et al., 2009) at neutral pH. Moreover, MnO_2 can oxidize E2 in aqueous solution to yield 2-OH-E2 as a final product in addition to E1 under neutral pH and room temperature (Jiang et al., 2009). According to the reducible Mn levels (Table 3) measured in the present two soils, Mn oxides were abundant and likely played a significant role in the rapid hydroxylation of E2-17S.

The aqueous concentrations of the hydroxyl metabolites (Fig. 7c−7f) increased rapidly within the first 72 h followed by an apparent steady-state or slight decline in most cases. During MnO_2 oxidation, the released Mn^{2+} can bind to the MnO_2 surface (Li et al., 2003; Jiang et al., 2009), and the active reaction sites are occupied and become unavailable for E2-17S so that the hydroxylation rates are reduced at late stages of the batch experiments. In addition, higher concentrations of the hydroxyl metabolites were found in the topsoil compared to the subsoil. For example, at 336 h the two hydroxyl E2-17S metabolites were detected at 9−25% and 6−7% of the applied dose for the topsoil and subsoil, respectively (Fig. 7c−7f). The greater hydroxylation in the topsoil is likely due to the enhanced MnO_2 oxidation by the more abundant humic substances compared to the subsoil (Xu et al., 2008). Furthermore, Jiang et al. (2009)

proposed a mechanism of E2 to form 2-OH-E2, from which a plausible hydroxylation pathway for E2-17S to form OH-E2-17S can be derived (Fig. 10).

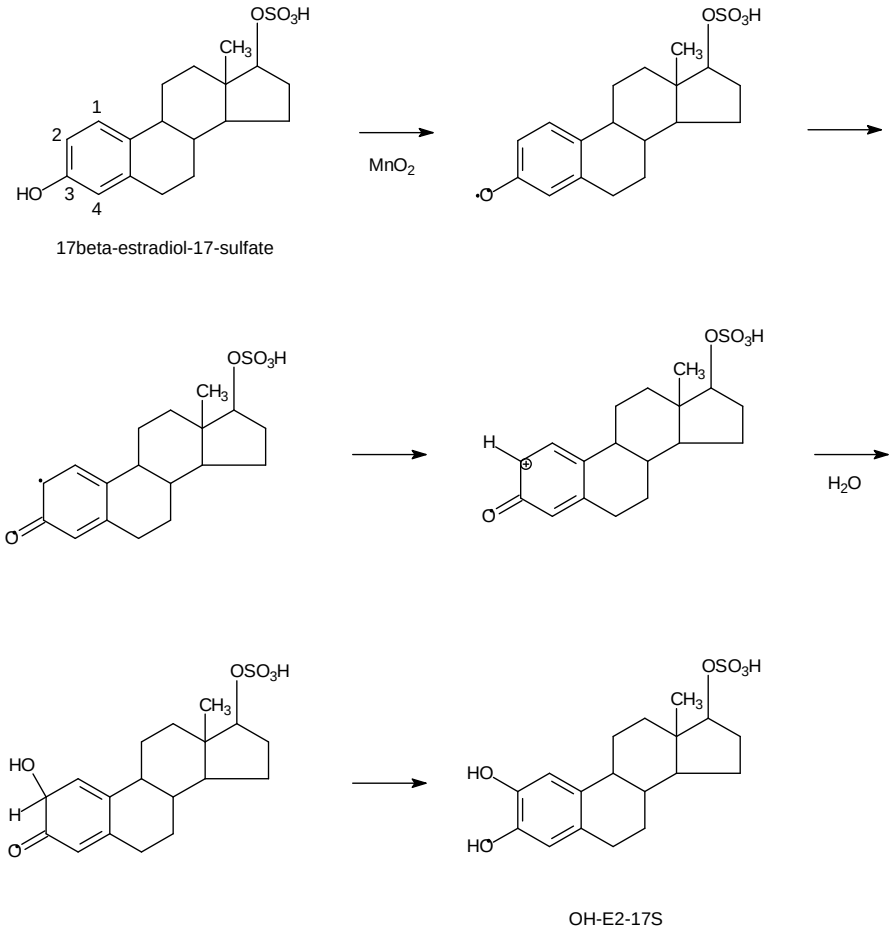

Figure 10. Proposed transformation pathway and estimated intermediate structures for hydroxylation of [^{14}C]17β-estradiol-17-sulfate.

Similarly, a hydroxyl was assumed to be attached to the unsaturated phenolic ring of E2-17S. Adsorption to MnO_2 surface to form a precursor complex is the initial step for the oxidation of substrates (Li et al., 2003). The proposed pathways include: 1) the hydroxyl (C-3) of E2-17S loses an electron and is subsequently oxidized into a free radical; 2) the free radical is transferred to the adjacent site (C-2); and 3) after the combination with water and a loss of hydrogen, 2-OH-E2-17S is formed.

Hydroxylation of E2-17S catalyzed by other metal oxides is also possible. Oxidation by Fe oxides are much less thermodynamically favorable than by Mn oxides in an aerobic environment; and Fe oxides generally do not play a significant role in redox processes until the environment becomes sufficiently reducing (Li et al., 2003). Many studies have found that Fe(III) is reduced to release Fe(II) during soil irradiation (Gournis et al., 2000; Bank et al., 2008), but soil Mn levels do not change much at irradiation doses of 2–10 kGy (McNamara et al., 2003). Li et al. (2003) also suggested that oxidation of aromatic amines by Mn would occur first, but the contribution of Fe was plausible. As a result, Fe may participate in the hydroxylation of E2-17S when the thermodynamic condition is favorable. Another possible mechanism causing hydroxylation of E2-17S is enzyme catalysis. Enzymes may remain active for weeks in soil after irradiation (McNamara et al., 2003), and extracellular enzymes can be bound to and protected by soil colloids (Lensi et al., 1991). Herein, it cannot be ruled out that lysed enzymes in the irradiated soil-water systems were still active.

Deconjugation

Free estrogens E2 and E1 were observed in the reversibly sorbed phase of the soil (Fig. 8), but they were not found in the aqueous phase. The free E2 and E1 were measured up to 4.0% and 12% (data not shown) of the soil extractable radioactivity from the “stop vials”, respectively. The presence of free estrogens demonstrated that deconjugation of E2-17S occurred in the sterilized soils. Deconjugation of E2-17S is a hydrolysis process that may be promoted by clay minerals. Numerous studies have reported that clay surfaces, especially smectites, are effective in catalyzing hydrolysis of organic pollutants, e.g. pesticides (Sanchezcamazano and Sanchezmartin, 1991; Pusino et al., 1996). After hydrolysis of E2-17S, the released E2 was subsequently oxidized into E1, which has been observed in similar sterilized soil-water systems

previously (Zitnick et al., 2011; Shrestha et al., 2013) and can be attributed to MnO_2 oxidation (Sheng et al., 2009). Given the relatively hydrophobic nature, the released free E2 and E1 tend to bind to smectite clay and/or metal oxide surfaces, which explains no detections of the free estrogens from the aqueous phase and the residue radioactivity in the irreversibly sorbed phase of the soil. In addition, the HPLC analysis indicated the presence of an unknown metabolite with polarity between E2-17S and E2. Although the metabolite could not be characterized due to the low recovered radioactivity, it can be presumed to be hydroxyl E2, another product of E2 oxidation that has been identified previously (Jiang et al., 2009).

Conclusions

The present study investigated the sorption and metabolism processes that govern the dissipation of E2-17S in sterilized soil-water systems. Sorption of E2-17S to the sterile soils was found limited with a reduced sorption affinity compared to E2, which explains the frequent detection and high levels of E2-17S in surface runoff nearby agricultural fields with manure application. In real-world conditions, soil microorganisms and manure-borne chemicals would compete for sorption sites within soil organic matter, which may further limit the sorption capacity and enhance the mobility of E2-17S. The present sorption parameters can be useful information to serve in fate and transport modeling and risk assessment studies related to estrogens and estrogen conjugates.

In the sterilized soil-water systems, E2-17S underwent complex metabolism pathways forming multiple metabolites, e.g. hydroxyl metabolites, E2, and E1. These results demonstrate that soil microorganisms do not necessarily play a role in the transformation of E2-17S. Abiotic transformations for E2-17S can be attributed to manganese oxides and clay surfaces promoted reactions. The present and previous study (Bai et al., 2013) both imply that manure-borne E2-

17S may not be a major contributor to the frequently detected free estrogens in the surface and subsurface soil systems after liquid manure application.

Acknowledgments

The authors sincerely thank Mrs. Colleen Pfaff (Biosciences Research Laboratory, USDA-ARS, Fargo, ND) and Mr. Nathan Derby (Soil Science, North Dakota State University, Fargo, ND) for their assistance on collecting data. We would like to acknowledge Dr. Jane Schuh (Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo, ND) for her support on the soil sterilization procedure. This project is supported by Agriculture and Food Research Initiative Competitive Grant No. 2010-65102-20400 from the USDA National Institute of Food and Agriculture. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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PAPER 4. MODELING COUPLED SORPTION AND TRANSFORMATION OF 17ß-ESTRADIOL-17-SULFATE IN SOIL-WATER SYSTEMS

Abstract

Animal manure is a primary source of exogenous free estrogens in the environment, which are known to be endocrine-disrupting chemicals. Conjugated estrogens can act as precursors to free estrogens, which may increase the total estrogenicity in the environment. In this study, a comprehensive model was used to simultaneously simulate the coupled sorption and transformation of a sulfate estrogen conjugate, 17β-estradiol-17-sulfate (E2-17S), in various soil-water systems. The simulated processes included multiple transformation pathways (i.e. hydroxylation, hydrolysis, and oxidation) and mass transfer between the aqueous, reversibly sorbed, and irreversibly sorbed phases of the soils for E2-17S and its metabolites. The conceptual model was conceived based on a series of linear sorption and first-order transformation expressions. The model was inversely solved using finite difference to estimate process parameters. A global optimization method was applied for the inverse analysis along with variable restrictions to estimate the total 36 parameters. The model provided a satisfactory simulation (R^2_{adj} = 0.93 and d = 0.87) to the experimental data and reliable parameter estimates. The modeling study improved the understanding of fate and transport of estrogen sulfate conjugate under various soil-water conditions.

Introduction

Estrogenic hormones are excreted from human and animals as intact molecules, which are mostly in the forms of glucuronide and sulfate conjugates (Johnson and Williams, 2004). Unconjugated or free estrogenic hormones are known as potent endocrine-disrupting chemicals,

because they can cause adverse effects to reproduction systems of aquatic wildlife at part-per-trillion levels (Jobling et al., 1998; Panter et al., 1998; Irwin et al., 2001). Estrogen conjugates (glucuronide and sulfate) can act as precursors to free estrogens and increase the total estrogen load in the environment via deconjugation. 17β-Estradiol (E2) is the most potent natural estrogen that is frequently detected in the environment, and E2 conjugates in sulfate (Scherr et al., 2009; Bai et al., 2013a) and glucuronide (Shrestha et al., 2012b) forms have drawn increasing attention as potential precursors to E2. Compared to other estrogen conjugates, the sulfate conjugate, 17β-estradiol-17-sulfate (E2-17S), is of particular importance because it is detected at relatively high concentrations in animal manure lagoons (87 ng L^{-1}) (Hutchins et al., 2007) and surface runoff after manure application (107 ng L^{-1}) (Dutta et al., 2012). In a previous study on behavior of E2-17S in agricultural soils, Bai et al. (2013a) found that E2-17S underwent competitive sorption and metabolism pathways to form multiple metabolites including free estrogens. However, to fully understand the processes governing the fate and transport of E2-17S in soils, mathematical models are necessary.

Mathematical models have been used to predict the behavior of reactive steroid hormones in soil batch and column studies. Das et al. (2004) applied forward modeling approach (predictive mode) to simulate sorption and degradation of several steroid hormones in soil columns. This two-region modeling approach consisted of advective-dispersive transport with non-equilibrium, two-site sorption, and first-order transformation mechanisms. Their results suggested that the first-order kinetic process was sufficient for modeling hormone degradation, but not accurate. Casey et al. (2003) used two convective-dispersive transport models, with and without transformation, and two-site kinetic Freundlich sorption to fit the breakthrough curves of E2. The results provided good description of the experimental data, but solutions were non-unique, and

parameter estimates had low confidence. Another study by Casey et al. (2004) applied a one-site fully kinetic convective-dispersive model with sorbed phase transformation and Freundlich sorption to simulate the fate of testosterone in soils, which resulted in a satisfactory fit and reasonable parameter estimates.

These previous studies considered a two-phase system (aqueous and reversibly sorbed phase) for sorption and degradation of hormones; however, later studies reported that a significant fraction of the applied steroid hormones could be irreversibly bound to soil (Colucci et al., 2001; Fan et al., 2007b). Without considering irreversible sorption, the models may give rise to inaccurate descriptions. Additionally, the previous models provided parameter estimates of relatively low confidence. To improve the modeling techniques for steroid hormones, Fan et al. (2008) developed a one-site, kinetic sorption and first-order transformation model to simulate the distribution of E2 in the aqueous, reversibly sorbed, and irreversibly sorbed phases simultaneously. The model was solved inversely using a global optimization method, the stochastic ranking evolutionary strategy (SRES) (Runarsson and Yao, 2000), instead of the traditional local optimization parameter estimation method, and the one-site model resulted in satisfactory fits and unique solutions (Fan et al., 2008).

To date models have not been applied to predict the behavior of estrogen conjugates in the environment. Modeling approaches can help identify the fate and transport, and furthermore, understand key processes that control deconjugation of conjugates to release potent free estrogens. However, it is challenging to simulate the fate of E2-17S in agricultural soils because of the simultaneous and complex governing processes. Herein, the objective of this study was to apply an integrated modeling approach (Fan et al., 2008) to fit the experimental data for E2-17S obtained from soil batch studies. The present modeling study attempted to discern and quantify

the coupled sorption and transformation mechanisms of E2-17S and its multiple metabolites in various soil-water systems.

Experiment and Model Development

Soil batch experiments

Soil samples were collected from southeastern North Dakota at two depths, topsoil (0−15 cm) and subsoil (46−61 cm). The soil samples were from a Hecla-Hamar Series (sandy, mixed, frigid Oxyaquic Hapludolls). Most of the soil properties were similar except for the soil organic carbon (OC) content (Table 3). To conduct the batch experiments, synthesized radiolabeled [^{14}C]E2-17S (Shrestha et al., 2011) was injected into non-sterile and sterile soil-water slurries to reach an initial concentration of 0.5 mg L^{-1}. The slurries contained 1.6 g soil and 8 mL of 0.1 M $CaCl_2$ solution. For sample sterilization, the soil-water slurries were irradiated for 14 h using a 7.6 kGy gamma source (M38-4 Gammator, Radiation Machinery Corp., Parsippany, NJ) before added with [^{14}C]E2-17S. At sampling time 4, 8, 24, 48, 72, 168, and 336 h of the batch study, metabolites were analyzed by a combined high performance liquid chromatography (HPLC; Beckman Coulter Inc., Fullerton, CA) and liquid scintillation counting (LSC; 1900 CA, Packard, Downers Grove, IL) method for the aqueous and reversibly sorbed phases. The detected metabolites were then characterized by liquid chromatography with tandem mass spectroscopy (LC-MS/MS; Waters, Beverly, MA). For the irreversibly sorbed phase, non-extractable radioactive residue was measured by soil combustion using a Packard Model 307 Oxidizer (Downers Grove, IL). All sampling procedure and radiometric analytical methods have been thoroughly described in previous studies (Shrestha et al., 2012a; Bai et al., 2013a).

Experimental results

The experimental data showed that the parent compound E2-17S dissipated more quickly from the aqueous phase of the topsoil (Fig. 11a and 11b) compared to the subsoil (Fig. 11c and 11d) regardless of soil sterility, which demonstrated the significant role that soil OC played. The primary transformation pathway for E2-17S was found to be hydroxylation, forming mono-hydroxy-E2-17S (OH-E2-17S) and di-hydroxy-E2-17S (diOH-E2-17S) as major metabolites. The aqueous concentrations of the hydroxylated metabolites were higher in the topsoil (Fig. 11a and 11b) compared to the subsoil (Fig. 11c and 11d) under both non-sterile and sterile conditions. For the solid phase (Fig. 11e−11h), since the reversibly sorbed radioactivity remained at relatively low levels (<15% of applied dose) for all soils, individual species were not shown in the figures. Free estrogens, E2 and estrone (E1) were observed from the reversibly sorbed phase in addition to the hydroxylated metabolites. The irreversibly sorbed radioactivity was found greater for topsoil (Fig. 11e and 11f) than subsoil (Fig. 11g and 11h), and irreversible sorption appeared to be the predominant sorption process under all conditions. The overall radioactivity recoveries were more than 90% for all soils, which were acceptable for the following model simulation.

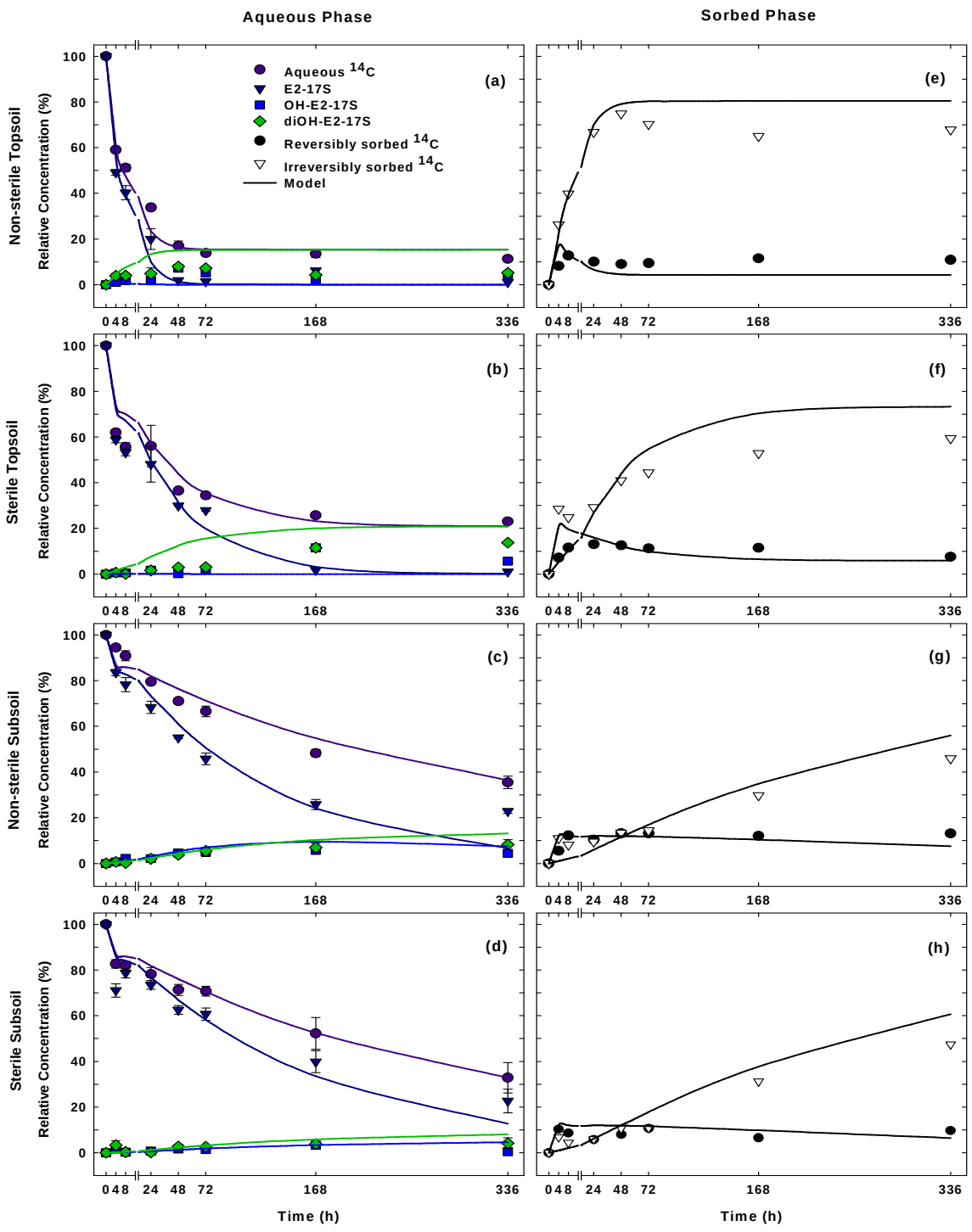

Figure 11. Measured and simulated concentrations for [^{14}C]17β-estradiol-17-sulfate, the hydroxylated metabolites, and the total radioactive residue in the aqueous, reversibly sorbed, and irreversibly sorbed phases of the non-sterile and sterile topsoil and subsoil through time. Dots represent the measured data, and solid lines represent the simulated data.

Model development

In order to describe the distribution of all observed compounds in the aqueous, reversibly sorbed, and irreversibly sorbed phases simultaneously, a linear kinetic sorption model combined with first-order transformation was applied. Linear sorption isotherms have been applied to efficiently describe hormone sorption in soils (Das et al., 2004; Fan et al., 2007a); and first-order kinetic transformation was sufficient for modeling hormone transport (Das et al., 2004; Fan et al., 2008). Given the complexity of the present observations, the one-site kinetic model was chosen to reduce the total numbers of parameters.

The schematic conceptual model is shown in Fig. 12, where several assumptions were applied to conceive the model. First, the hydroxyl positions could not be characterized for OH-E2-17S and diOH-E2-17S, and the hydroxylation mechanisms were not known. One possible mechanism for E2-17S hydroxylation is enzymatic catalysis. Mono- and di-oxygenases (e.g. cytochromes P450) can add one and two hydroxyl groups to steroids, respectively (Ullrich and Hofrichter, 2007). Also, oxidation catalyzed by metal oxides (e.g. MnO_2) is likely to be another hydroxylation mechanism (Bai et al., 2013b). However, the interactions between enzyme and metal oxides mediated hydroxylation are not understood. The conceptual model assumed that the formation of OH-E2-17S and diOH-E2-17S occurred simultaneously following parallel reactions. To further verify the hypothesis, two conceptual models were developed using either a parallel or stepwise hydroxylation pattern, and the model fit was more satisfactory with the parallel reaction rather than the stepwise pattern, meaning a more reasonable assumption.

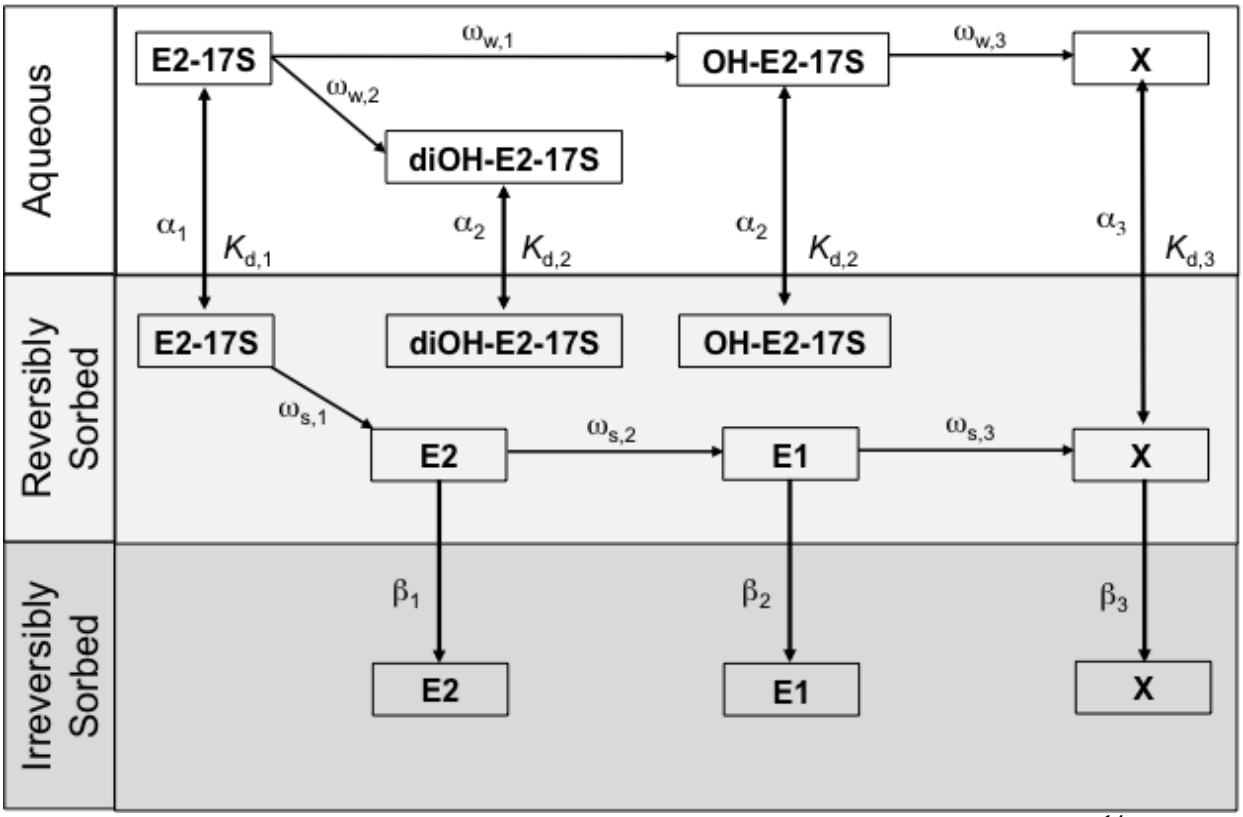

Figure 12. Schematic conceptual model for sorption and transformation processes of [^{14}C]17β-estradiol-17-sulfate and its multiple metabolites in the aqueous, reversibly sorbed, and irreversibly sorbed phases of the soil. Parameters include linear sorption coefficient K_d (L g^{-1}); first-order transformation rate coefficients in the aqueous and reversibly sorbed phase ω_w and ω_s (h^{-1}); mass transfer rate constant between the aqueous and reversibly sorbed phase α (h^{-1}); and mass transfer rate constant between the reversibly and irreversibly sorbed phase β (h^{-1}).

Second, free estrogens, E2 and E1, were not detected in the aqueous phase during the entire batch experiments, and they were only observed in the reversibly sorbed phase for all soils. These observations suggested that deconjugation/hydrolysis of E2-17S occurred on the soil surface. After E2-17S hydrolysis, the released E2 can be subsequently oxidized to yield E1, which is demonstrated to be a surface process (Sheng et al., 2009). Several studies have also provided evidence for sorbed phase transformation of E2 (Layton et al., 2000; Casey et al., 2003). Therefore, it was appropriate to assume that hydrolysis of E2-17S and oxidation of E2 both occurred on the solid phase in the present conceptual model.

Third, metabolites could not be characterized in the irreversibly sorbed phase, but only total radioactive residue was measured. According to Fan et al. (2008), most of the hydrophobic compounds would be associated with irreversible sorption sites, but polar metabolites would bind to reversible sites only. In the current conceptual model the less hydrophobic compounds, E2-17S, OH-E2-17S, and diOH-E2-17S, were assumed to be present in the reversibly sorbed phase only. The more hydrophobic metabolites, E2 and E1, were considered to be bound both reversibly and irreversibly to the soil surface. Additionally, in previous studies with similar soil-water systems, mineralization of E2 (Fan et al., 2007b) and E2 glucuronide conjugate (Shrestha et al., 2012b) did not occur, and thus gas phase distribution was not considered in the present model.

Based on the three major assumptions above, the following one-site kinetic sorption and first-order transformation model was developed to describe the fate of E2-17S and its metabolites as a series of differential equations (Eq. [6−8]):

$$\begin{cases} \dfrac{dC_{E2S}}{dt} = -\omega_{w,1}C_{E2S} - \omega_{w,2}C_{E2S} - \dfrac{M}{V}\alpha_1\left(K_{d,1}C_{E2S} - S_{E2S}\right) \\ \dfrac{dC_{OHE2S}}{dt} = \omega_{w,1}C_{1E2S} - \omega_{w,3}C_{OHE2S} - \dfrac{M}{V}\alpha_2(K_{d,2}C_{OHE2S} - S_{OHE2S}) \\ \dfrac{dC_{diOHE2S}}{dt} = \omega_{w,2}C_{E2S} - \dfrac{M}{V}\alpha_2(K_{d,2}C_{diOHE2S} - S_{diOHE2S}) \\ \dfrac{dC_X}{dt} = \omega_{w,3}C_{OHE2S} - \dfrac{M}{V}\alpha_3(K_{d,3}C_X - S_X) \end{cases}$$

[6]

$$\begin{cases} \dfrac{dS_{E2S}}{dt} = \alpha_1\left(K_{d,1}C_{E2S} - S_{E2S}\right) - \omega_{s,1}S_{E2S} \\ \dfrac{dS_{OHE2S2}}{dt} = \alpha_2\left(K_{d,2}C_{OHE2S} - S_{OHE2S}\right) \\ \dfrac{dS_{diOHE2S}}{dt} = \alpha_2\left(K_{d,2}C_{diOHE2S} - S_{diOHE2S}\right) \\ \dfrac{dS_{E2}}{dt} = \omega_{s,1}S_{E2S} - \omega_{s,2}S_{E2} - \beta_1 S_{E2} \\ \dfrac{dS_{E1}}{dt} = \omega_{s,2}S_{E2} - \omega_{s,3}S_{E1} - \beta_2 S_{E1} \\ \dfrac{dS_X}{dt} = \omega_{s,3}S_{E1} + \alpha_3(K_{d,3}C_X - S_X) - \beta_3 S_X \end{cases}$$

[7]

$$\begin{cases} \dfrac{d\bar{S}_{E2}}{dt} = \beta_1 S_{E2} \\ \dfrac{d\bar{S}_{E1}}{dt} = \beta_2 S_{E1} \\ \dfrac{d\bar{S}_X}{dt} = \beta_3 S_X \end{cases}$$

[8]

In the differential equations, C represents the aqueous concentration; S and $\bar{S}$ are the concentration on the reversibly and irreversibly sorbed phase, respectively; K_d (L g^{-1}) is the linear sorption coefficient between the aqueous and reversibly sorbed phase; ω_w and ω_s (h^{-1}) are the first-order transformation rate coefficient in the aqueous and reversibly sorbed phase, respectively; α (h^{-1}) is the mass transfer rate constant between the aqueous and reversibly sorbed phase; β (h^{-1}) is the mass transfer rate constant between the reversibly and irreversibly sorbed phase; X represents unknown scatters detected during the HPLC-LSC analysis, and M/V is the mass to volume ratio (soil:$CaCl_2$ solution = 200 g L^{-1}) in the batch vials. The batch studies were conducted under four soil conditions (non-sterile topsoil, sterile topsoil, non-sterile subsoil, and sterile subsoil), and 13 compounds were assumed to be present in the three phases of each soil, which resulted in 52 equations to be solved and 36 parameters to be estimated. In this case, the mass balance of the model was expressed as follows (Eq. [9]):

$$\begin{cases} C_{total} = C_{E2S} + C_{OHE2S} + C_{diOHE2S} + C_X \\ S_{total} = S_{E2S} + S_{OHE2S} + S_{diOHE2S} + S_{E2} + S_{E1} + S_X + \bar{S}_{E2} + \bar{S}_{E1} + \bar{S}_X \\ -M\dfrac{dS_{total}}{dt} = V\dfrac{dC_{total}}{dt} \end{cases} \quad [9]$$

Model solution

The differential equations (Eq. [6]−[8]) were inversely solved using a finite difference method, CVODE (Cohen and Hindmarsh, 1994) that was written in C using libSRES (Ji and Xu, 2006), and a spatial increment of 1 cm and time step of 6.0 sec were applied for computation. In the present study, local optimization was not suitable due to the nonlinearity of the mathematical

model and the large numbers of parameters (Fan and Casey, 2008). The parameters were thus optimized with a global optimization method, SRES, which has been successfully used for parameter estimation in previous studies (Fan et al., 2007a; Fan and Casey, 2008; Fan et al., 2008). Using the global optimization method, the following objective function (Eq. [10]) was minimized:

$$J = \sum_{i=1}^{l}\sum_{j=1}^{m}\sum_{k=1}^{n}\left[(O-P)_{ijk}\right]^2 \qquad [10]$$

In the objective function, O is the observed data, P is the simulated data, l is the number of soil sets, m is the number of datasets for each experiment, and n is the number of total compounds. Herein, l = 4 indicating four soils, m = 7 indicating seven sampling time points (i.e. 4, 8, 24, 48, 72, 168, and 336 h), and n = 13 representing all compounds in the three phases of each soil, which was equal to the number of differential equations for each soil. Additionally, the ratio of reversibly sorbed radioactivity to irreversibly sorbed radioactivity was added to the objective function, and all data were treated equally with weight of unity.

Constraints on model process and parameters

The model simulated all governing processes in the aqueous, reversibly sorbed, and irreversibly sorbed phases of all soils simultaneously, which reduces confidence intervals of optimized parameters and provides more reliable determination of governing processes (Casey and Simunek, 2001). Simultaneous fit for all soil conditions also allows additional constraints that improve uniqueness of parameter estimates (Casey and Simunek, 2001). The objective function (Eq. [10]) was thus subjected to the following constraints (Eq. [11−13]) to ensure that the appropriate processes were modeled and that unique sets of parameters were optimized.

$$K_{d,topsoil} > K_{d,subsoil} \quad [11]$$

$$K_{d,E2S} > K_{d,OHE2S} = K_{d,diOHE2S} \quad [12]$$

$$\omega_{w,s,topsoil} > \omega_{w,s,subsoil} \quad [13]$$

The observed faster aqueous dissipation of E2-17S in the topsoil compared to the subsoil was attributed to the greater sorption and transformation rates (Bai et al., 2013a; Bai et al., 2013b). Soil OC is the primary sorption domain for estrogenic compounds (Sarmah et al., 2008). As a result of the soil OC effects on estrogen sorption, the K_d values for E2-17S and its metabolites were constrained to be greater in the topsoil of higher OC (Eq. [11]). Furthermore, sorption of steroid hormones in soil is governed by hydrophobic interaction (Das et al., 2004; Yu et al., 2004). The K_d value is considered inversely correlated to the polarity of the compound (Eq. [12]). The polarities of the hydroxylated E2-17S were greater than E2-17S as indicated by the reverse-phase HPLC elution time, which was, in order of polarity from highest to lowest, the following: diOH-E2-17S, OH-E2-17S, and E2-17S. Additionally, the two hydroxylated metabolites have similar molecular structure, and moreover, the HPLC elution times were close (approximate 4 and 6 min for diOH-E2-17S and OH-E2-17S, respectively). The K_d values for the two hydroxylated metabolites were thus assumed to be identical (Eq. [12]), which further reduced the total numbers of parameters and increased the parameter confidence.

Restrictions were also given to the transformation rate constants, ω_w and ω_s, in the aqueous and reversibly sorbed phase, respectively. The applied E2-17S in the soil-water systems underwent different pathways, i.e. hydroxylation, hydrolysis, and oxidation. Compared to subsoil, topsoil has higher OC and higher microbial and enzymatic activities (Watts et al., 2010), which would cause greater transformation rates for E2-17S. Also, the measured manganese level

was higher for topsoil than subsoil (Table 3), which would cause more rapid metal oxides promoted reactions. Moreover, the measured metabolite concentrations were consistently higher for the topsoil than the subsoil; therefore, both ω_w and ω_s values were set greater for the topsoil (Eq. [13]). In addition to these major constraints, the K_d, ω_s, α and β values were set to be equal for non-sterile and sterile soils because gamma irradiation would not alter the total soil OC content or soil minerals.

Parameter optimization and goodness-of-fit

In order to optimize the computational time, the program was initially run with a lower bound of 1.00×10^{-8} and an upper bound of 1.00 until the parameter values stabilized, and then the parameters were refined by new upper and lower bounds as 3 and 0.3 times the old values till no more changes in the parameter estimates (Shrestha, 2011). The final simulation was run 30 times to compute the 95% confidence intervals, which was two times of the standard deviation of the 30 optimized values. For model evaluation, two statistical indices were used to determine the goodness-of-fit, the adjusted coefficient of determination (R^2_{adj}) and the modified index of agreement *d* (Willmott et al., 1985), which was calculated as follows (Eq. [14]):

$$d = 1 - \frac{\sum_i |O_i - P_i|}{\sum_i (|P_i - \bar{O}| + |O_i - \bar{O}|)} \quad [14]$$

where O_i is the observed value; P_i is the predicted value; and $\bar{O}$ is the mean of all observed values. The *d* value ranges from 0 to 1, and 1 indicates a perfect fit (Legates and McCabe, 1999; Helmke et al., 2004; Shrestha, 2011).

Modeling Results

Model fit

In general, the model provided a good fit for the measured data under all soil conditions. The goodness-of-fit was shown by the R^2_{adj} value (0.93) and the *d* value (0.87), which were calculated from all data modeled for the multiple species in different phases of all soils. The satisfactory fit demonstrated that the assumptions made in the conceptual model were suitable to describe the governing processes of all compounds. The model successfully captured the overall trends of E2-17S in the aqueous phase for all soils (Fig. 11a−11d); but it slightly underestimated the aqueous levels of E2-17S at the end time point (336 h) for the two subsoil (Fig. 11c and 11d). It suggested that sorption kinetics was more significant than metabolism because the aqueous concentrations of E2-17S converged rather than rapidly decreasing as the model results showed. Additionally, the model simulated the concentrations of the hydroxylated E2-17S fairly well (Fig. 11a−11d), except for the non-sterile and sterile topsoil (Fig. 11a and 11b), where the concentrations of diOH-E2-17S were overestimated, and the concentrations of OH-E2-17S were slightly underestimated at the late stages (after 168 h). These deviations may be caused by the assumption that the unidentified scatters (X) were produced from OH-E2-17S rather than from diOH-E2-17S (Fig. 12), and as a result, the predicted concentrations of OH-E2-17S were lower than the observed data.

For the sorbed phase (Fig. 11e−11h), the model could describe the total radioactive residue distribution under all soil conditions. Deviations were found for the irreversibly sorbed phase, where the model appeared to overestimate the irreversibly sorbed radioactivity at the late stages for all soils (72 to 336 h). During the batch experiments, there may be radioactive residue that

were associated with colloidal organic matter fraction (Zitnick et al., 2011) in the soil-water slurries, which was not taken in to account by the model as a sink of the applied radioactivity. Moreover, the one-site fully kinetic sorption did not consider instantaneous sorption, which likely resulted in inaccurate description of the sorbed radioactive residue at the initial stage (4−8 h; Fig. 11f−11h) of the batch experiments.

Parameter estimates

All estimated parameters are listed in Table 8 with their 95% confidence intervals. The parameter estimates had narrow 95% confidence intervals for K_d, ω_{w1}, ω_{w2}, and ω_s, indicating high confidence. The estimated K_d values for E2-17S and its hydroxylated metabolites were similar, which was reasonable because the polarities for the parent and metabolite compounds were similar as indicated by the HPLC elution times. According to the estimated hydroxylation rate constants (ω_{w1} and ω_{w2}), the corresponding transformation half-lives of E2-17S were 7.53, 99.0, 231, and 693 h for the non-sterile topsoil, sterile topsoil, non-sterile subsoil, and sterile subsoil, respectively. Fan et al. (2008) estimated the half-life of free E2 to be less than 5 h in the non-sterile topsoil using the one-site kinetic model. The greater estimated half-life of E2-17S compared to E2 demonstrated that E2-17S was more persistent in the soil-water systems than its free counterpart.

Table 8. Parameter estimates for [^{14}C]17β-estradiol-17-sulfate and its metabolites in various soil-water systems. The values inside parentheses represent the 95% confidence interval of the estimated parameter.

	E2-17S	OH-E2-17S	diOH-E2-17S	E2	E1
Non-sterile Topsoil					
K_d (L g^{-1})	2.8 (2.7, 2.9) (×10^{-1})	2.8 (2.7, 2.9) (×10^{-1})	2.8 (2.7, 2.9) (×10^{-1})		
$\omega_{w,1}$ (h^{-1})	7.1 (6.8, 7.4) (×10^{-2})				
$\omega_{w,2}$ (h^{-1})	2.1 (2.0, 2.3) (×10^{-2})				
ω_s (h^{-1})	2.8 (2.6, 3.0) (×10^{-4})			4.4 (2.0, 6.7)	
α (h^{-1})	0.92 (0.27, 1.57)	0.87 (−0.04, 1.78)	0.87 (−0.04, 1.78)		
β (h^{-1})				0.16 (−0.01, 0.33)	0.62 (−0.03, 1.27)
Sterile Topsoil					
K_d (L g^{-1})	2.8 (2.7, 2.9) (×10^{-1})	2.8 (2.7, 2.9) (×10^{-1})	2.8 (2.7, 2.9) (×10^{-1})		
$\omega_{w,1}$ (h^{-1})	1.1 (0.1, 2.2) (×10^{-3})				
$\omega_{w,2}$ (h^{-1})	6.5 (6.1, 6.8) (×10^{-3})				
ω_s (h^{-1})	2.8 (2.6, 3.0) (×10^{-4})			4.4 (2.0, 6.7)	
α (h^{-1})	0.92 (0.27, 1.57)	0.87 (−0.04, 1.78)	0.87 (−0.04, 1.78)		
β (h^{-1})				0.16 (−0.01, 0.33)	0.62 (−0.03, 1.27)
Non-sterile Subsoil					
K_d (L g^{-1})	1.4 (1.3, 1.4) (×10^{-1})	1.3 (1.3, 1.4) (×10^{-1})	1.3 (1.3, 1.4) (×10^{-1})		
$\omega_{w,1}$ (h^{-1})	1.9 (1.7, 2.2) (×10^{-3})				
$\omega_{w,2}$ (h^{-1})	1.4 (1.3, 1.5) (×10^{-3})				
ω_s (h^{-1})	1.7 (1.6, 1.7) (×10^{-4})			1.8 (0.7, 2.9)	
α (h^{-1})	1.2 (0.5, 1.9)	0.71 (0.13, 1.3)	0.71 (0.13, 1.3)		
β (h^{-1})				0.89 (0.42, 1.4)	1.7 (0.7, 2.8)
Sterile Subsoil					
K_d (L g^{-1})	1.4 (1.3, 1.4) (×10^{-1})	1.3 (1.3, 1.4) (×10^{-1})	1.3 (1.3, 1.4) (×10^{-1})		
$\omega_{w,1}$ (h^{-1})	4.3 (3.6, 5.1) (×10^{-4})				
$\omega_{w,2}$ (h^{-1})	6.5 (5.8, 7.3) (×10^{-4})				
ω_s (h^{-1})	1.7 (1.6, 1.7) (×10^{-4})			1.8 (0.7, 2.9)	
α (h^{-1})	1.2 (0.5, 1.9)	0.71 (0.13, 1.3)	0.71 (0.13, 1.3)		
β (h^{-1})				0.89 (0.42, 1.4)	1.7 (0.7, 2.8)

K_d is the linear sorption coefficient. ω_w and ω_s are the first-order transformation rate coefficients in the aqueous and reversibly sorbed phase respectively. α and β are the mass transfer rate constants between the aqueous and reversibly sorbed phase, and the reversibly and irreversibly sorbed phase, respectively.

The estimated deconjugation/hydrolysis rate constants (ω_s) for E2-17S on the solid phase were much smaller than the oxidation rate constants of E2 to yield E1. Previous studies have reported that hydrolysis is not a primary transformation pathway for the sulfate conjugates, E2-17S (Bai et al., 2013a) and E2-3S (Scherr et al., 2009), which can explain the low hydrolysis rates estimated by the present model. However, oxidation of E2 is reported to be a very rapid process on the soil surface (Colucci et al., 2001), and E1 is known as a more common and persistent estrogen than E2 in the environment (Hanselman et al., 2003), which is consistent with the current modeling results with the great transformation rates for E2.

The confidence in the mass transfer rate constants, α and β, were lower compared to other parameters in Table 2. The α values of E2-17S were greater than the two hydroxylated metabolites, indicating a faster mass exchange between the aqueous and reversibly sorbed phase for the parent compound E2-17S. This is likely because that E2-17S was at higher levels in the aqueous phase than the hydroxylated metabolites, especially at the early stages of the batch studies, which facilitated the mass transfer to the sorbed phase. Also, the α values for E2-17S were greater than those estimated for free E2 (~0.4 h^{-1}) by Fan et al. (2008), which may be due to the higher mobility of E2-17S in the soil-water systems that enhanced the mass exchange between the aqueous and solid phase.

Conclusions

This study developed a comprehensive model to simulate the coupled sorption and transformation of E2-17S in the aqueous, reversibly sorbed, and irreversibly sorbed phases of various soil-water systems. The model could successfully identify and quantify the multiple governing processes based on the experimental data. Given that all of the parameters were estimated simultaneously, the parameters were of acceptable confidence and uniqueness. Moreover, compared to the previous study to estimate 12 parameters (Fan et al., 2008), the one-site kinetic model was demonstrated to be able to simulate more complex scenarios with larger numbers (36) of parameters. Although the assumptions may provide challenges in applying the model to conditions such as column studies and field scale studies, the model has given significant insights in understanding the complex processes governing the environmental fate of manure-borne estrogen conjugates.

Acknowledgments

The authors sincerely thank Mrs. Colleen Pfaff (Biosciences Research Laboratory, USDA-ARS, Fargo, ND) and Mr. Nathan Derby (Department of Soil Science, North Dakota State University, Fargo, ND) for their assistance on the research. We would like to acknowledge Dr. Jane Schuh (Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo, ND) for her support on the sterilization procedure. This project is supported by Agriculture and Food Research Initiative Competitive Grant No. 2010-65102-20400 from the USDA National Institute of Food and Agriculture. Mention of trade names or commercial

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GENERAL CONCLUSIONS

This study hypothesized that the sulfate conjugated estrogen, 17β-estradiol-17sulfate (E2-17S) could be a precursor to free estrogens in the environment. The objectives of this study were to investigate the sorption and transformation of E2-17S in various soil-water systems, under the context of understanding the fate and transport of manure-borne estrogens. The soil batch experimental results showed that soil organic carbon content significantly influenced the aqueous dissipation of E2-17S under both non-sterile and sterile soil conditions. Sorption of E2-17S to the soils was found limited with a reduced sorption affinity compared to 17β-estradiol (E2), which explains the frequent detection and high levels of E2-17S in surface runoff nearby agricultural fields with manure application. The present sorption parameters can be useful information to serve in fate and transport modeling and risk assessment studies related to estrogens and estrogen conjugates. For metabolism pathways, hydroxylation was found to be the primary transformation process for E2-17S in all soils. Deconjugation/hydrolysis of E2-17S did occur, but it was a minor transformation pathway compared to hydroxylation, with low concentrations of free estrogens (i.e. E2 and estrone) being released on the reversibly sorbed phase. At last, the integrated one-site kinetic model along with reasonable assumptions provided reliable parameter estimates and gave significant insights in understanding the complex governing processes of E2-17S the soil-water systems.

In conclusion, although E2-17S cannot be excluded as a precursor of free estrogens in the environment, the present study implied that manure-borne E2-17S may not be a major

contributor to the frequently detected free estrogens in the surface and subsurface soil profile after liquid manure application.

Further Studies

The present study utilized soil batch experiments to investigate the coupled sorption and transformation of E2-17S in soil-water slurries. The results indicated that deconjugation of E2-17S occurred on the solid phase; however, under field or soil column conditions with greater soil to water ratios, the deconjugation may be facilitated. Further studies may need to elucidate the sorption, degradation, and mobility of E2-17S in soil columns to mimic the real-world conditions.

Additionally, the present study stated that both biotic and abiotic metabolism of E2-17S could occur in the soil-water systems. To further identify the governing processes, studies are needed to characterize the specific roles that soil enzymes (e.g. sulfatase and cytochrome p450), metal oxides (MnO_2), and clay minerals (smectite) play in the metabolism especially deconjugation of E2-17S.

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APPENDIX A. THE EXPERIMENTAL DATA FOR SYNTHESIS OF RADIOLABLED MATERIALS

Table A1. Chemical shift assignments of NMR spectra of 17β-estradiol-3-glucuronide and 17*β*-estradiol-17-sulfate.

Carbon/Proton	**E2**[a]	**E2-3-B**	**E2-3B-17S**	**E2-17S**	**Remark**
Carbon					E2-3B = E2-3-Benzoate
1	127.22	127.472	127.52	127.26	E2-3B-17S = E2-3-Benzoate-17-Sulfate
2	113.72	119.791	119.839	113.762	E2-17S = E2-17-Sulfate
3	155.84	150.127	150.105	155.892	
4	116.05	122.626	122.638	116.053	[a]Carbon assignments based on the values provided by Dionne et al. (1997) for 17â-estradiol
5	138.80	139.490	139.424	138.762	
6	30.72[b]	134.857	134.947	132.535	[b]The signal for C6 is masked under solvent peaks (Acetone –d6) in values provided by Dionne et al. (1997). The assignment for C6 is based on the NMR spectrum obtained under MeOH solvent.
7	28.83	28.268	28.177	28.477	
8	40.50	40.144	40.292	40.335	
9	45.34	45.553	45.408	45.297	
10	132.32	30.694	30.533	30.71	
11	27.53	27.480	27.471	27.48	
12	38.00	37.992	37.981	38.00	
13	44.35	44.343	44.225	44.236	
14	51.26	51.327	50.779	50.778	s= singlet; d = dplt; t= triplet
15	24.03	24.043	24.106	24.1	
16	30.68	30.560	29.223	29.219	
17	82.49	82.455	88.216	88.186	
18	11.71	11.677	12.238	12.192	
Protons[d]					
H_1	6.527(d,a)	6.9375(d)	6.9035 (d)	6.527 (d)	
H_2	7.058(d,b)	7.4405(d)	7.3015 (d)	7.0605 (d)	
H_4	6.467(s,c)	6.8920(d)	6.855 (s)	6.4665 (s)	
H_{17}		3.669 (t)	4.311 (t)	4.295 (t)	
Benzoate moiety carbons[c]					
B-1		165.936	166.953		
B-2		130.995	130.853		
B-3		130.995	130.853		
B-4		129.827	129.892		
B-5B-6		129.827	129.892		
		139.385	139.265		

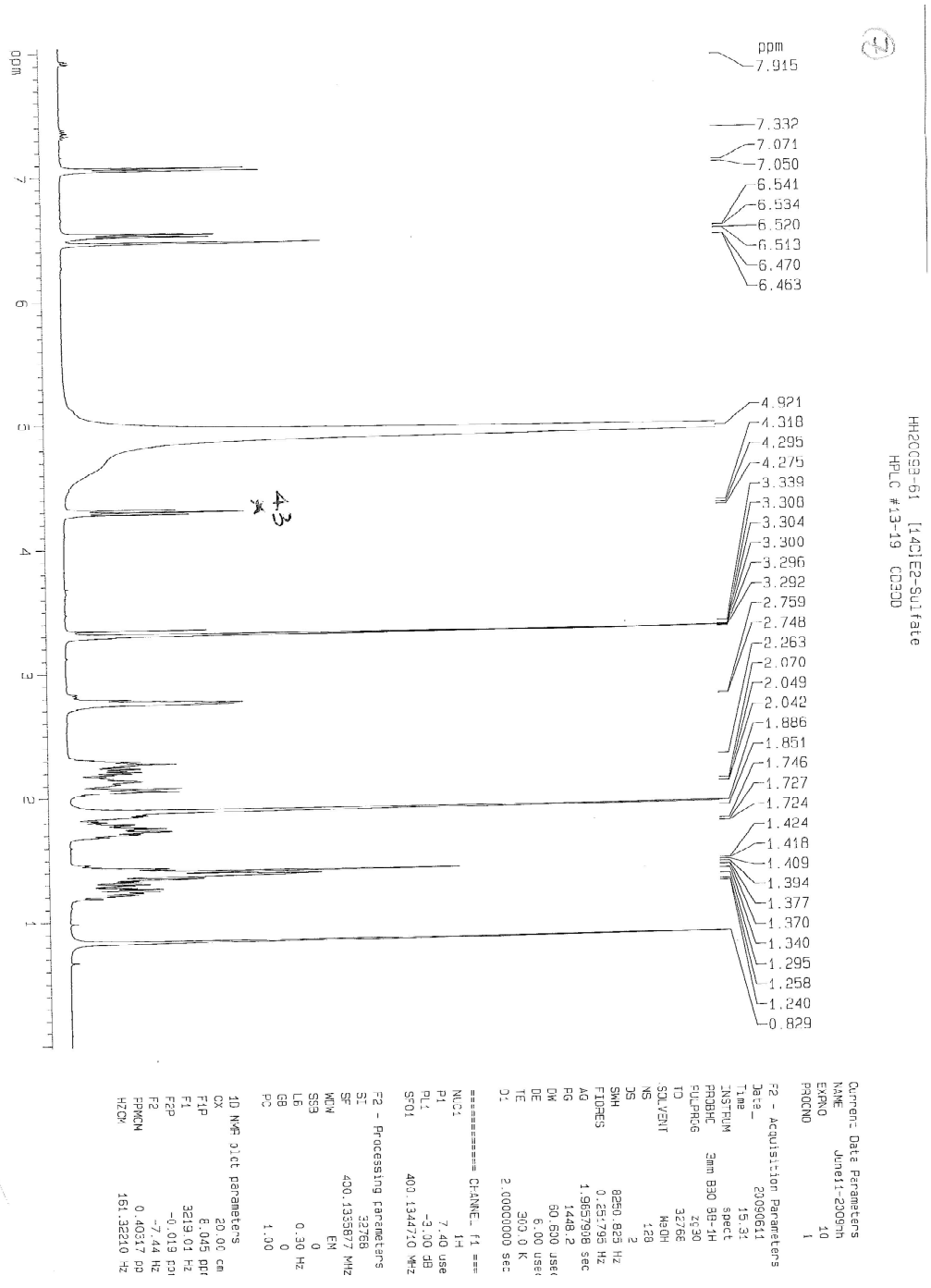

Figure A1. NMR Spectra for 17β-estradiol-17-sulfate synthesis (Proton).

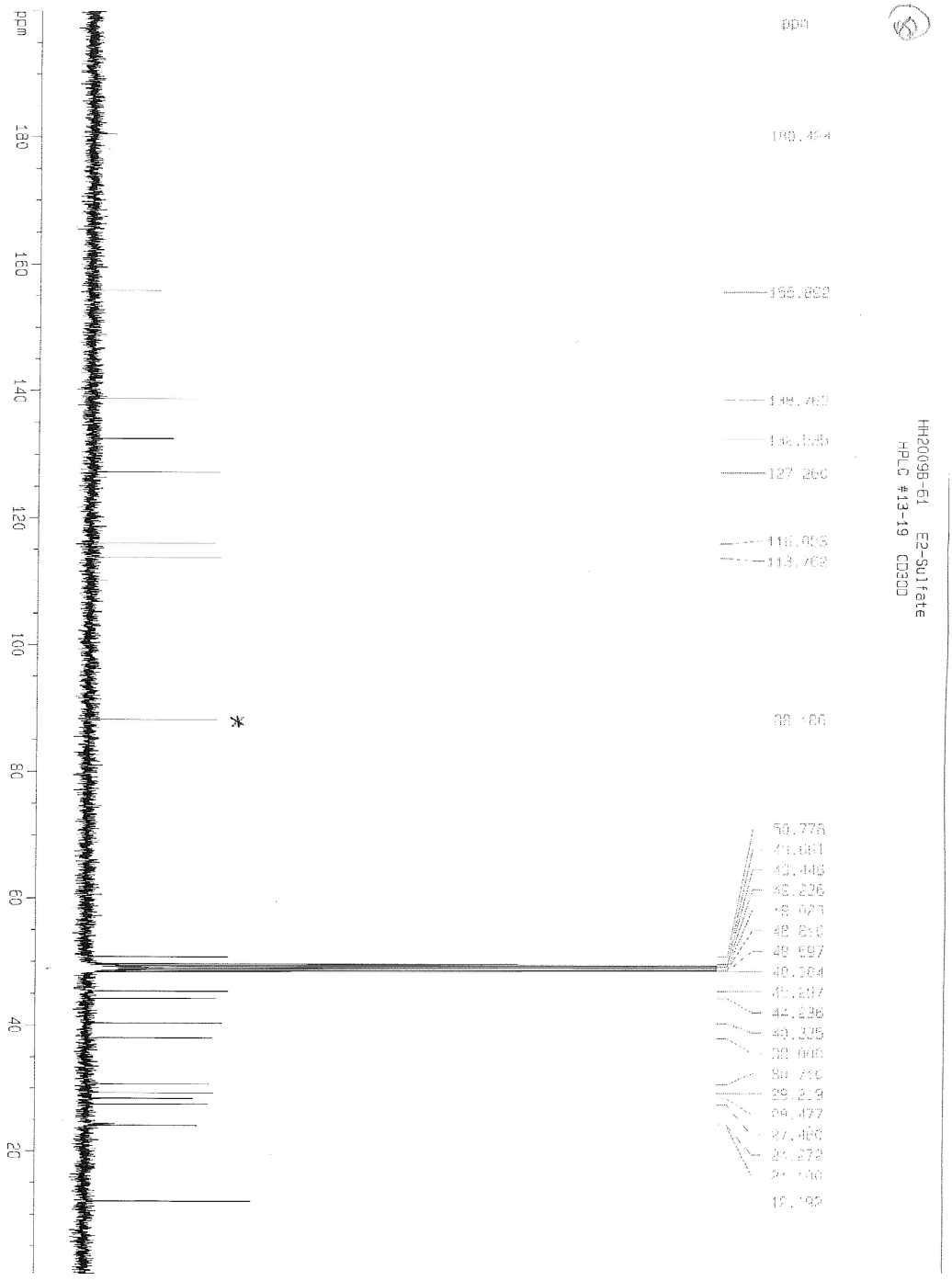

Figure A2. NMR Spectra for 17β-estradiol-17-sulfate synthesis (Carbon).

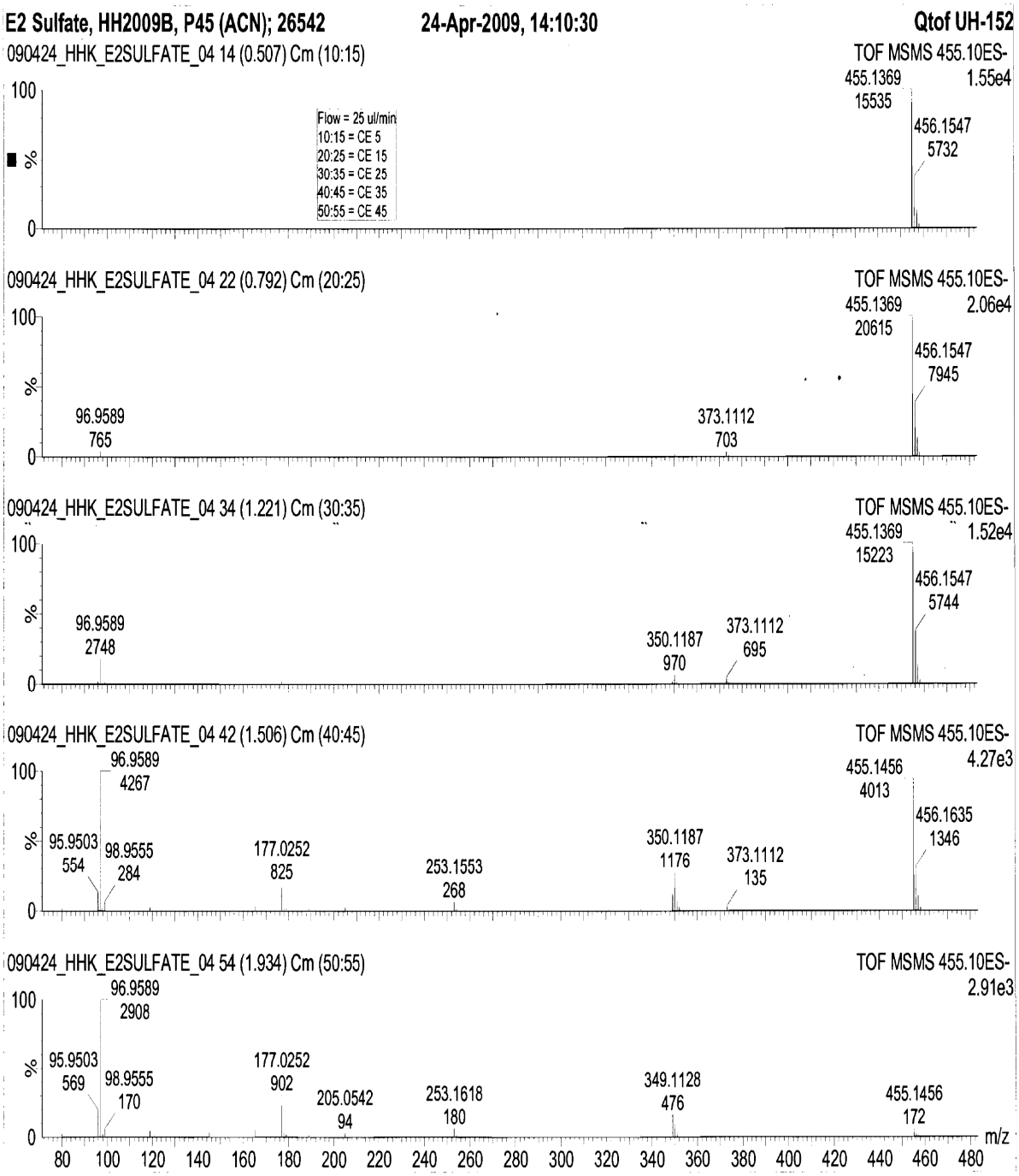

Figure A3. LC-MS/MS Spectra for 17β-estradiol-17-sulfate synthesis (1).

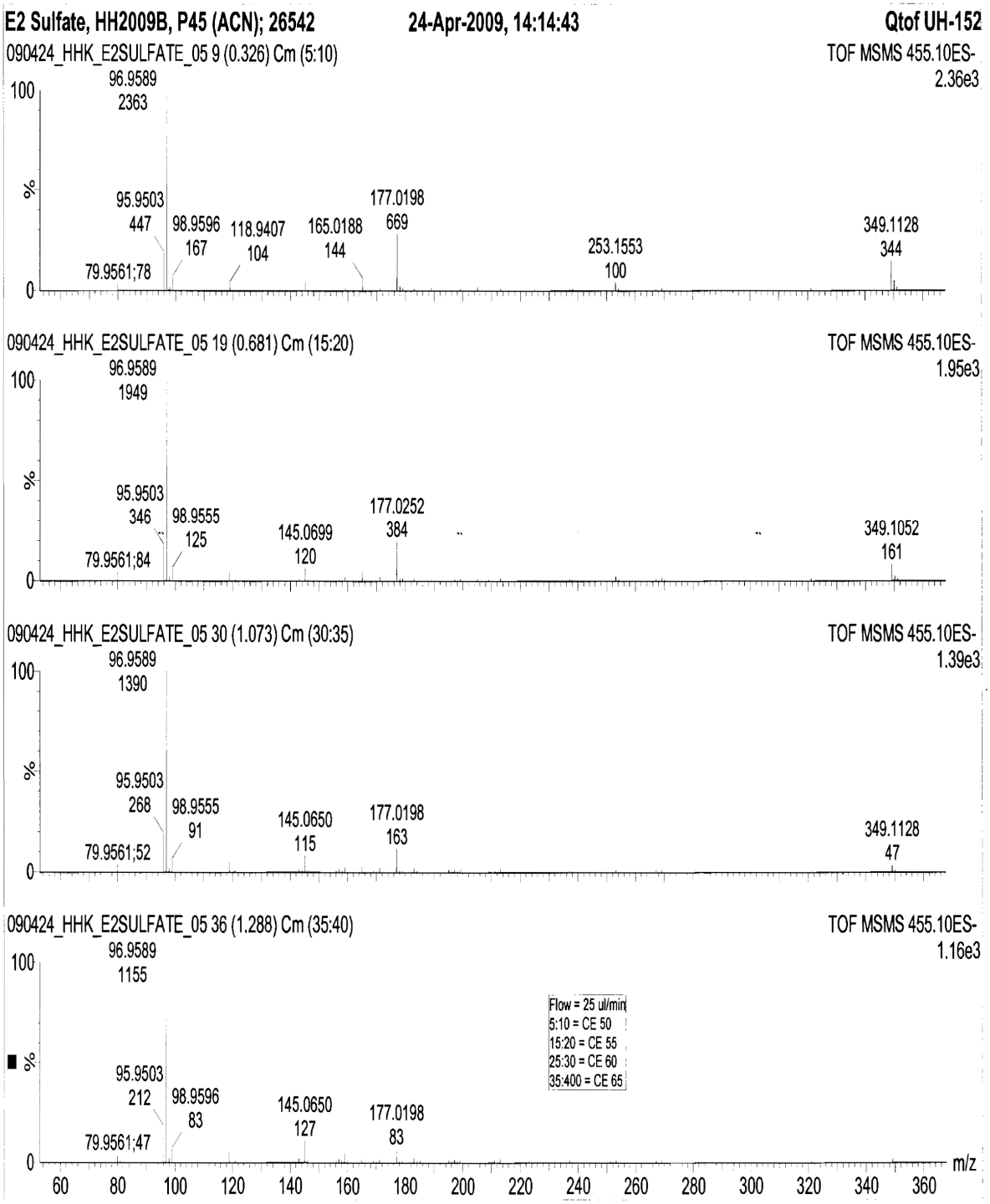

Figure A4. LC-MS/MS Spectra for 17β-estradiol-17-sulfate synthesis (2).

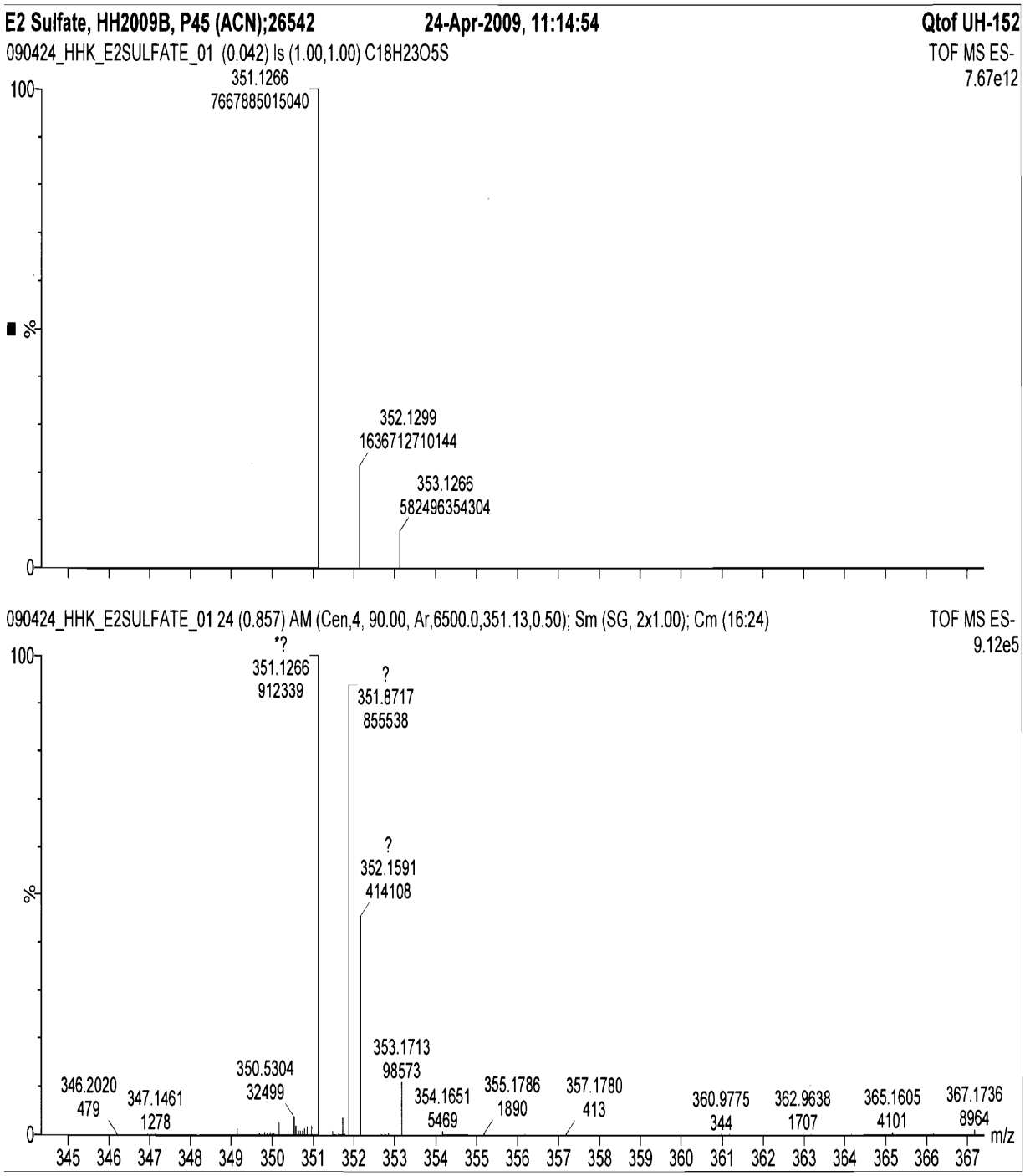

Figure A5. LC-MS/MS Spectra for 17β-estradiol-17-sulfate synthesis (3).

APPENDIX B. THE EXPERIMENTAL DATA FOR BATCH EXPERIMENTS

Table B1. The experimental data from liquid scintillation counting analysis for the radioactive residue in the aqueous phase of non-sterile topsoil.

Time (h)	0	4	8	24	48	72	168	336
Concentration[†]	mg L^{-1}							
Control A	0.55	0.60	0.60	0.62	0.59	0.60	0.59	0.61
Control B	0.55	0.60	0.60	0.62	0.59	0.59	0.63	0.60
Control C	0.55	0.60	0.58	0.58	0.59	0.60	0.59	0.60
Low A	0.55	0.32	0.29	0.20	0.11	0.09	0.08	0.07
Low B	0.55	0.33	0.28	0.18	0.08	0.07	0.06	0.06
Low C	0.55	0.33	0.27	0.18	0.08	0.07	0.06	0.06
Medium 2 A	2.89	2.18	2.00	1.52	0.89	0.60	0.44	0.45
Medium 2 B	2.89	2.58	2.40	1.83	1.08	0.70	0.61	0.54
Medium 2 C	2.89	2.73	2.50	1.92	1.15	0.65	0.48	0.48
Medium 1 A	8.92	7.00	6.49	5.14	3.17	2.02	1.58	1.38
Medium 1 B	8.92	7.13	6.68	5.33	3.29	2.16	1.72	1.49
Medium 1 C	8.92	7.31	6.73	5.53	3.37	2.20	1.58	1.54
High A	30.34	24.55	23.19	19.01	11.53	7.73	7.02	6.77
High B	30.34	25.33	24.26	20.20	12.11	8.18	7.37	7.14
High C	30.34	25.67	24.37	20.55	12.27	8.44	6.94	6.89

[†] Control = 0.6 mg L^{-1}, Low = 0.6 mg L^{-1}, Medium 2 = 2.9 mg L^{-1}, Medium 1 = 8.9 mg L^{-1}, High = 30 mg L^{-1}

Table B2. The experimental data from liquid scintillation counting analysis for the radioactive residue in the aqueous phase of non-sterile subsoil.

Time (h)	0	4	8	24	48	72	168	336
Concentration[†]	mg L^{-1}							
Control A	0.55	0.57	0.54	0.59	0.57	0.57	0.55	0.55
Control B	0.55	0.56	0.56	0.56	0.59	0.56	0.58	0.56
Control C	0.55	0.58	0.57	0.57	0.56	0.58	0.55	0.57
Low A	0.55	0.53	0.51	0.43	0.39	0.38	0.27	0.17
Low B	0.55	0.52	0.51	0.45	0.39	0.37	0.27	0.15
Low C	0.55	0.52	0.49	0.44	0.40	0.35	0.25	0.19
Medium 2 A	2.89	2.87	2.87	2.55	2.19	2.00	1.48	0.96
Medium 2 B	2.89	2.90	2.88	2.56	2.26	2.01	1.47	1.00
Medium 2 C	2.89	2.85	2.84	2.51	2.26	2.02	1.53	1.04
Medium 1 A	8.92	9.21	9.15	8.02	7.06	6.48	5.14	4.05
Medium 1 B	8.92	9.13	9.06	7.76	7.04	6.12	4.83	4.18
Medium 1 C	8.92	8.96	8.63	7.79	6.98	6.28	5.05	4.03
High A	30.34	28.75	28.19	25.84	24.21	22.66	20.89	20.01
High B	30.34	29.26	28.38	25.67	24.44	22.95	21.38	19.93
High C	30.34	29.46	29.39	26.28	24.37	23.02	21.47	19.69

[†] Control = 0.6 mg L^{-1}, Low = 0.6 mg L^{-1}, Medium 2 = 2.9 mg L^{-1}, Medium 1 = 8.9 mg L^{-1}, High = 30 mg L^{-1}

Table B3. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for 17β-estradiol-17-sulfate in the aqueous phase of non-sterile topsoil.

Time (h)	0	4	8	24	48	72	168	336
Concentration[†]	mg L^{-1}							
Control A	0.55	0.57	ND	0.52	0.55	0.58	0.55	0.58
Control B	0.55	0.58	0.55	0.55	0.56	0.59	0.60	0.57
Control C	0.55	0.58	0.66	0.55	0.57	0.58	0.54	0.57
Low A	0.55	0.28	0.36	0.12	ND	ND	ND	ND
Low B	0.55	0.26	0.22	0.11	ND	ND	ND	ND
Low C	0.55	0.27	0.26	0.14	ND	ND	ND	ND
Medium 2 A	2.89	1.90	1.71	1.20	0.58	0.27	0.19	0.19
Medium 2 B	2.89	2.23	2.14	1.60	0.75	0.33	0.27	0.25
Medium 2 C	2.89	2.36	2.24	1.65	0.77	0.20	0.14	ND
Medium 1 A	8.92	6.69	6.53	4.42	2.23	1.16	1.05	0.95
Medium 1 B	8.92	6.89	6.12	4.71	2.35	1.25	1.22	0.96
Medium 1 C	8.92	6.97	6.42	4.94	2.50	1.24	1.06	0.99
High A	30.34	23.63	22.20	17.27	8.37	3.75	1.37	1.32
High B	30.34	24.76	23.31	18.54	8.90	3.61	1.36	1.43
High C	30.34	25.08	23.46	18.64	9.12	3.50	1.11	1.50

[†] Control = 0.6 mg L^{-1}, Low = 0.6 mg L^{-1}, Medium 2 = 2.9 mg L^{-1}, Medium 1 = 8.9 mg L^{-1}, High = 30 mg L^{-1}

[‡] ND = not detected

Table B4. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for monohydroxy-17β-estradiol-17-sulfate in the aqueous phase of non-sterile topsoil.

Time (h)	0	4	8	24	48	72	168	336
Concentration[†]				mg L^{-1}				
Control A	0.00	0.00	ND	0.00	0.00	0.00	0.01	0.01
Control B	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Control C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Low A	0.00	0.01	0.01	0.02	ND	ND	ND	ND
Low B	0.00	0.00	0.01	0.01	ND	ND	ND	ND
Low C	0.00	0.01	0.02	0.01	ND	ND	ND	ND
Medium 2 A	0.00	0.05	0.05	0.11	0.07	0.06	0.04	0.05
Medium 2 B	0.00	0.00	0.01	0.07	0.07	0.08	0.07	0.05
Medium 2 C	0.00	0.19	0.03	0.07	0.09	0.11	0.06	ND
Medium 1 A	0.00	0.07	0.14	0.20	0.29	0.25	0.12	0.12
Medium 1 B	0.00	0.04	0.09	0.17	0.18	0.21	0.13	0.12
Medium 1 C	0.00	0.04	0.07	0.16	0.16	0.21	0.15	0.15
High A	0.00	0.15	0.25	0.54	1.06	1.13	1.59	1.87
High B	0.00	0.14	0.20	0.48	0.94	1.34	1.73	1.97
High C	0.00	0.10	0.25	0.66	0.89	1.31	1.77	2.01

[†] Control = 0.6 mg L^{-1}, Low = 0.6 mg L^{-1}, Medium 2 = 2.9 mg L^{-1}, Medium 1 = 8.9 mg L^{-1}, High = 30 mg L^{-1}

[‡] ND = not detected

Table B5. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for dihydroxy-17β-estradiol-17-sulfate in the aqueous phase of non-sterile topsoil.

Time (h)	0	4	8	24	48	72	168	336
Concentration[†]	mg L^{-1}							
Control A	0.00	0.00	ND	0.00	0.00	0.01	0.00	0.00
Control B	0.00	0.00	0.03	0.00	0.01	0.00	0.00	0.01
Control C	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.00
Low A	0.00	0.01	0.02	0.04	ND	ND	ND	ND
Low B	0.00	0.02	0.03	0.02	ND	ND	ND	ND
Low C	0.00	0.03	0.03	0.02	ND	ND	ND	ND
Medium 2 A	0.00	0.10	0.12	0.17	0.20	0.20	0.09	0.10
Medium 2 B	0.00	0.05	0.06	0.15	0.25	0.23	0.15	0.09
Medium 2 C	0.00	0.07	0.11	0.16	0.28	0.28	0.17	ND
Medium 1 A	0.00	0.15	0.19	0.43	0.58	0.54	0.31	0.24
Medium 1 B	0.00	0.19	0.22	0.37	0.59	0.62	0.29	0.26
Medium 1 C	0.00	0.17	0.22	0.33	0.55	0.67	0.32	0.30
High A	0.00	0.54	0.63	1.03	2.00	2.67	3.65	3.12
High B	0.00	0.33	0.58	1.00	2.14	2.99	3.58	3.18
High C	0.00	0.39	0.50	1.04	2.03	3.38	3.71	3.00

[†] Control = 0.6 mg L^{-1}, Low = 0.6 mg L^{-1}, Medium 2 = 2.9 mg L^{-1}, Medium 1 = 8.9 mg L^{-1}, High = 30 mg L^{-1}

[‡] ND = not detected

Table B6. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for 17β-estradiol-17-sulfate in the aqueous phase of non-sterile subsoil.

Time (h)	0	4	8	24	48	72	168	336
Concentration[†]	mg L^{-1}							
Control A	0.55	0.57	0.00	0.52	0.55	0.58	0.55	0.58
Control B	0.55	0.58	0.55	0.55	0.56	0.59	0.60	0.57
Control C	0.55	0.58	0.66	0.55	0.57	0.58	0.54	0.57
Low A	0.55	0.47	0.00	0.39	0.30	0.27	0.13	0.04
Low B	0.55	0.45	0.45	0.39	0.37	0.27	0.16	0.00
Low C	0.55	0.46	0.42	0.36	0.32	0.28	0.16	0.08
Medium 2 A	2.89	2.77	2.74	2.40	1.96	1.57	1.04	0.26
Medium 2 B	2.89	2.80	2.75	2.41	1.99	1.64	1.08	0.27
Medium 2 C	2.89	2.75	2.70	2.38	2.00	1.67	1.06	0.30
Medium 1 A	8.92	8.81	8.80	7.54	6.23	5.35	3.45	1.55
Medium 1 B	8.92	8.72	8.73	7.26	6.35	5.12	3.12	1.30
Medium 1 C	8.92	8.65	8.36	7.39	6.28	5.20	3.52	1.48
High A	30.34	28.08	27.53	23.88	22.58	20.40	15.76	11.65
High B	30.34	28.57	27.60	24.32	22.56	19.86	15.68	11.25
High C	30.34	28.77	28.64	24.95	21.86	20.50	15.54	11.31

[†] Control = 0.6 mg L^{-1}, Low = 0.6 mg L^{-1}, Medium 2 = 2.9 mg L^{-1}, Medium 1 = 8.9 mg L^{-1}, High = 30 mg L^{-1}

[‡] ND = not detected

Table B7. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for monohydroxy-17β-estradiol-17-sulfate in the aqueous phase of non-sterile subsoil.

Time (h)	0	4	8	24	48	72	168	336
Concentration[†]	mg L^{-1}							
Control A	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01
Control B	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Control C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Low A	0.00	0.01	0.00	0.00	0.02	0.01	0.04	0.03
Low B	0.00	0.00	0.01	0.01	0.03	0.04	0.03	0.00
Low C	0.00	0.00	0.02	0.02	0.03	0.04	0.02	0.02
Medium 2 A	0.00	0.01	0.01	0.06	0.06	0.18	0.16	0.33
Medium 2 B	0.00	0.02	0.04	0.02	0.07	0.14	0.15	0.26
Medium 2 C	0.00	0.03	0.03	0.03	0.13	0.14	0.19	0.32
Medium 1 A	0.00	0.06	0.10	0.16	0.35	0.42	0.61	0.97
Medium 1 B	0.00	0.05	0.05	0.18	0.26	0.41	0.84	1.36
Medium 1 C	0.00	0.04	0.09	0.14	0.34	0.40	0.71	0.95
High A	0.00	0.20	0.18	1.19	0.66	1.25	2.43	4.04
High B	0.00	0.19	0.22	0.49	0.79	1.56	2.75	4.20
High C	0.00	0.25	0.24	0.53	1.27	1.07	3.47	3.90

[†] Control = 0.6 mg L^{-1}, Low = 0.6 mg L^{-1}, Medium 2 = 2.9 mg L^{-1}, Medium 1 = 8.9 mg L^{-1}, High = 30 mg L^{-1}

[‡] ND = not detected

Table B8. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for dihydroxy-17β-estradiol-17-sulfate in the aqueous phase of non-sterile subsoil.

Time (h)	0	4	8	24	48	72	168	336
Concentration[†]	mg L^{-1}							
Control A	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
Control B	0.00	0.00	0.03	0.00	0.01	0.00	0.00	0.01
Control C	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.00
Low A	0.00	0.00	0.00	0.01	0.02	0.04	0.05	0.06
Low B	0.00	0.00	0.00	0.00	0.03	0.03	0.04	0.00
Low C	0.00	0.01	0.00	0.02	0.02	0.03	0.03	0.03
Medium 2 A	0.00	0.02	0.01	0.04	0.09	0.20	0.21	0.31
Medium 2 B	0.00	0.03	0.02	0.07	0.11	0.15	0.19	0.37
Medium 2 C	0.00	0.02	0.01	0.06	0.10	0.14	0.23	0.33
Medium 1 A	0.00	0.10	0.09	0.17	0.40	0.54	0.94	1.18
Medium 1 B	0.00	0.11	0.10	0.19	0.33	0.48	0.71	1.17
Medium 1 C	0.00	0.08	0.10	0.17	0.24	0.47	0.65	1.23
High A	0.00	0.22	0.26	0.57	0.78	0.80	2.13	3.18
High B	0.00	0.21	0.23	0.64	0.85	1.31	2.34	3.14
High C	0.00	0.16	0.30	0.63	1.02	1.09	1.75	3.44

[†] Control = 0.6 mg L^{-1}, Low = 0.6 mg L^{-1}, Medium 2 = 2.9 mg L^{-1}, Medium 1 = 8.9 mg L^{-1}, High = 30 mg L^{-1}

[‡] ND = not detected

Table B9. The experimental data from liquid scintillation counting analysis for the radioactive residue in the aqueous phase of sterile topsoil.

Time (h)	0	4	8	24	48	72	168	336
Concentration[†]	mg L^{-1}							
Control A	0.56	0.51	0.49	0.52	0.50	0.54	0.51	0.50
Control B	0.56	0.52	0.53	0.50	0.51	0.54	0.50	0.51
Control C	0.56	0.50	0.52	0.52	0.53	0.54	0.50	0.51
Low A	0.56	0.19	0.15	0.07	0.07	0.07	0.07	0.06
Low B	0.47	0.29	0.25	0.31	0.17	0.16	0.12	0.11
Low C	0.47	0.30	0.27	0.22	0.17	0.17	0.12	0.11
Medium 2 A	3.14	1.33	0.80	0.50	0.44	0.47	0.36	0.38
Medium 2 B	3.14	1.41	0.82	0.52	0.46	0.46	0.34	0.38
Medium 2 C	3.14	1.35	0.93	0.53	0.47	0.45	0.34	0.37
Medium 1 A	6.56	4.12	2.88	1.51	1.39	1.30	1.02	0.92
Medium 1 B	6.56	4.46	3.22	1.77	1.69	1.67	1.64	1.59
Medium 1 C	6.56	3.55	2.55	1.42	1.29	1.22	0.91	0.88
High A	17.77	14.01	11.25	6.38	5.98	6.19	6.49	5.91
High B	17.77	14.08	11.13	5.99	5.72	5.79	5.92	5.32
High C	17.77	13.46	11.88	6.57	5.61	5.89	6.15	5.93

[†] Control = 0.5 mg L^{-1}, Low = 0.5 mg L^{-1}, Medium 2 = 3.2 mg L^{-1}, Medium 1 = 7.4 mg L^{-1}, High = 18 mg L^{-1}

Table B10. The experimental data from liquid scintillation counting analysis for the radioactive residue in the aqueous phase of sterile subsoil.

Time (h)	0	4	8	24	48	72	168	336
Concentration[†]	mg L^{-1}							
Control A	0.56	0.51	0.49	0.52	0.50	0.54	0.51	0.50
Control B	0.56	0.52	0.53	0.50	0.51	0.54	0.50	0.51
Control C	0.56	0.50	0.52	0.52	0.53	0.54	0.50	0.51
Low A	0.56	0.46	0.45	0.46	0.39	0.41	0.34	0.23
Low B	0.56	0.46	0.47	0.42	0.40	0.38	0.29	0.19
Low C	0.56	0.48	0.47	0.44	0.42	0.40	0.25	0.14
Medium 2 A	3.35	2.97	2.95	2.99	2.85	2.79	2.66	2.67
Medium 2 B	3.35	3.05	3.07	3.06	3.05	3.00	2.78	2.71
Medium 2 C	3.35	3.15	3.13	3.11	3.10	3.01	2.95	3.07
Medium 1 A	6.56	6.80	6.54	6.44	6.03	5.60	4.28	3.13
Medium 1 B	8.18	7.60	7.63	7.58	7.44	7.37	7.36	7.18
Medium 1 C	8.18	7.76	7.80	7.78	7.96	7.55	7.38	7.22
High A	18.06	16.75	16.62	16.33	16.61	15.90	15.54	15.40
High B	18.06	16.89	17.12	19.07	17.12	16.28	16.46	14.85
High C	18.06	17.11	17.23	16.24	17.30	16.49	16.64	15.62

[†] Control = 0.5 mg L^{-1}, Low = 0.5 mg L^{-1}, Medium 2 = 3.2 mg L^{-1}, Medium 1 = 7.4 mg L^{-1}, High = 18 mg L^{-1}

Table B11. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for 17β-estradiol-17-sulfate in the aqueous phase of sterile topsoil.

Time (h)	0	4	8	24	48	72	168	336
Concentration[†]	mg L^{-1}							
Control A	0.56	0.50	0.48	0.52	0.45	0.53	0.48	0.46
Control B	0.56	0.50	0.53	0.48	0.48	0.51	0.48	0.49
Control C	0.56	0.49	0.53	0.49	0.51	0.53	0.47	0.49
Low A	0.56	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Low B	0.47	0.27	0.24	0.26	0.14	0.13	0.00	0.00
Low C	0.47	0.28	0.26	0.19	0.14	0.13	0.00	0.00
Medium 2 A	3.14	0.95	0.44	0.06	0.08	0.02	0.03	0.02
Medium 2 B	3.14	0.98	0.42	0.07	0.01	0.04	0.05	0.06
Medium 2 C	3.14	0.97	0.49	0.04	0.08	0.02	0.05	0.02
Medium 1 A	6.56	3.40	2.06	0.36	0.20	0.18	0.15	0.14
Medium 1 B	6.56	3.80	2.44	0.48	0.24	0.26	0.22	0.23
Medium 1 C	6.56	2.92	1.71	0.23	0.18	0.14	0.10	0.12
High A	17.77	12.18	9.37	2.79	1.32	1.15	1.03	1.01
High B	17.77	12.57	9.40	2.33	1.15	1.40	0.86	0.89
High C	17.77	12.07	10.51	3.45	1.05	1.21	1.10	0.97

[†]Control = 0.5 mg L^{-1}, Low = 0.5 mg L^{-1}, Medium 2 = 3.2 mg L^{-1}, Medium 1 = 7.4 mg L^{-1}, High = 18 mg L^{-1}

[‡] ND = not detected

Table B12. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for monohydroxy-17β-estradiol-17-sulfate in the aqueous phase of sterile topsoil.

Time (h)	0	4	8	24	48	72	168	336
Concentration[†]				mg L^{-1}				
Control A	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Control B	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Control C	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01
Low A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Low B	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00
Low C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Medium 2 A	0.00	0.13	0.09	0.08	0.06	0.06	0.04	0.04
Medium 2 B	0.00	0.16	0.15	0.10	0.06	0.08	0.06	0.04
Medium 2 C	0.00	0.13	0.21	0.12	0.06	0.07	0.03	0.06
Medium 1 A	0.00	0.34	0.30	0.28	0.23	0.22	0.16	0.14
Medium 1 B	0.00	0.26	0.25	0.30	0.38	0.34	0.41	0.29
Medium 1 C	0.00	0.31	0.26	0.27	0.37	0.27	0.14	0.12
High A	0.00	0.93	0.72	1.22	1.39	1.53	1.40	1.89
High B	0.00	0.78	0.80	1.20	1.36	1.93	1.29	1.01
High C	0.00	0.67	0.37	0.95	1.30	1.44	1.50	1.43

[†]Control = 0.5 mg L^{-1}, Low = 0.5 mg L^{-1}, Medium 2 = 3.2 mg L^{-1}, Medium 1 = 7.4 mg L^{-1}, High = 18 mg L^{-1}

[‡] ND = not detected

Table B13. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for dihydroxy-17β-estradiol-17-sulfate in the aqueous phase of sterile topsoil.

Time (h)	0	4	8	24	48	72	168	336
Concentration[†]				mg L^{-1}				
Control A	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Control B	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
Control C	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
Low A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Low B	0.00	0.01	0.00	0.01	0.02	0.01	0.00	0.00
Low C	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.00
Medium 2 A	0.00	0.21	0.23	0.34	0.28	0.33	0.25	0.27
Medium 2 B	0.00	0.23	0.22	0.34	0.33	0.29	0.21	0.24
Medium 2 C	0.00	0.19	0.20	0.34	0.29	0.31	0.23	0.25
Medium 1 A	0.00	0.36	0.46	0.78	0.93	0.81	0.64	0.59
Medium 1 B	0.00	0.34	0.44	0.84	0.96	0.92	0.91	0.99
Medium 1 C	0.00	0.29	0.54	0.75	0.66	0.80	0.66	0.58
High A	0.00	0.83	1.06	2.17	2.69	2.97	3.50	2.66
High B	0.00	0.63	0.80	2.29	2.61	1.99	3.34	3.03
High C	0.00	0.58	0.73	1.62	2.39	2.41	3.04	3.15

[†]Control = 0.5 mg L^{-1}, Low = 0.5 mg L^{-1}, Medium 2 = 3.2 mg L^{-1}, Medium 1 = 7.4 mg L^{-1}, High = 18 mg L^{-1}

[‡] ND = not detected

Table B14. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for 17β-estradiol-17-sulfate in the aqueous phase of sterile subsoil.

Time (h)	0	4	8	24	48	72	168	336
Concentration[†]	mg L^{-1}							
Control A	0.56	0.50	0.48	0.52	0.45	0.53	0.48	0.46
Control B	0.56	0.50	0.53	0.48	0.48	0.51	0.48	0.49
Control C	0.56	0.49	0.53	0.49	0.51	0.53	0.47	0.49
Low A	0.56	0.39	0.43	0.43	0.34	0.36	0.26	0.16
Low B	0.56	0.39	0.46	0.40	0.36	0.33	0.23	0.13
Low C	0.56	0.42	0.45	0.42	0.36	0.33	0.19	0.09
Medium 2 A	3.35	2.84	2.79	2.90	2.68	2.59	2.42	2.51
Medium 2 B	3.35	2.97	2.91	2.94	2.69	2.78	2.49	2.47
Medium 2 C	3.35	3.04	2.97	2.97	2.81	2.79	2.73	2.77
Medium 1 A	6.56	6.50	6.21	6.16	5.58	5.01	3.11	1.33
Medium 1 B	8.18	7.15	7.17	7.02	6.68	6.58	6.46	6.46
Medium 1 C	8.18	7.35	7.46	7.22	6.85	6.93	6.34	6.53
High A	18.06	16.21	15.69	15.77	15.44	14.55	14.35	14.11
High B	18.06	16.22	16.69	18.32	15.73	15.26	15.30	13.60
High C	18.06	16.51	16.43	15.59	16.08	15.90	15.45	14.75

[†]Control = 0.5 mg L^{-1}, Low = 0.5 mg L^{-1}, Medium 2 = 3.2 mg L^{-1}, Medium 1 = 7.4 mg L^{-1}, High = 18 mg L^{-1}

[‡] ND = not detected

Table B15. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for monohydroxy-17β-estradiol-17-sulfate in the aqueous phase of sterile subsoil.

Time (h)	0	4	8	24	48	72	168	336
Concentration[†]				mg L^{-1}				
Control A	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Control B	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Control C	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01
Low A	0.00	0.02	0.00	0.01	0.02	0.00	0.02	0.01
Low B	0.00	0.01	0.00	0.00	0.00	0.01	0.03	0.00
Low C	0.00	0.01	0.00	0.00	0.01	0.01	0.01	0.00
Medium 2 A	0.00	0.07	0.09	0.05	0.09	0.10	0.12	0.11
Medium 2 B	0.00	0.04	0.10	0.07	0.11	0.10	0.23	0.15
Medium 2 C	0.00	0.03	0.07	0.09	0.17	0.13	0.12	0.21
Medium 1 A	0.00	0.22	0.22	0.14	0.24	0.28	0.41	0.56
Medium 1 B	0.00	0.30	0.35	0.44	0.46	0.62	0.68	0.49
Medium 1 C	0.00	0.27	0.23	0.36	0.93	0.46	0.79	0.40
High A	0.00	0.32	0.67	0.35	0.91	1.06	0.87	0.83
High B	0.00	0.49	0.25	0.47	1.15	0.68	0.72	0.65
High C	0.00	0.34	0.59	0.46	0.95	0.28	0.74	0.62

[†]Control = 0.5 mg L^{-1}, Low = 0.5 mg L^{-1}, Medium 2 = 3.2 mg L^{-1}, Medium 1 = 7.4 mg L^{-1}, High = 18 mg L^{-1}

[‡] ND = not detected

Table B16. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for dihydroxy-17β-estradiol-17-sulfate in the aqueous phase of sterile subsoil.

Time (h)	0	4	8	24	48	72	168	336
Concentration[†]				mg L^{-1}				
Control A	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Control B	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
Control C	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
Low A	0.00	0.00	0.00	0.00	0.01	0.01	0.03	0.02
Low B	0.00	0.03	0.00	0.00	0.01	0.02	0.02	0.04
Low C	0.00	0.03	0.00	0.00	0.02	0.02	0.02	0.01
Medium 2 A	0.00	0.01	0.01	0.01	0.03	0.03	0.05	0.05
Medium 2 B	0.00	0.01	0.03	0.02	0.01	0.04	0.03	0.07
Medium 2 C	0.00	0.02	0.02	0.01	0.02	0.02	0.04	0.06
Medium 1 A	0.00	0.05	0.04	0.08	0.17	0.26	0.70	1.19
Medium 1 B	0.00	0.10	0.05	0.08	0.17	0.11	0.13	0.12
Medium 1 C	0.00	0.07	0.03	0.14	0.12	0.11	0.14	0.20
High A	0.00	0.13	0.18	0.13	0.19	0.18	0.19	0.33
High B	0.00	0.11	0.12	0.19	0.14	0.19	0.29	0.51
High C	0.00	0.10	0.13	0.12	0.15	0.14	0.32	0.19

[†]Control = 0.5 mg L^{-1}, Low = 0.5 mg L^{-1}, Medium 2 = 3.2 mg L^{-1}, Medium 1 = 7.4 mg L^{-1}, High = 18 mg L^{-1}

[‡] ND = not detected

APPENDIX C. THE EXPERIMENTAL DATA FOR BATCH EXPERIMENTS OF STOP VIALS

Table C1. The experimental data for the stop vials using non-sterile topsoil with 0.6 mg L^{-1} of 17β-estradiol-17-sulfate.

Time (h)	Aqueous Phase	Sorbed Phase			Total	Recovery (%)
		Water Extract	Acetone Extract	Non-extractable		
	^{14}C (dpm) assayed by LSC					
0	28820	0	0	0	28820	100
4	16241	1276	430	7569	25516	89
8	14356	1744	1246	11469	28815	100
24	6425	1459	995	19256	28136	98
48	5087	2284	1129	21591	30091	104
72	3772	1459	1218	20249	26696	93
168	5223	1588	1457	18578	26845	93
336	2509	1191	1877	17055	22631	79

Table C2. The experimental data for the stop vials using non-sterile subsoil with 0.6 mg L^{-1} of 17β-estradiol-17-sulfate.

Time (h)	Aqueous Phase	Sorbed Phase			Total	Recovery (%)
		Water Extract	Acetone Extract	Non-extractable		
	^{14}C (dpm) assayed by LSC					
0	28820	0	0	0	28820	100
4	26660	614	325	3176	30775	107
8	25900	2976	354	2345	31575	110
24	24156	2617	922	2730	30424	106
48	20315	2640	675	3854	27484	95
72	17157	3048	674	4142	25021	87
168	10357	2326	1452	6250	20386	71
336	3818	1290	566	10792	16466	57

Table C3. The experimental data for the stop vials using sterile topsoil with 0.5 mg L^{-1} of 17β-estradiol-17-sulfate.

Time (h)	Aqueous Phase	Sorbed Phase			Total	Recovery (%)
		Water Extract	Acetone Extract	Non-extractable		
	^{14}C (dpm) assayed by LSC					
0	24550	0	0	0	24550	100
4	12914	748	281	8373	22316	76
8	13959	715	703	6094	21472	87
24	11359	893	749	7167	20167	82
48	8743	1026	432	7684	17885	73
72	8012	809	745	9703	19269	78
168	5659	724	540	9593	16516	67
336	3721	414	1058	11603	16796	68

Table C4. The experimental data for the stop vials using sterile subsoil with 0.5 mg L^{-1} of 17β-estradiol-17-sulfate.

Time (h)	Aqueous Phase	Sorbed Phase			Total	Recovery (%)
		Water Extract	Acetone Extract	Non-extractable		
	^{14}C (dpm) assayed by LSC					
0	29420	0	0	0	29420	100
4	23814	1591	298	2015	27719	94
8	23417	722	281	1294	25714	87
24	23739	725	235	1691	26389	90
48	20984	983	275	1977	24219	82
72	19642	1238	437	3210	24528	83
168	19723	773	260	3139	23895	81
336	11681	716	795	3913	17104	58

Table C5. The high performance liquid chromatography and liquid scintillation counting data for the aqueous metabolites in stop vials with non-sterile topsoil.

Time (h)	E2-17S	OH-E2-17S	diOH-E2-17S	Total
		^{14}C (dpm) assayed by LSC		
0	28820			
4	13333	2396	463	16192
8	5235	7301	1526	14062
24	902	3647	1829	6377
48	571	2146	2368	5085
72	371	1376	1968	3716
168	2367	812	1639	4818
336	232	908	1154	2294

Table C6. The high performance liquid chromatography and liquid scintillation counting data for the aqueous metabolites in stop vials with non-sterile subsoil.

Time (h)	E2-17S	OH-E2-17S	diOH-E2-17S	Total
		^{14}C (dpm) assayed by LSC		
0	28820			
4	18371	6391	916	25678
8	5730	18789	993	25512
24	7373	15563	1074	24009
48	3420	15268	1541	20229
72	2660	12673	1681	17014
168	2267	5621	1967	9855
336	341	1112	2364	3818

Table C7. The high performance liquid chromatography and liquid scintillation counting data for the aqueous metabolites in stop vials with sterile topsoil.

Time (h)	E2-17S	OH-E2-17S	diOH-E2-17S	Total
		^{14}C (dpm) assayed by LSC		
0	24550			
4	1153	8095	3389	12638
8	2127	9583	2175	13885
24	1981	6426	2664	11070
48	1051	4868	2803	8722
72	506	3686	3770	7962
168	385	2618	2629	5632
336	185	1021	2507	3713

Table C8. The high performance liquid chromatography and liquid scintillation counting data for the aqueous metabolites in stop vials with sterile subsoil.

Time (h)	E2-17S	OH-E2-17S	diOH-E2-17S	Total
		^{14}C (dpm) assayed by LSC		
0	29420			
4	8140	14286	1224	23650
8	5818	16427	1064	23308
24	4268	18128	1039	23434
48	2286	16884	1612	20781
72	4659	13208	1630	19497
168	2702	14831	2048	19581
336	1302	7791	2483	11576

Table C9. The high performance liquid chromatography and liquid scintillation counting data for the reversibly sorbed phase in stop vials with non-sterile topsoil.

Time (h)	E2-17S	OH-E2-17S	diOH-E2-17S	E2	E1	Unknown	Total
			^{14}C (dpm) assayed by LSC				
4	313	158	779	17	158	22	1446
8	1094	850	792	26	31	44	2837
24	675	286	1249	21	91	41	2363
48	380	413	2019	57	158	82	3110
72	346	200	1416	33	178	89	2261
168	1146	464	749	58	213	201	2832
336	1064	335	768	164	340	225	2896

Table C10. The high performance liquid chromatography and liquid scintillation counting data for the reversibly sorbed phase in stop vials with non-sterile subsoil.

Time (h)	E2-17S	OH-E2-17S	diOH-E2-17S	E2	E1	Unknown	Total
			^{14}C (dpm) assayed by LSC				
4	198	132	352	10	9	110	812
8	1311	1206	603	9	4	83	3216
24	1217	970	873	4	46	314	3424
48	1283	775	832	23	33	282	3227
72	1518	973	679	17	27	296	3511
168	1028	673	800	76	150	684	3411
336	440	189	542	41	80	204	1496

Table C11. The high performance liquid chromatography and liquid scintillation counting data for the reversibly sorbed phase in stop vials with sterile topsoil.

Time (h)	E2-17S	OH-E2-17S	diOH-E2-17S	E2	E1	Unknown	Total
			^{14}C (dpm) assayed by LSC				
4	95	121	728	0	1	3	948
8	750	137	460	9	13	4	1373
24	584	190	784	14	13	3	1588
48	795	228	480	4	15	ND	1521
72	896	222	393	9	44	3	1567
168	543	159	408	16	60	3	1189
336	579	105	481	17	165	9	1357

Table C12. The high performance liquid chromatography and liquid scintillation counting data for the reversibly sorbed phase in stop vials with sterile subsoil.

Time (h)	E2-17S	OH-E2-17S	diOH-E2-17S	E2	E1	Unknown	Total
			^{14}C (dpm) assayed by LSC				
4	1017	634	23	2	19	44	1740
8	408	61	281	36	16	25	828
24	497	259	224	7	0	1	988
48	564	250	400	4	40	10	1269
72	671	579	653	22	41	6	1971
168	397	284	443	7	10	6	1147
336	769	244	255	46	183	5	1503

APPENDIX D. METABOLITES SPECIATION AND CHARACTERIZATION BY HIGH PERFORMANCE LIQUID CHROMOTOGRAPHY

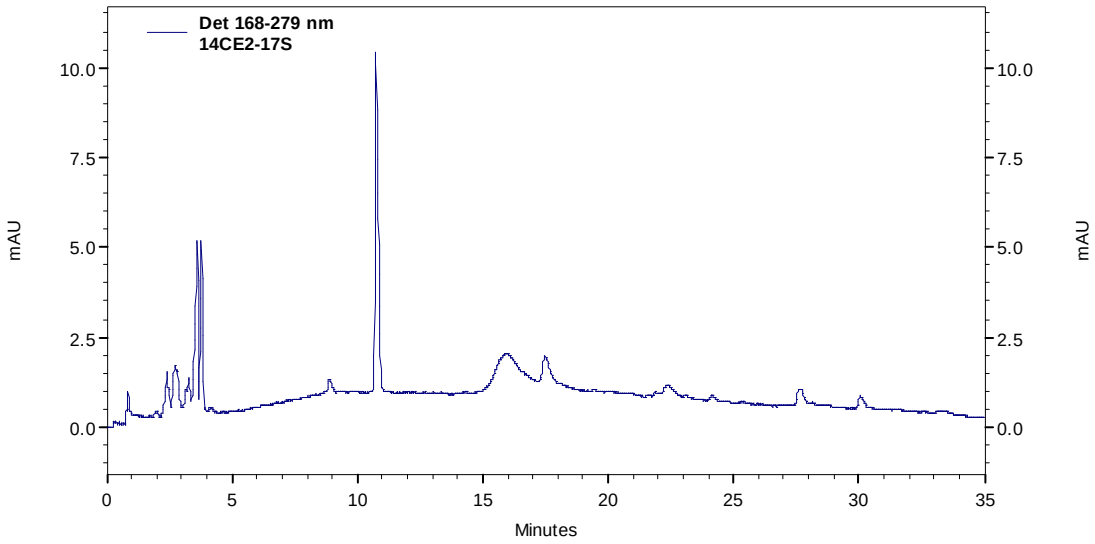

Figure D1. Chromatogram of aqueous phase speciation in the non-sterile subsoil at 4 h.

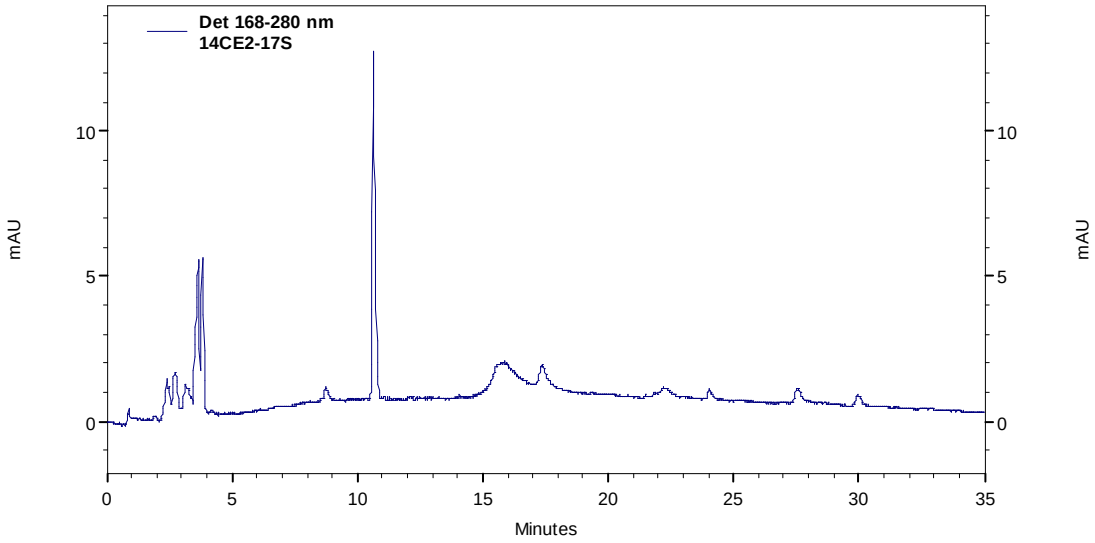

Figure D2. Chromatogram of aqueous phase speciation in the non-sterile subsoil at 8 h.

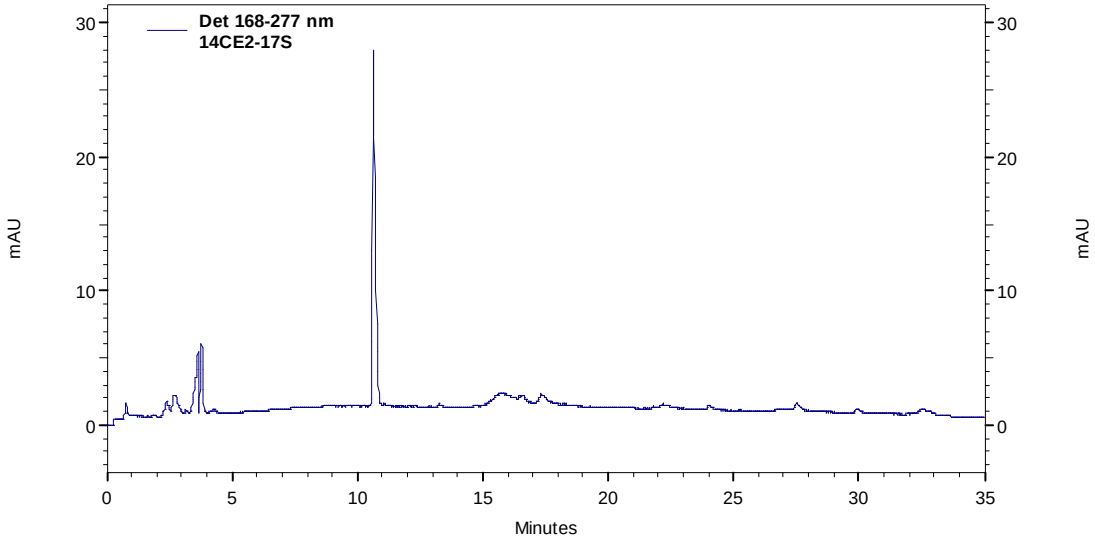

Figure D3. Chromatogram of aqueous phase speciation in the non-sterile subsoil at 24 h.

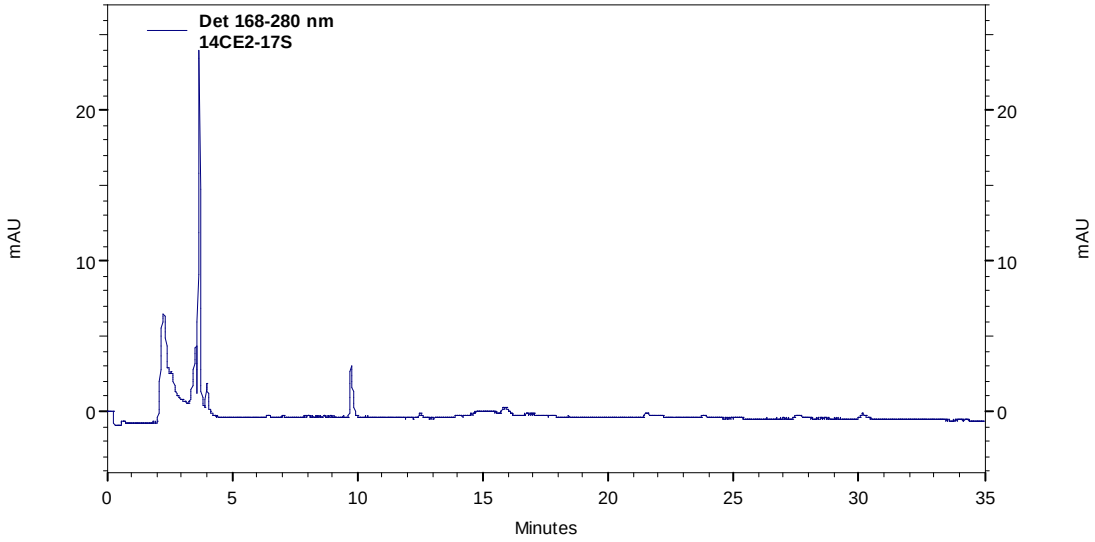

Figure D4. Chromatogram of aqueous phase speciation in the non-sterile topsoil at 24 h.

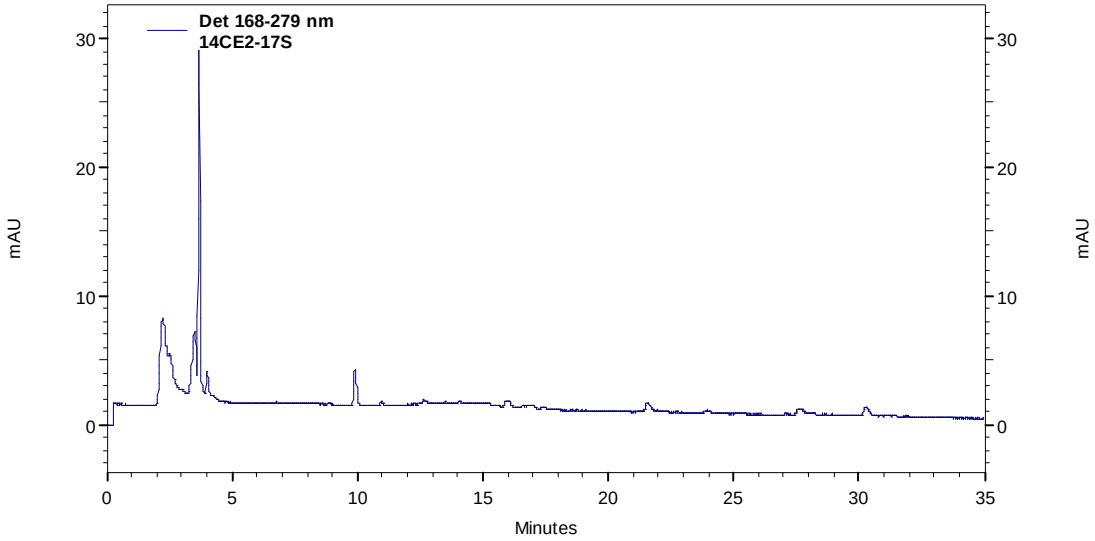

Figure D5. Chromatogram of aqueous phase speciation in the non-sterile topsoil at 48 h.

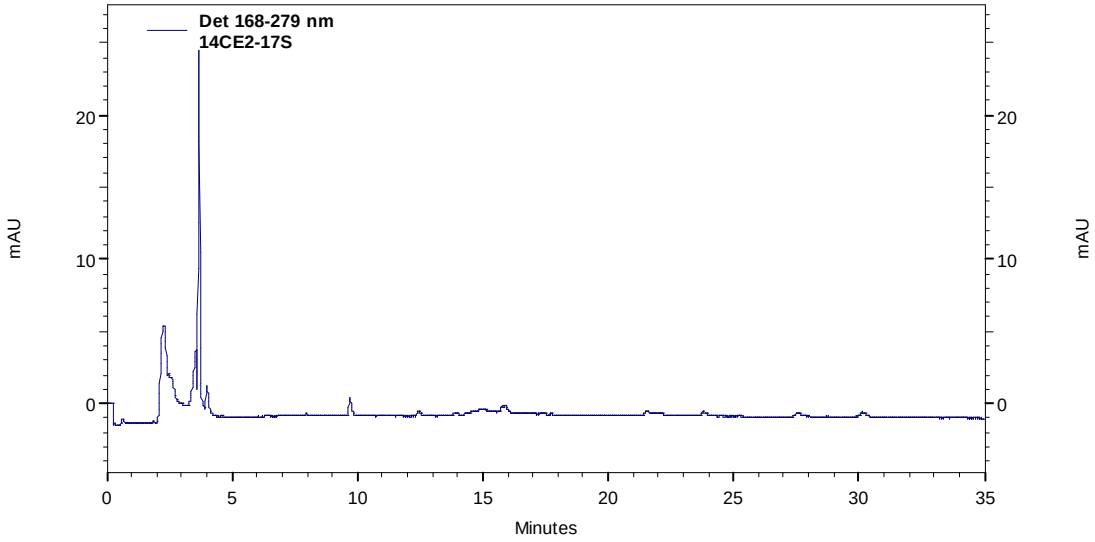

Figure D6. Chromatogram of aqueous phase speciation in the non-sterile topsoil at 72 h.

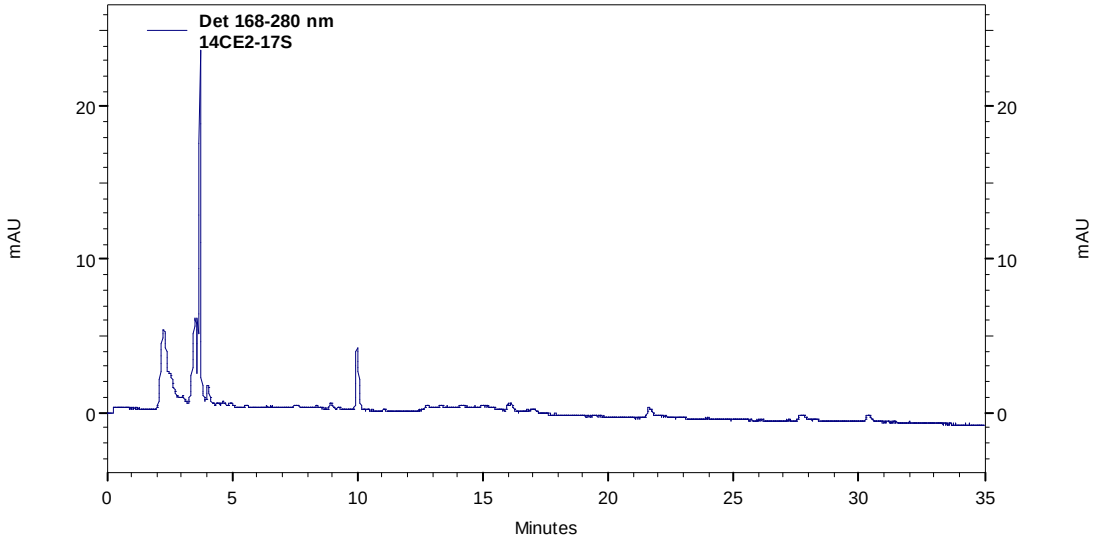

Figure D7. Chromatogram of aqueous phase speciation in the non-sterile topsoil at 336 h.

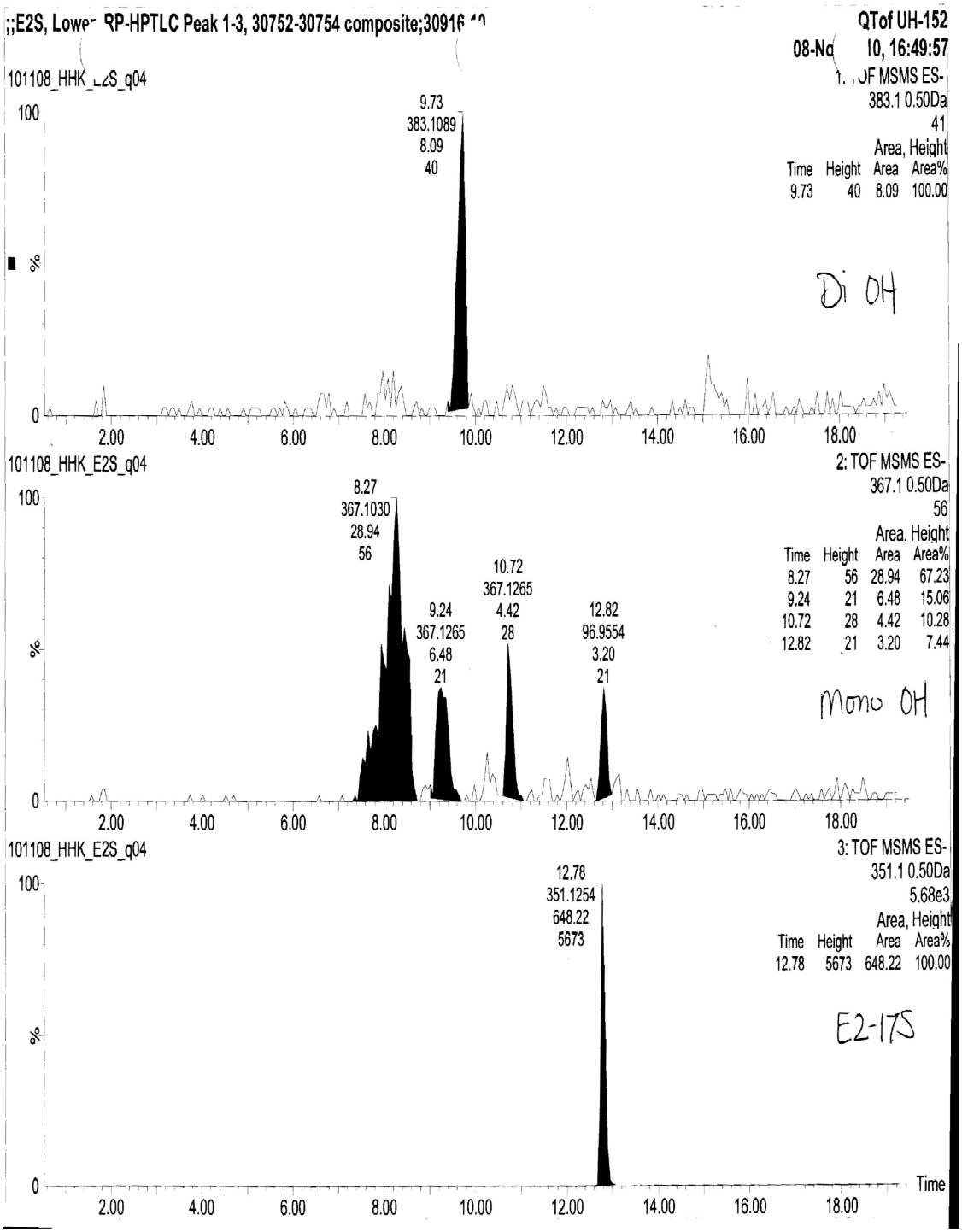

Figure D8. LC-MS/MS Spectra for hydroxyl metabolites characterization (1).

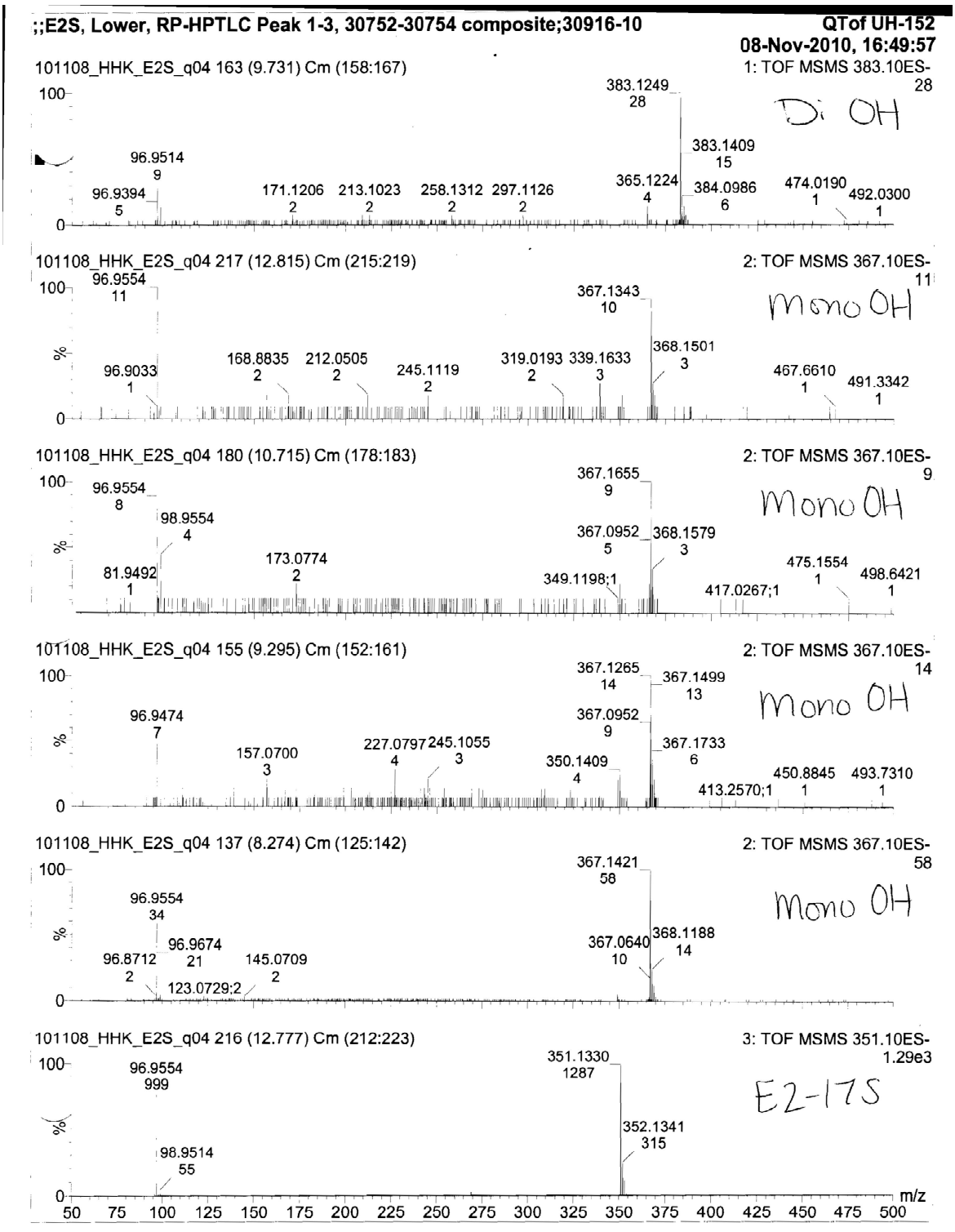

Figure D9. LC-MS/MS Spectra for hydroxyl metabolites characterization (2).

APPENDIX E. THE C SOURCE CODE FOR PAPER 4

```
#include <stdio.h>
#include <stdlib.h>
#include <math.h>

#include "sharefunc.h"
#include "ESSRSort.h"
#include "ESES.h"

#include "llnltyps.h"
#include "cvode.h"
#include "cvdense.h"
#include "nvector.h"
#include "dense.h"

#define Ith(v,i) N_VIth(v,i-1)
#define IJth(A,i,j) DENSE_ELEM(A,i-1,j-1)
#define SIM2

#undef OUTPUT
#undef REFINE

#define MV 200.0
#define WT1 10000.0

int NEQ, tn, dim;

double RTOL, ATOL;
double T0, T1, Tm;

ESfcnTrsfm *trsfm;
double w1un, w2un, w3un;
double w1us, w2us, w3us;
double w1ln, w2ln, w3ln;
double w1ls, w2ls, w3ls;
double s1u, s2u, s3u;
double a1u, a2u, a3u;
double b1u, b2u, b3u;
```

```
double kd1u, kd2u, kd3u;
double s1l, s2l, s3l;
double a1l, a2l, a3l;
double b1l, b2l, b3l;
double kd1l, kd2l, kd3l;

double **w_mun, **w_mus, **w_mln, **w_mls;
double **a_mun, **a_mus, **a_mln, **a_mls;
double *c_mun, *c_mus, *c_mln, *c_mls;

double transform(double x);
void fitness(double *, double *, double *);
static void difeq(integer N, real t, N_Vector y, N_Vector ydot, void *f_data);
double square(double xxx);
double **ReadWA(const char file[], const int iRow, const int iCol);
double *ReadC(const char file[], const int iRow);

int main(int argc, char **argv) {
        int i, es, constraint, miu, lambda, gen, retry;
        unsigned int seed;
        double *ub, *lb, gamma, alpha, varphi, pf;
        double *sim_para;

        ESParameter *param;
        ESPopulation *population;
        ESStatistics *stats;

        seed = shareDefSeed;
        gamma = esDefGamma;
        alpha = esDefAlpha;
        varphi = esDefVarphi;
        retry = esDefRetry;
        pf = essrDefPf;
        es = esDefESSlash;

        constraint = 1;
        dim = 36;
        miu = 300;
        lambda = 3500;
        gen = 100000000;
```

```
	ub = NULL;
	lb = NULL;

	ub = ShareMallocM1d(dim);
	lb = ShareMallocM1d(dim);
	sim_para = ShareMallocM1d(dim);

	trsfm = (ESfcnTrsfm *)ShareMallocM1c(dim * sizeof(ESfcnTrsfm));

	for (i = 0; i < dim; i++)
		trsfm[i] = transform;

	for (i = 0; i < dim; i++) {
		lb[i] = 1e-8;
		ub[i] = 1.0;
	}

#ifdef SIM2

#ifdef OUTPUT
	sim_para = ReadC("sim2_para.txt", dim);

	for (i = 0; i < dim; i++) {
		ub[i] = sim_para[i];
		lb[i] = ub[i];
	}

#endif

#ifndef OUTPUT

#ifdef REFINE
	sim_para = ReadC("sim2_para.txt", dim);

	for (i = 0; i < dim; i++) {
		ub[i] = sim_para[i] * 3.0;
		lb[i] = sim_para[i] * 0.3;
	}
#endif

#ifndef REFINE
```

```
#endif
#endif
#endif

        NEQ = 52;
        RTOL = 1e-4;
        ATOL = 1e-4;
        T0 = 0.0;
        T1 = 0.1;
        Tm = 338;

        w_mun = ShareMallocM2d(7, 4);
        w_mus = ShareMallocM2d(7, 4);
        w_mln = ShareMallocM2d(7, 4);
        w_mls = ShareMallocM2d(7, 4);

        a_mun = ShareMallocM2d(7, 6);
        a_mus = ShareMallocM2d(7, 6);
        a_mln = ShareMallocM2d(7, 6);
        a_mls = ShareMallocM2d(7, 6);

        c_mun = ShareMallocM1d(7);
        c_mus = ShareMallocM1d(7);
        c_mln = ShareMallocM1d(7);
        c_mls = ShareMallocM1d(7);

        w_mun = ReadWA("w_mun.txt", 7, 4);
        w_mus = ReadWA("w_mus.txt", 7, 4);
        w_mln = ReadWA("w_mln.txt", 7, 4);
        w_mls = ReadWA("w_mls.txt", 7, 4);

        a_mun = ReadWA("a_mun.txt", 7, 6);
        a_mus = ReadWA("a_mus.txt", 7, 6);
        a_mln = ReadWA("a_mln.txt", 7, 6);
        a_mls = ReadWA("a_mls.txt", 7, 6);

        c_mun = ReadC("c_mun.txt", 7);
        c_mus = ReadC("c_mus.txt", 7);
        c_mln = ReadC("c_mln.txt", 7);
        c_mls = ReadC("c_mls.txt", 7);
```

```
ESInitial(seed, &param, trsfm, fitness, es, constraint, dim, ub, lb,
      miu, lambda, gen, gamma, alpha, varphi, retry, &population,
      &stats);
while (stats->curgen < param->gen)
        ESStep(population, param, stats, pf);
ESDeInitial(param, population, stats);
ShareFreeM1c((char *) trsfm);

ShareFreeM1d(ub);
ub = NULL;
ShareFreeM1d(lb);
lb = NULL;
ShareFreeM1d(sim_para);
sim_para = NULL;
ShareFreeM1d(c_mun);
c_mun = NULL;
ShareFreeM1d(c_mus);
c_mus = NULL;
ShareFreeM1d(c_mln);
c_mln = NULL;
ShareFreeM1d(c_mls);
c_mls = NULL;

ShareFreeM2d(w_mun, 7);
w_mun = NULL;
ShareFreeM2d(w_mus, 7);
w_mus = NULL;
ShareFreeM2d(w_mln, 7);
w_mln = NULL;
ShareFreeM2d(w_mls, 7);
w_mls = NULL;

ShareFreeM2d(a_mun, 7);
a_mun = NULL;
ShareFreeM2d(a_mus, 7);
a_mus = NULL;
ShareFreeM2d(a_mln, 7);
a_mln = NULL;
ShareFreeM2d(a_mls, 7);
a_mls = NULL;
```

```
	return 0;
}

void fitness(double *x, double *f, double *g) {
	real ropt[OPT_SIZE], reltol, t, tout;
	long int iopt[OPT_SIZE];
	N_Vector y;
	real abstol;
	double sum1, sum2, sum3, sum4,
	sumr_mun=0.0, sumr_mus=0.0, sumr_mln=0.0, sumr_mls=0.0,
	aceton_mun=0.0, aceton_mus=0.0, aceton_mln=0.0, aceton_mls=0.0,
	combus_mun=0.0, combus_mus=0.0, combus_mln=0.0, combus_mls=0.0;
	void *cvode_mem;
	int iout, flag, i, iPos = -1;

#ifdef OUTPUT
	FILE *mun, *mus, *mln, *mls;

#ifdef SIM2

	if ((mun = fopen("mun_output2.txt", "w")) == NULL) {
		printf("fopen %s failed!\n", "mun_output.txt");
		exit(-1);
	}
	if ((mus = fopen("mus_output2.txt", "w")) == NULL) {
		printf("fopen %s failed!\n", "mus_output.txt");
		exit(-1);
	}
	if ((mln = fopen("mln_output2.txt", "w")) == NULL) {
		printf("fopen %s failed!\n", "mln_output.txt");
		exit(-1);
	}
	if ((mls = fopen("mls_output2.txt", "w")) == NULL) {
		printf("fopen %s failed!\n", "mls_output.txt");
		exit(-1);
	}
#endif
#endif

	sum1 = 0.0;
```

```
sum2 = 0.0;
sum3 = 0.0;
sum4 = 0.0;
sumr_mun= 0.0;
sumr_mus= 0.0;
sumr_mln= 0.0;
sumr_mls= 0.0;

w1un = (trsfm[0])(x[0]);
w2un = (trsfm[1])(x[1]);
w3un = (trsfm[2])(x[2]);
w1us = (trsfm[3])(x[3]);
w2us = (trsfm[4])(x[4]);
w3us = (trsfm[5])(x[5]);
w1ln = (trsfm[6])(x[6]);
w2ln = (trsfm[7])(x[7]);
w3ln = (trsfm[8])(x[8]);
w1ls = (trsfm[9])(x[9]);
w2ls = (trsfm[10])(x[10]);
w3ls = (trsfm[11])(x[11]);
s1u = (trsfm[12])(x[12]);
s2u = (trsfm[13])(x[13]);
s3u = (trsfm[14])(x[14]);
s1l = (trsfm[15])(x[15]);
s2l = (trsfm[16])(x[16]);
s3l = (trsfm[17])(x[17]);
a1u = (trsfm[18])(x[18]);
a2u = (trsfm[19])(x[19]);
a3u = (trsfm[20])(x[20]);
b1u = (trsfm[21])(x[21]);
b2u = (trsfm[22])(x[22]);
b3u = (trsfm[23])(x[23]);
kd1u = (trsfm[24])(x[24]);
kd2u = (trsfm[25])(x[25]);
kd3u = (trsfm[26])(x[26]);
a1l = (trsfm[27])(x[27]);
a2l = (trsfm[28])(x[28]);
a3l = (trsfm[29])(x[29]);
b1l = (trsfm[30])(x[30]);
b2l = (trsfm[31])(x[31]);
b3l = (trsfm[32])(x[32]);
```

```
        kd1l = (trsfm[33])(x[33]);
        kd2l = (trsfm[34])(x[34]);
        kd3l = (trsfm[35])(x[35]);

        if (kd1u <= kd1l || kd2u <= kd2l || kd3u <= kd3l) {
                (*f) = 800000000000.0;
                g[0] = 0.0;
                return ;
        }

        if (w1un <= w1ln || w1us <= w1ls) {
                (*f) = 800000000000.0;
                g[0] = 0.0;
                return ;
        }
if (w2un <= w2ln || w2us <= w2ls) {
                (*f) = 800000000000.0;
                g[0] = 0.0;
                return ;
        }
        if (s1u <= s1l || s2u <= s2l || s3u >= s3l) {
                (*f) = 800000000000.0;
                g[0] = 0.0;
                return ;
        }
        if (kd3u < kd1u || kd1u < kd2u) {
                g[0] = 800000000000.0;
                return ;
        }
        if (kd3l < kd1l || kd1l < kd2l) {
                (*f) = 800000000000.0;
                g[0] = 0.0;
                return ;
        }
        y = N_VNew(NEQ, NULL);

        for (i = 1; i <= NEQ; i++)
                Ith(y, i) = 0.0;
```

```
Ith(y, 1) = 1.0;
Ith(y, 14) = 1.0;
Ith(y, 27) = 1.0;
Ith(y, 40) = 1.0;

reltol = RTOL;
abstol = ATOL;

cvode_mem =
   CVodeMalloc(NEQ, difeq, T0, y, BDF, NEWTON, SS, &reltol,
         &abstol, NULL, NULL, FALSE, iopt, ropt, NULL);

if (cvode_mem == NULL) {
         printf("CVodeMalloc failed.\n");
         exit(1);
}
CVDense(cvode_mem, NULL, NULL);

for (iout = 1, tout = T1; tout <= Tm;  iout++, tout = iout * T1) {
         flag = CVode(cvode_mem, tout, y, &t, NORMAL);

         if (flag != SUCCESS) {
                  (*f) = 800000000000.0;
                  g[0] = 0.0;
                  return ;
         }
         iPos = -1;

         if (iout == 40.0)
                  iPos = 0;
         if (iout == 80.0)
                  iPos = 1;
         if (iout == 240.0)
                  iPos = 2;
         if (iout == 480.0)
                  iPos = 3;
         if (iout == 720.0)
                  iPos = 4;
         if (iout == 1680.0)
                  iPos = 5;
         if (iout == 3360.0)
```

```
                iPos = 6;

        if (iPos == 0 || iPos == 1 || iPos == 2 || iPos == 3 || iPos == 4 || iPos == 5 || iPos ==
6) {

        aceton_mun = Ith(y,5)+Ith(y,6)+Ith(y,7)+Ith(y,8)+Ith(y,9)+Ith(y,10);
        combus_mun = Ith(y,11) + Ith(y,12)+Ith(y,13);

                if (iPos == 0)
                sumr_mun = sumr_mun+(aceton_mun/combus_mun - 0.3105) * WT1;

                if (iPos == 1)
                sumr_mun = sumr_mun + (aceton_mun/combus_mun - 0.3215) * WT1;

                if (iPos == 2)
                sumr_mun = sumr_mun + (aceton_mun/combus_mun - 0.1502) * WT1;

                if (iPos == 3)
                sumr_mun = sumr_mun + (aceton_mun/combus_mun - 0.1206) * WT1;

                if (iPos == 4)
                sumr_mun = sumr_mun + (aceton_mun/combus_mun - 0.1341) *WT1;

                if (iPos == 5)
                sumr_mun = sumr_mun + (aceton_mun/combus_mun - 0.1769) * WT1;

                if (iPos == 6)
                sumr_mun = sumr_mun + (aceton_mun/combus_mun - 0.1594) * WT1;


sum1 = sum1 + square(Ith(y, 1) - w_mun[iPos][0]) + square(Ith(y, 2) - w_mun[iPos][1]) +
square(Ith(y, 3) - w_mun[iPos][2]) + square(Ith(y, 4) - w_mun[iPos][3]) + (square(Ith(y, 5) -
a_mun[iPos][0]) + square(Ith(y, 6) - a_mun[iPos][1]) + square(Ith(y, 7) - a_mun[iPos][2]) +
square(Ith(y, 8) - a_mun[iPos][3]) + square(Ith(y, 9) - a_mun[iPos][4]) + square(Ith(y, 10) -
a_mun[iPos][5]))+ square(Ith(y, 11) + Ith(y, 12) + Ith(y, 13) - c_mun[iPos]);

#ifdef OUTPUT

        fprintf(mun, "%d\t", iout);
        fprintf(mun, "%f\t %f\t %f\t %f\t\t", Ith(y, 1), Ith(y, 2), Ith(y, 3), Ith(y, 4));
```

```
                fprintf(mun, "%f\t %f\t %f\t %f\t\t\t", w_mun[iPos][0], w_mun[iPos][1],
w_mun[iPos][2], w_mun[iPos][3]);
                fprintf(mun, "%f\t %f\t %f\t %f\t %f\t %f\t\t", Ith(y, 5), Ith(y, 6), Ith(y, 7), Ith(y,
8), Ith(y, 9), Ith(y, 10));
                fprintf(mun, "%f\t %f\t %f\t %f\t %f\t %f\t\t\t", a_mun[iPos][0], a_mun[iPos][1],
a_mun[iPos][2], a_mun[iPos][3], a_mun[iPos][4], a_mun[iPos][5]);
                fprintf(mun, "%f\t %f\n", Ith(y,11)+Ith(y, 12)+Ith(y, 13), c_mun[iPos]);

#endif
                aceton_mus = Ith(y,18)+Ith(y,19)+Ith(y,20)+Ith(y,21)+Ith(y,22)+Ith(y,23);
                combus_mus = Ith(y,24)+Ith(y,25)+Ith(y,26);


                        if (iPos == 0)
                        sumr_mus = sumr_mus+(aceton_mus/combus_mus - 0.2504) * WT1;

                        if (iPos == 1)
                        sumr_mus = sumr_mus + (aceton_mus/combus_mus - 0.4657) * WT1;

                        if (iPos == 2)
                        sumr_mus = sumr_mus + (aceton_mus/combus_mus - 0.4456) * WT1;

                        if (iPos == 3)
                        sumr_mus = sumr_mus + (aceton_mus/combus_mus - 0.3057) * WT1;

                        if (iPos == 4)
                        sumr_mus = sumr_mus + (aceton_mus/combus_mus - 0.2532) *WT1;

                        if (iPos == 5)
                        sumr_mus = sumr_mus + (aceton_mus/combus_mus - 0.2167) * WT1;

                        if (iPos == 6)
                        sumr_mus = sumr_mus + (aceton_mus/combus_mus - 0.1275) * WT1;


sum2 = sum2 + square(Ith(y, 14) - w_mus[iPos][0]) + square(Ith(y, 15) - w_mus[iPos][1]) +
square(Ith(y, 16) - w_mus[iPos][2])+ square(Ith(y, 17) - w_mus[iPos][3])+ (square(Ith(y, 18) -
a_mus[iPos][0]) + square(Ith(y, 19) - a_mus[iPos][1]) + square(Ith(y, 20) - a_mus[iPos][2]) +
square(Ith(y, 21) - a_mus[iPos][3]) + square(Ith(y, 22) - a_mus[iPos][4])+ square(Ith(y, 23) -
a_mus[iPos][5]))+ square(Ith(y, 24) + Ith(y, 25) + Ith(y, 26) - c_mus[iPos]);
```

```
#ifdef OUTPUT
		fprintf(mus, "%d\t", iout);
		fprintf(mus, "%f\t %f\t %f\t %f\t\t", Ith(y, 14), Ith(y, 15), Ith(y, 16), Ith(y, 17));
		fprintf(mus, "%f\t %f\t %f\t %f\t\t\t", w_mus[iPos][0], w_mus[iPos][1],
w_mus[iPos][2], w_mus[iPos][3]);

		fprintf(mus, "%f\t %f\t %f\t %f\t %f\t %f\t\t", Ith(y, 18), Ith(y, 19), Ith(y, 20),
Ith(y, 21), Ith(y, 22), Ith(y, 23));
		fprintf(mus, "%f\t %f\t %f\t %f\t %f\t %f\t\t\t", a_mus[iPos][0], a_mus[iPos][1],
a_mus[iPos][2], a_mus[iPos][3], a_mus[iPos][4], a_mus[iPos][5]);

		fprintf(mus, "%f\t %f\n", Ith(y, 24) + Ith(y, 25) + Ith(y, 26), c_mus[iPos]);

#endif
		aceton_mln = Ith(y,31)+Ith(y,32)+Ith(y,33)+Ith(y,34)+Ith(y,35)+Ith(y,36);
		combus_mln = Ith(y,37)+Ith(y,38)+Ith(y,39);

			if (iPos == 0)
			sumr_mln = sumr_mln + (aceton_mln/combus_mln - 0.4992) * WT1;

			if (iPos == 1)
			sumr_mln = sumr_mln + (aceton_mln/combus_mln - 1.4979) * WT1;

			if (iPos == 2)
			sumr_mln = sumr_mln + (aceton_mln/combus_mln - 1.0989) * WT1;

			if (iPos == 3)
			sumr_mln = sumr_mln + (aceton_mln/combus_mln - 0.9825) * WT1;

			if (iPos == 4)
			sumr_mln = sumr_mln + (aceton_mln/combus_mln - 0.9152) *WT1;

			if (iPos == 5)
			sumr_mln = sumr_mln + (aceton_mln/combus_mln - 0.4043) * WT1;

			if (iPos == 6)
			sumr_mln = sumr_mln + (aceton_mln/combus_mln - 0.2851) * WT1;

sum3 = sum3 + square(Ith(y, 27) - w_mln[iPos][0]) + square(Ith(y, 28) - w_mln[iPos][1]) +
square(Ith(y, 29) - w_mln[iPos][2]) + square(Ith(y, 30) - w_mln[iPos][3]) + (square(Ith(y, 31) -
a_mln[iPos][0]) + square(Ith(y, 32) - a_mln[iPos][1]) + square(Ith(y, 33) - a_mln[iPos][2]) +
```

```
square(Ith(y, 34) - a_mln[iPos][3]) + square(Ith(y, 35) - a_mln[iPos][4])+ square(Ith(y, 36) - a_mln[iPos][5]))+ square(Ith(y, 37) + Ith(y, 38) + Ith(y, 39) - c_mln[iPos]);

#ifdef OUTPUT
		fprintf(mln, "%d\t", iout);
		fprintf(mln, "%f\t %f\t %f\t %f\t\t", Ith(y, 27), Ith(y, 28), Ith(y, 29), Ith(y, 30));
		fprintf(mln, "%f\t %f\t %f\t %f\t\t\t", w_mln[iPos][0], w_mln[iPos][1], w_mln[iPos][2], w_mln[iPos][3]);

		fprintf(mln, "%f\t %f\t %f\t %f\t %f\t %f\t\t", Ith(y, 31), Ith(y, 32), Ith(y, 33), Ith(y, 34), Ith(y, 35), Ith(y, 36));
		fprintf(mln, "%f\t %f\t %f\t %f\t %f\t %f\t\t\t", a_mln[iPos][0], a_mln[iPos][1], a_mln[iPos][2], a_mln[iPos][3], a_mln[iPos][4], a_mln[iPos][5]);

		fprintf(mln, "%f\t %f\n", Ith(y, 37) + Ith(y, 38) + Ith(y, 39) , c_mln[iPos]);

#endif

		aceton_mls = Ith(y,44)+Ith(y,45)+Ith(y,46)+Ith(y,47)+Ith(y,48)+Ith(y,49);
		combus_mls = Ith(y,50)+Ith(y,51)+Ith(y,52);


			if (iPos == 0)
			sumr_mls = sumr_mls + (aceton_mls/combus_mls - 1.4963) * WT1;

			if (iPos == 1)
			sumr_mls = sumr_mls + (aceton_mls/combus_mls - 1.9657) * WT1;

			if (iPos == 2)
			sumr_mls = sumr_mls + (aceton_mls/combus_mls - 0.9650) * WT1;

			if (iPos == 3)
			sumr_mls = sumr_mls + (aceton_mls/combus_mls - 0.7484) * WT1;

			if (iPos == 4)
			sumr_mls = sumr_mls + (aceton_mls/combus_mls - 0.9771) *WT1;

			if (iPos == 5)
			sumr_mls = sumr_mls + (aceton_mls/combus_mls - 0.2088) * WT1;

			if (iPos == 6)
```

```
                    sumr_mls = sumr_mls + (aceton_mls/combus_mls - 0.2049) * WT1;

sum4= sum4 + square(Ith(y, 40) - w_mls[iPos][0]) + square(Ith(y, 41) - w_mls[iPos][1]) +
square(Ith(y, 42) - w_mls[iPos][2]) + square(Ith(y, 43) - w_mls[iPos][3]) + (square(Ith(y, 44) -
a_mls[iPos][0]) +square(Ith(y, 45) - a_mls[iPos][1]) + square(Ith(y, 46) - a_mls[iPos][2])+
square(Ith(y, 47) - a_mls[iPos][3]) + square(Ith(y, 48) - a_mls[iPos][4])+ square(Ith(y, 49) -
a_mls[iPos][5]))+ square(Ith(y, 50) + Ith(y, 51) + Ith(y, 52) - c_mls[iPos]);

#ifdef OUTPUT
                    fprintf(mls, "%d\t", iout);
                    fprintf(mls, "%f\t %f\t %f\t %f\t\t", Ith(y, 40), Ith(y, 41), Ith(y, 42), Ith(y, 43));
                    fprintf(mls, "%f\t %f\t %f\t %f\t\t\t", w_mls[iPos][0], w_mls[iPos][1],
w_mls[iPos][2], w_mls[iPos][3]);
                    fprintf(mls, "%f\t %f\t %f\t %f\t %f\t %f\t\t", Ith(y, 44), Ith(y, 45), Ith(y, 46),
Ith(y, 47), Ith(y, 48), Ith(y, 49));
                    fprintf(mls, "%f\t %f\t %f\t %f\t %f\t %f\t\t\t", a_mls[iPos][0], a_mls[iPos][1],
a_mls[iPos][2], a_mls[iPos][3], a_mls[iPos][4], a_mls[iPos][5]);
                    fprintf(mls, "%f\t %f\n", Ith(y, 50) + Ith(y, 51) + Ith(y, 52), c_mls[iPos]);

#endif
}
          }

          g[0] = 0.0;
          N_VFree(y);
          CVodeFree(cvode_mem);
          (*f) = sum1 + sum2 + sum3 + sum4 + sumr_mun + sumr_mus + sumr_mln + sumr_mls;

#ifdef OUTPUT
          fclose(mun);
          fclose(mus);
          fclose(mln);
          fclose(mls);
          printf("%f\t %f\t %f\t %f\t %f\n", sum1, sum2, sum3, sum4, sum1 + sum2 + sum3 +
sum4);
          exit(0);
#endif
          return;
}

static void difeq(integer N, real t, N_Vector y, N_Vector ydot, void *f_data) {
```

```
double C1un, C2un, C3un, C4un;
double S1un, S2un, S3un, S4un, S5un, S6un;
double SS1un, SS2un, SS3un;

double C1us, C2us, C3us, C4us;
double S1us, S2us, S3us, S4us, S5us, S6us;
double SS1us, SS2us, SS3us;

double C1ln, C2ln, C3ln, C4ln;
double S1ln, S2ln, S3ln, S4ln, S5ln, S6ln;
double SS1ln, SS2ln, SS3ln;

double C1ls, C2ls, C3ls, C4ls;
double S1ls, S2ls, S3ls, S4ls, S5ls, S6ls;
double SS1ls, SS2ls, SS3ls;

C1un = Ith(y, 1);
C2un = Ith(y, 2);
C3un = Ith(y, 3);
C4un = Ith(y, 4);

S1un = Ith(y, 5);
S2un = Ith(y, 6);
S3un = Ith(y, 7);
S4un = Ith(y, 8);
S5un = Ith(y, 9);
S6un = Ith(y, 10);

SS1un = Ith(y, 11);
SS2un = Ith(y, 12);
SS3un = Ith(y, 13);

C1us = Ith(y, 14);
C2us = Ith(y, 15);
C3us = Ith(y, 16);
C4us = Ith(y, 17);

S1us = Ith(y, 18);
S2us = Ith(y, 19);
S3us = Ith(y, 20);
S4us = Ith(y, 21);
```

```
S5us = Ith(y, 22);
S6us = Ith(y, 23);

SS1us = Ith(y, 24);
SS2us = Ith(y, 25);
SS3us = Ith(y, 26);

C1ln = Ith(y, 27);
C2ln = Ith(y, 28);
C3ln = Ith(y, 29);
C4ln = Ith(y, 30);

S1ln = Ith(y, 31);
S2ln = Ith(y, 32);
S3ln = Ith(y, 33);
S4ln = Ith(y, 34);
S5ln = Ith(y, 35);
S6ln = Ith(y, 36);

SS1ln = Ith(y, 37);
SS2ln = Ith(y, 38);
SS3ln = Ith(y, 39);

C1ls = Ith(y, 40);
C2ls = Ith(y, 41);
C3ls = Ith(y, 42);
C4ls = Ith(y, 43);

S1ls = Ith(y, 44);
S2ls = Ith(y, 45);
S3ls = Ith(y, 46);
S4ls = Ith(y, 47);
S5ls = Ith(y, 48);
S6ls = Ith(y, 49);

SS1ls = Ith(y, 50);
SS2ls = Ith(y, 51);
SS3ls = Ith(y, 52);

Ith(ydot, 1) = -w1un * C1un - w2un * C1un  - MV * a1u * (kd1u * C1un - S1un);
Ith(ydot, 2) = w1un * C1un - w3un * C2un - MV * a2u * (kd2u * C2un - S4un);
```

```
Ith(ydot, 3) = w2un * C1un  - MV * a2u * (kd2u * C3un - S5un);
Ith(ydot, 4) = w3un * C2un - MV * a3u * (kd3u * C4un - S6un);

Ith(ydot, 5) = (a1u * (kd1u * C1un - S1un) - s1u * S1un) * MV;
Ith(ydot, 6) = (s1u * S1un - s2u * S2un - b1u * S2un) * MV;
Ith(ydot, 7) = (s2u * S2un - s3u * S3un - b2u * S3un) * MV;
Ith(ydot, 8) = (a2u * (kd2u * C2un - S4un)) * MV;
Ith(ydot, 9) = (a2u * (kd2u * C3un - S5un)) * MV;
Ith(ydot, 10) = (a3u * (kd3u * C4un - S6un) + s3u * S3un - b3u * S6un) * MV;

Ith(ydot, 11) = b1u * S2un * MV;
Ith(ydot, 12) = b2u * S3un * MV;
Ith(ydot, 13) = b3u * S6un * MV;

Ith(ydot, 14) = -w1us * C1us - w2us * C1us - MV * a1u * (kd1u * C1us - S1us);
Ith(ydot, 15) = w1us * C1us - w3us * C2us - MV * a2u * (kd2u * C2us - S4us);
Ith(ydot, 16) = w2us * C1us -  MV * a2u * (kd2u * C3us - S5us);
Ith(ydot, 17) = w3us * C2us - MV * a3u * (kd3u * C4us - S6us);

Ith(ydot, 18) = (a1u * (kd1u * C1us - S1us) - s1u * S1us) * MV;
Ith(ydot, 19) = (s1u * S1us - s2u * S2us - b1u * S2us) * MV;
Ith(ydot, 20) = (s2u * S2us - s3u * S3us - b2u * S3us) * MV;
Ith(ydot, 21) = (a2u * (kd2u * C2us - S4us)) * MV;
Ith(ydot, 22) = (a2u * (kd2u * C3us - S5us)) * MV;
Ith(ydot, 23) = (a3u * (kd3u * C4us - S6us) + s3u * S3us- b3u * S6us) * MV;

Ith(ydot, 24) = b1u * S2us * MV;
Ith(ydot, 25) = b2u * S3us * MV;
Ith(ydot, 26) = b3u * S6us * MV;

Ith(ydot, 27) = -w1ln * C1ln - w2ln * C1ln -  MV * a1l * (kd1l * C1ln - S1ln);
Ith(ydot, 28) = w1ln * C1ln - w3ln * C2ln - MV * a2l * (kd2l * C2ln - S4ln);
Ith(ydot, 29) = w2ln * C1ln - MV * a2l * (kd2l * C3ln - S5ln);
Ith(ydot, 30) = w3ln * C2ln - MV * a3l * (kd3l * C4ln - S6ln);

Ith(ydot, 31) = (a1l * (kd1l * C1ln - S1ln) - s1l * S1ln) * MV;
Ith(ydot, 32) = (s1l * S1ln - s2l * S2ln - b1l * S2ln) * MV;
Ith(ydot, 33) = (s2l * S2ln - s3l * S3ln - b2l * S3ln) * MV;
Ith(ydot, 34) = (a2l * (kd2l * C2ln - S4ln)) * MV;
Ith(ydot, 35) = (a2l * (kd2l * C3ln - S5ln)) * MV;
Ith(ydot, 36) = (a3l * (kd3l * C4ln - S6ln) + s3l * S3ln - b3l * S6ln) * MV;
```

```
	Ith(ydot, 37) = b1l * S2ln * MV;
	Ith(ydot, 38) = b2l * S3ln * MV;
	Ith(ydot, 39) = b3l * S6ln * MV;

	Ith(ydot, 40) = -w1ls * C1ls - w2ls * C1ls - MV * a1l * (kd1l * C1ls - S1ls);
	Ith(ydot, 41) = w1ls * C1ls - w3ls * C2ls - MV * a2l * (kd2l * C2ls - S4ls);
	Ith(ydot, 42) = w2ls * C1ls -  MV * a2l * (kd2l * C3ls - S5ls);
	Ith(ydot, 43) = w3ls * C2ls - MV * a3l * (kd3l * C4ls - S6ls);

	Ith(ydot, 44) = (a1l * (kd1l * C1ls - S1ls) - s1l * S1ls) * MV;
	Ith(ydot, 45) = (s1l * S1ls - s2l * S2ls - b1l * S2ls) * MV;
	Ith(ydot, 46) = (s2l * S2ls - s3l * S3ls - b2l * S3ls) * MV;
	Ith(ydot, 47) = (a2l * (kd2l * C2ls - S4ls)) * MV;
	Ith(ydot, 48) = (a2l * (kd2l * C3ls - S5ls)) * MV;
	Ith(ydot, 49) = (a3l * (kd3l * C4ls - S6ls) + s3l * S3ls - b3l * S6ls) * MV;

	Ith(ydot, 50) = b1l * S2ls * MV;
	Ith(ydot, 51) = b2l * S3ls * MV;
	Ith(ydot, 52) = b3l * S6ls * MV;

}

double transform(double x) {
	double y;
	y = x;
	return y;
}
double square(double xxx) {
	return xxx * xxx * 10000.0 * 10000.0;
}
double **ReadWA(const char file[], const int iRow, const int iCol) {
	char buf[shareDefMaxLine];
	char **sl;
	FILE *fp;
	int i = 0, n, k = 0;
	double **pData = NULL;

	if ((fp = fopen(file, "r")) == NULL) {
		printf("fopen %s failed!\n", file);
```

```
			exit(-1);
		}
		pData = ShareMallocM2d(iRow, iCol);

		while (fgets(buf, shareDefMaxLine, fp) != NULL) {
			ShareChop(buf);
			sl = ShareSplitStr(buf, "\t", &n, shareDefNullNo);

			if (n != iCol) {
				printf("line failed: %s\n", buf);
				exit(-1);
			}
			for (k = 0; k < iCol; k++)
				pData[i][k] = atof(sl[k]);

			i = i + 1;
		}
		return pData;
}

double *ReadC(const char file[], const int iRow) {
		char buf[shareDefMaxLine];
		char **sl;
		FILE *fp;
		int i = 0, n, k = 0;
		double *pData = NULL;

		if ((fp = fopen(file, "r")) == NULL) {
			printf("fopen %s failed!\n", file);
			exit(-1);
		}

		pData = ShareMallocM1d(iRow);

		while (fgets(buf, shareDefMaxLine, fp) != NULL) {
			ShareChop(buf);
			sl = ShareSplitStr(buf, "\t", &n, shareDefNullNo);

			if (n > 1) {
				printf("line failed: %s\n", buf);
				exit(-1);
```

```
                }
                pData[i] = atof(sl[0]);
                i = i + 1;
        }
        return pData;
}
```