INTEGRATING ENGINEERED WETLANDS WITH CROP IRRIGATION: AN EVALUATION OF CHEMICAL UPTAKE

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ABSTRACT

RAMÓN DC ALATORRE: Integrating Engineered Wetlands with Crop Irrigation: An Evaluation of Chemical Uptake
(Under the direction of Dr. Howard Weinberg)

The potential for ELISAs (Enzyme Linked Immunosorbant Assays) to track the fate of micropollutants in crops grown through irrigation with wastewater from a decentralized, engineered wetland-type reclamation system, was realized in a controlled greenhouse study. Caffeine was observed within sweet potato tissue between 0 and 9 ng/g when irrigated with tap water and between 16 and 21ng/g when irrigated with reclaimed (unmodified and spiked with elevated levels of chemical) wastewater sources, indicating uptake of between 2 and 10% of the total estimated mass applied. Analysis of a sweet potato from a local grocery store detected caffeine between 6 and 8ng/g. Triclosan was only observed within the sweet potato tissue of crops grown with the wastewater spiked with elevated levels of the chemical and, even then, only between 4 and 11ng/g, indicating less than 2% uptake of the estimated mass applied. The results indicate limited uptake of the target compounds.

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LIST OF SYMBOLS AND ABBREVIATIONS

μg	microgram
μL	microliter
CAN	acetonitrile
AF	acre feet
BAC	biologically active compounds
BSTFA	N,O-bis(trimethylsilyl) trifluoroacetamide
CAS	Chemical Abstract Service
CE	capillary electrophoresis
CI	confidence interval
DBP	disinfection byproducts
dSPE	dispersive Solid Phase Extraction
E2	17-β estradiol
E3	estriol
EC	emerging contaminant
ECD	electron capture detector
EDC	endocrine disrupting compound
EE2	17α-ethinyl estradiol
EI	electron ionization
ELISA	enzyme linked immunosorbant assay
EPA	Environmental Proctection Agency
g	gram
GC	gas chromatography

gpd	gallons per day
HDPE	high-density polyethylene
HRP	horseradish peroxidase
IDL	instrument detection limit
IWS	Integrated Water Strategies
Koc	organic carbon-water partition coefficient
Kow	octanol-water partition coefficient
LC	liquid chromatography
LGW	laboratory grade water
L	liter
LOD	limit of detection
m/z	mass to charge ratio
MeOH	methanol
mg	milligram
$MgSO_4$	magnesium sulfate
mL	milliliter
mm	millimeter
MS	mass spectrometry
Na_2SO_4	sodium sulfate
NC	North Carolina
NCSU	North Carolina State University
ng	nanogram
PBT	persistent bioaccumulative toxins

PDL	practical detection limit
pg	picogram
POP	persistent organic pollutants
PPCP	pharmaceuticals and personal care products
PSA	primary secondary amine
PTFE	polytetrafluoroethylene
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe extraction method
rpm	revolutions per minute
S/N	signal to noise ratio
TMCS	trimethylchlorosilane
TSCF	transpiration stream concentration factor
UNC	University of North Carolina at Chapel Hill
USDA	United States Department of Agriculture
VSA	virgin sand sample
VSF	vegetative sand filter
VSO	virgin soil sample

CHAPTER 1: BACKGROUND INTRODUCTION AND LITERATURE REVIEW

1.1 Anthropogenic Influence on Water Sources

The occurrence of anthropogenic influence on the constituency of surface water is unquestioned. The extent and significance of that influence are constantly evolving areas of study. Indeed, tens of millions of organic and inorganic substances have been indexed by American Chemical Society's Chemical Abstracts Service (CAS) in their CAS registry. As of March 2012 over 65 million are indexed, with more than 63 million being commercially available. Less than 300,000 of these are currently inventoried or regulated. For reference, consider that in 2004 C. G. Daughton reported that the CAS registry had nearly 23 million indexed chemicals and that *only* 7 million were commercially available (Daughton 2004). Daughton went on to point out that the "universe" of potential organic and inorganic chemicals (those existing that have yet to be identified and those that could be synthesized) is astoundingly large to the point of being essentially limitless.

Anthropogenic chemicals, whether synthesized or naturally occurring, may enter waterways from countless point sources (including commercial, industrial, and municipal waste releases) as well as nonpoint sources (highly dispersed which largely enter waterways through runoff). Indeed, with the ever growing volume of research, and the constant development and improvement of analytical techniques, it is hardly surprising that the perception of water purity has had to be reassessed. An incomplete glance at the acronymic wealth of research topics involving the anthropogenic influence on water content include:

ECs (emerging contaminants), PPCPs (pharmaceuticals and personal care products), POPs (persistent organic pollutants), PBTs (persistent bioaccumulative toxins), EDCs (endocrine disrupting compounds), DBPs (disinfection byproducts), BACs (biologically active compounds) and many more. Admittedly there is some overlap between many of these research areas, with some classifications being intentionally broad and others seeking to narrow their scope. Still, the fact remains that the more and deeper we look, the more apparent the anthropogenic influence on our water.

1.1.1 PPCPs & EDCs: Their Presence in the Environment and Their Repercussions

Endocrine disrupting compounds (EDCs) have been defined as "exogenous agents(s) that interfere with the synthesis, storage/release, transport, metabolism, binding, action or elimination of natural blood-borne hormones responsible for the regulation of homeostasis and regulation of developmental processes" (Cooper & Kavlock, 1997). The consequences of exposure to EDCs will be further outlined below. Pharmaceuticals are compounds that have been expressly designed to have some biological effect on their target when consumed or applied and many pharmaceuticals can be sub classified specifically as EDCs. Numerous non-pharmaceutical personal care products as well as compounds present in commercial, industrial and biological wastes are also known to be endocrine disrupting.

Pharmaceuticals that are incompletely metabolized by their intended target are subsequently excreted, and typically enter a waste stream that is ultimately bound for release into an aquatic system. In the event that the treatment processes between excretion and release are insufficient to degrade or deactivate the pharmaceuticals, they can enter into these water sources in a still biologically active and often (depending on the design of the pharmaceutical) endocrine disrupting state (Calderón-Preciado et al. 2011; Snyder et al.

2003; Focazio et al. 2008). The same fate (release into aquatic environments in a still active state) has been observed for many other PPCPs and EDCs that enter various waste streams as waste treatment processes are not optimized for their removal (Westerhoff et al. 2005; Thomas and Foster 2005; Kim et al 2007). Many PPCPs and EDCs have been detected in surface and irrigation waters at trace concentrations (µg/L and ng/L) for more than 10 years (Ternes et al 1998; Kolpin et al. 2002; Moldovan 2006; Loos et al. 2009).

The repercussions of PPCPs and EDCs in water sources are layered. Their introduction to the aquatic environment can significantly impact individual organisms as well as having broader ecosystem ramifications (Segner et al. 2003, Munoz; Thorpe et a. 2003; Kidd et al. 2007; Oetken et al. 2004; Mills and Chichester 2005). Mills and Chichester Review of Evidence is particularly insightful. On the individual species level, fish can be exposed to EDCs in water by a number of routes including aquatic respiration and osmoregulation. Disruption of the endocrine system by EDCs manifests itself by hindering normal development and reproduction. The impacts can be multigenerational, as progeny of exposed parents can also suffer developmental and reproductive issues. There are considerable concerns over bioaccumulation and transfer of these compounds through the food chain as developmental and reproductive anomalies have been cited from invertebrates, to fish, reptiles, birds, mammals and humans (Cooper and Kavlock 1997). Segner et al. (2003) point out that little attention has been given to understanding the effect of EDCs on invertebrates, a sobering insight given that invertebrates constitute 95% of all living species and play an essential role in the health of ecosystems.

Beyond the concerns attributable to aquatic species and broader ecosystems, PPCPs and EDCs in water sources can have very direct human health impacts as well. Water

sources with PPCPs and EDCs may be used for recreation, irrigation, and drinking water, all of which represent potential routes of exposure to humans. Westerhoff et al. (2005) showed that the degree of removal of PPCPs and EDCs during drinking water treatment is largely dependent on the processes being used. Conventional treatment using coagulation and chlorine had low removal of many PPCPs and EDCs. More advanced treatment processes proved capable of increasing the removal of many compounds, yet others had low removal rates regardless the treatment process. Additionally, it should be emphasized that disinfection processes during drinking water treatment have the potential to transform compounds and that "removal" of PPCPs and EDCs does not necessarily ensure deactivation. Perhaps the conclusion to be made then is that if PPCPs and EDCs are present in source water (as they are known to be), then water treatment processes are not presently capable of removing or deactivating all PPCPs and EDCs and chronic low dose exposure to some of these compounds in our drinking water is a likely reality. Additional studies on the fate of PPCPs and EDCs in simulated drinking water processes, pilot and at scale plants (Esplugas et al. 2007; Boyd et al. 2003; Tunkanen et al 2007) also demonstrate differences in removal performance based on the treatment process utilized but ultimately conclude that complete removal of PPCPs is not achieved.

1.2 Water Reclamation and Reuse: A "Keeping the Horse Before the Cart" Solution

Much energy has and should continue to be devoted to developing advanced drinking and waste water technologies to achieve better treatment performance of anthropogenic waste from a holistic perspective, beyond even just the lens of PPCPs and EDCs. While technology and engineering certainly have their place in addressing water quality concerns, progressive management strategies may also prove to be an effective component in what is

surely going to need to be a diverse portfolio of solutions. Water reclamation and reuse is a management strategy that may contribute to the portfolio and also has benefits that go beyond water quality implications.

Redirecting treated waste water for productive non-potable use diverts PPCPs, EDCs and other anthropogenic wastes from water sources and sensitive ecosystems. Diversion by means of water reclamation can thus circumvent the environmental and human health issues associated with direct release of these compounds into aquatic environments. In contrast to engineering technological solutions to solve the multi-faceted issues associated with waste release into aquatic sources, diversion strategies lessen the extent of the initial problem. The U.S. Environmental Protection Agency (U.S. EPA) recognizes water reclamation and reuse as having a number of benefits including: decreasing diversion of freshwater from sensitive ecosystems, diversion of waste from sensitive ecosystems, decreasing discharge to sensitive water bodies, creating or enhancing wetland and riparian habitats, and reducing and preventing pollution (EPA 2009).

1.3 Additional Benefits Attributable to Water Reclamation and Reuse

1.3.1 Water Quantity: Primary and Secondary Benefits

In October 2011 the United Nations Department of Economic and Social Affairs (UNDESA) estimated that the global population had reached 7 billion. It only took 12 years to make the increase from 6 to 7 billion. Additionally the UNDESA reported that as of 2010 more people live in urban areas than rural. With urbanization and rural migration projected to continue, geographic population densities are increasing and putting greater demand on local natural resources including fresh water. Indeed, a paleo perspective of the droughts of the 20th century and beyond (Woodhouse et al. 2003) indicate that these drought events are of

only moderate historical severity (see the National Oceanic and Atmospheric Administration and National Climate Data Center paleoclimatology website at www.ncdc.noaa.gov/paleo) and that droughts of similar severity should be anticipated several times per century. Yet the environmental and social impacts of these historically unremarkable droughts have been increasingly detrimental as increasing population and population densities have exacerbated their effects.

Incorporating water reclamation and reuse into the water management and supply portfolio could ease the local burdens of providing water during times of natural scarcity. Reclamation and reuse allows for less withdrawal from water sources for non-potable productivity. Reclamation and reuse systems might draw comparisons to introducing or increasing reservoir capacity in terms of providing a buffer against variability. In fact many large scale projects incorporate significant storage capacity (see PUB projects in Singapore at www.pub.gov.sg), but even with non-centralized system designs the distributed storage can provide some buffer against variability.

As with any practice that eases the burdens associated with water quantity issues, water reclamation and reuse could potentially be ascribed a number of secondary benefits, not least of which are conflict prevention and self-sufficiency. An often reiterated phrase among scientists and politicians alike is that "the next great war will be over water." Taking note of the heated domestic disputes that have arisen over water within the contiguous borders of the United States, it does not seem inconceivable that escalated conflict across international borders could arise especially given the exacerbated effects of drought being experienced as a result of the aforementioned increasing population densities. Indeed the list of events that could be referenced to demonstrate the growing link between water and

international dispute is astounding. One of particular relevance, however, because of its connection to water reclamation is that of Singapore.

Singapore is a country with a population of 5.1 million in a land area of just 637.5 km^2 (CIA World Factbook, 2011). Historically Singapore has imported the great majority of its water supply from neighboring country Malaysia. This dependence on water supply from Malaysia has proven to be an expensive and politically vulnerable position for Singapore as Malaysia has been willing to use the threat of turning off the tap during unrelated political dealings. Singapore's Public Utility Board (PUB) has initiated a strategy to become self-sufficient in its water supply by 2060 that includes developing a reclamation system as a cornerstone of its water supply portfolio (NEWater) which will capable of providing 50% of its total water supply. In achieving self-sufficiency in water supply, Singapore will greatly strengthen its political position with Malaysia and the potential for conflict will be greatly reduced. As of 2011, PUB reports that the percentage of water imported from Malaysia is down to just 40% of supply and that the five NEWater plants in operation are providing 30%.

1.3.2 Economic Benefits

Developing water reclamation as a component of the water supply portfolio could create local jobs in communities. Water reclamation and reuse can be pursued from centralized government and municipal planning and from independent water reclamation service businesses, with job creation being inherent in either model. Indeed, entrepreneurial businesses already design, install and maintain decentralized on-site water reclamation systems. On-site systems of various design and scope can be implemented in business buildings, schools, homes, subdivisions, communities etc. Considering that over 25% of the U.S population is served by septic systems (over 40% of new developments and over 48% in

North Carolina) (EPA 2012), decentralized on-site water reclamation services represent great entrepreneurial opportunity and job creation potential.

1.3.2.1 Green Infrastructure and the Green Economy

Water reclamation for non-potable reuse needn't be of an energy or chemically intensive design. Green infrastructure designs utilizing natural processes have been implemented that meet high water quality standards for non-potable reuse (North Carolina Administrative Code section 15A provides regulations for Biological Oxygen Demand, Total Suspended Solids, ammonia, fecal coliform and turbidity). Engineered wetlands, sand filtration, vegetative contact, retention ponds, and other designs create environments that expose waste water to a host of degradative microbes, processes and conditions within aerobic, anaerobic and hypoxic environs. The ability of green infrastructure designs to meet non-potable reuse standards further increases the entrepreneurial and job creation potential of water reclamation services.

1.3.3 Developing World Applications

Water quality and quantity solutions that are simple, cheap and capable of utilizing local resources and talent are highly desirable in a developing world context. Water reclamation can satisfy all of these traits, especially considering the performance of green infrastructure and decentralized on-site system designs outlined above. The opportunity for incorporating reclamation philosophy and systems into the portfolio of water services in the developing world, as well as in more remote communities in developed nations, is another area of potential inertia.

1.3.4 Water Reclamation and Irrigation: Proceed with Caution?

In the United States, agriculture accounts for an estimated 80% of consumptive water use (USDA), up to 90% in the western states, and thus the temptation to utilize reclaimed water for irrigation in order to achieve the aforementioned health/environmental/social benefits is great. Indeed, the use of recycled water for agricultural irrigation is gaining momentum in the United States and around the world. According to the 2009 Municipal California Wastewater Recycling Survey (EPA), 29% of their total volume of recycled water, approximately 210,000 acre feet (AF), was applied to agricultural irrigation. By 2020, California intends to double their current water recycling capacity. As of 2006, 82% of Australia's recycled water, approximately 343,000 AF, was used for agricultural irrigation (lwa.gov.au). The levels of treatment required for agricultural irrigation using reclaimed water in these developed nations vary from un-disinfected secondary treatment (biological) to disinfected tertiary treatment (chemical) depending on crop type and irrigation delivery system.

Irrigation with recycled water diverts large volumes of treated waste water from aquatic ecosystems and the terrestrial application could provide an opportunity for natural degradation processes (photo/microbial) to deactivate/eliminate/transform or otherwise degrade contaminants. Irrigation also provides the opportunity for plant uptake of these contaminants, and indeed PPCPs and EDCs have been observed in plant tissues available for consumption (i.e. not grown in laboratory setting) (Calderón-Preciado et al. 2009 and 2011, Hu et al. 2010.). This represents a pathway that that could again expose humans to these contaminants, relegating one of the major benefits associated with water reclamation. Still, the current practices of waste water treatment and release into aquatic ecosystems has

rendered surface and irrigation water contaminated with PPCPs and EDCs, and thus irrigation from these sources would likewise represent an opportunity for plant uptake and human exposure to these contaminants from plant tissues. Nonetheless, as compared to reclaimed water, surface water is expected to be less concentrated with PPCPs, EDCs and other contaminants such that critical consideration and research should be given to the practice of crop irrigation with this less dilute water source.

1.4 Crop Analysis

1.4.1 Presence of Anthropogenic Chemicals Including PPCPs and EDCs in Plants

The majority of research on PPCPs and EDCs in the environment has been focused on water and sewer sludge matrices (Calderón-Preciado et al. 2009). Analysis of plant matrices has mostly focused on pesticide residues and a number of hydrophobic contaminants. In a ten year study by the United States Department of Agriculture (USDA) found one or more detectable pesticide residues on 65% of the approximately 65,000 fruit and vegetable samples analyzed, all of which came from various points in the food distribution network and were intended for consumption (Punzi et al. 2005). A number of studies have focused on the uptake potential of PPCPs and EDCs by plants under various laboratory conditions (Migliore et al. 2003; Kumar et al. 2005; Kong et al. 2007; Herklotz et al. 2010; Karnjanopiboonwong et al. 2011; Wu et al. 2010; Shenker et al. 2011), but until recently few studies have been conducted on field-grown crops or grocery bound produce, and certainly none in similar scope to the pesticide studies. A method for determining organic pollutants in leafy vegetables reported finding ibuprofen in a lettuce sample from a local market (Calderon et al. 2008). Another method for quantifying mammalian steroid hormones in plants reported detecting 4 endogenous steroids in plants grown under

standardized and field conditions (Simersky et al 2009). In 2010, 10 antibiotics were detected within the tissues of 4 different organic vegetable bases grown under field conditions in China (Hu et al. 2010). More recently, five PPCPs (including caffeine, ibuprofen and naproxen) were identified in alfalfa and apple leaves grown under field conditions in Spain (Calderón-Preciado et al. 2011).

1.4.2 Techniques

In order to analyze crops for the presence of PPCPs, EDCs, pesticides and other organic pollutants, at a minimum an extraction and quantification technique are required. Extraction techniques for the compounds in fruit and vegetable tissues have included (and are often used in combination) the following processes: accelerated solvent/pressurized solvent/fluid extraction (Calderón-Preciado et al. 2009), pressurized liquid extraction (Herklotz et al 2010; Wu et al. 2010), microwave assisted extraction(Pylypiw et al. 1997), matrix solid phase dispersion (Fang et al. 2009), immunoaffinity chromatography solid phase extraction (Simersky et al. 2009), salt assisted liquid liquid extractions such as the Quick Easy Cheap Effective Rugged and Safe (QuEChERS) method (Anastassiades et al. 2003), solid phase microextraction (Rodriguez et al. 2003), stir-bar-sorptive extraction (Garcia et al. 2005) as well as many others. Separation and quantification techniques are typically gas or liquid chromatography methods in line with a number of different detection techniques including mass spectrometry (MS), but other analytical methods have included capillary electrophoresis (CE) and the Enzyme Linked Immunosorbent Assay (ELISA) (Watanabe et al. 2004 and 2006). Each method or combination of methods has their advantages and disadvantages, and overall the sensitivities of many of these methods allow for the analysis

of residues and compounds at µg/g and ng/g levels assuming high levels of analyte recovery are achievable.

1.4.2.1 ELISAs vs. Chromatography: Speed vs. Multiresidue Analysis

Perhaps the most significant comparison to be made when considering the quantification methods above is between the ELISAs and the group of chromatographic methods. ELISAs are bioassays that utilize antibodies that have been engineered with binding sites with shape and chemical properties specific to a target compound. As such, ELISAs are very specific, a characteristic that can be a boon in many analytical situations. ELISAs are sensitive, often with limits of detection (LODs) of parts per billion (µg/L) to parts per trillion (ng/L). ELISAs are commercially available, affordable, and have high throughput capabilities (hundreds of samples can be analyzed in a few hours). They are robust and perform well in complex matrices; some have been used to analyze water, urine and saliva samples with only filtration and dilution required for sample preparation. While most are designed to analyze aqueous samples, some have shown tolerances for up to 10%-20% solvent content including acetonitrile and methanol. Watanabe et al. (2004 and 2006) used ELISAs specifically designed for the pesticides imidacloprid and acetamiprid to analyze dilute vegetable extracts.

Like ELISAs, chromatography methods coupled with detection techniques can also be very sensitive. Unlike ELISAs, they are especially powerful for multiresidue analysis: rather than looking for a single specific pesticide in an extract as would be the case in an ELISA analysis, these techniques can analyze an extract for a myriad of pesticides and other compounds acquired during extraction. When mass spectrometry (MS) and tandem MS/MS techniques are used, the identification of compounds detected within an extract are

considered confirmed. By comparison, while ELISAs are designed to be very specific, almost all have some cross reactivity with compounds of similar structure and positive results are not considered to be as absolute as MS methods.

The analytical power gained by chromatography + detection techniques comes at considerable time and expense. While ELISAs can be used to analyze hundreds (if not thousands) of samples in a matter of hours, the same time may be required to analyze just two or three consecutive samples on a chromatography + detection instrument. Even with robotic autosamplers available to inject samples 24/7, the sample throughput cannot compare to that of ELISAs. Additionally the cost of the instrumentation and maintenance of chromatographic columns and detectors are orders of magnitude higher than commercially available ELISA kits.

1.4.3 Benefits of Thinking Faster/Cheaper: Screening and Public Health

The tradeoffs outlined above provide a framework for considering the best use of the methods available. ELISAs are sensitive, robust, and have throughput capabilities that chromatography MS techniques cannot approach. Chromatography MS methods are sensitive, confirmatory techniques with multiresidue capabilities the ELISAs are incapable of. ELISAs cost far less but their specificity gives them limited scope.

The combination of cost efficiency, sensitivity, and speed positions ELISAs ideally for screening analysis of large sample volumes, though its specificity necessitates careful consideration as to how to maximize the value of single compound analysis. ELISAs developed to analyze indicator species, compounds that if detected might be indicative of the presence of a wider class of compounds, could be used to maximize their screening value. High throughput indicator species' screening methods provide an important public health

service as they allow for the analysis of a quantity of samples that otherwise could not be accomplished, flagging individual samples for additional analysis. The slower, more expensive, more expansive and more powerful chromatography MS techniques may be more efficiently used for confirmation and follow up of indicator species screening or for samples whose origins merit immediate multiresidue analysis.

1.4.3.1 QuEChERS: An Ideal Extraction Method for Screening Analysis

In order to draw further comment on the advantages and disadvantages of some of the previously listed extraction techniques (and with the concept of high throughput screening in mind) the group of techniques will be considered in comparison to the highly prolific QuEChERS method (Anastassiades and Lehotay 2003). The benefits of QuEChERS, an acronym which stands for Quick, Easy, Cheap, Effective, Rugged and Safe can be derived from its name. The speed and cost effectiveness of this multiresidue technique arise from many aspects of the method. After thorough sample comminution, target analytes are extracted by a relatively simple solvent partitioning which relies on the use of salts to separate aqueous and organic phases, a method sometimes referred to salting-out liquid-liquid extraction (SALLE). This is a much less intensive process in terms of time, chemicals, and instrumentation than many of the previously listed techniques. The organic phase is then cleaned by a process coined dispersive solid phase extraction (dSPE) which adds bulk adsorbent directly to the extract and vortexes it rather than passing the extract over cartridges or relying on other specialized equipment or instrumentation inherent in the more intensive methods. By comparison to other techniques, QuEChERS extracts may be considered less "clean," yet they are often sufficient for placement directly onto LC or GC instruments

without further cleanup and have achieved sensitivities for pesticide analysis (ng/g) similar to the more intensive methods.

After initial sample comminution, the only instruments required to complete the extraction are a vortex and centrifuge and the only chemicals required are a very modest amount of solvent (much less used than many other methods), salts and bulk adsorbents. The method is very fast, capable of processing tissue homogenates into finished extracts in under an hour. Many aspects of the technique can and have been automated. In the field of pesticide analysis of crop tissues, QuEChERS has gained considerable attention and momentum (it is now an official AOAC method (2007.01)).

1.4.3.2 QuEChERS + ELISAs: Screening Match Made in Heaven?

While the QuEChERS extraction method allows for quick sample processing, the extracts can only be analyzed as quickly as the analytical instrumentation allows. As discussed in Section 1.4.2.1, chromatography MS methods are powerful multiresidue techniques, but lack in sample throughput. Additionally, while in practice QuEChERS extracts have been put directly onto these instruments, especially for instruments that have selective detection abilities (Majors 2009), the polar solvent used can lead to relatively rapid column degradation and many co-extracted contaminants can result in vapor overload of the insert liner due to the high thermal expansion coefficient (Cunha et al 2010).

The concept of pairing QuEChERS extracts with ELISA bioassays thus becomes particularly intriguing. Lost is the multiresidue analysis inherent in GC and LC analysis due to the specificity of the ELISA method. Gained is significant sample throughput. If the methods are found to be compatible, hundreds of rapidly processed QuEChERS extracts could be analyzed in a matter of hours rather than days/weeks/months. While the

QuEChERS extracts will require dilution for ELISA analysis (a minimum 10 fold dilution expected), the high sensitivity of the bioassays would still allow for detection of target compounds within the plant tissues at the µg/g and ng/g level.

1.4.4 Chemical Indicators

If using single compound ELISAs to screen crop tissues for waste water contaminants including PPCPs and EDCs, the choice of what compounds to focus on becomes significant. Are there chemicals that might be analyzed by an ELISA that might be indicative of exposure and uptake of a wider class of PPCP and EDC compounds? Fortunately the fields of water and waste water research provide a good starting point. A 10 year study of 139 U.S. streams by Kolpin et al. (2002) narrowed the universe of organic waste contaminants choosing to focus on 95 specific compounds because they are "expected to enter the environment through common wastewater pathways, are used in significant quantities, may have human or environmental implications, are representative or potential indicators of certain classes of compounds or sources and/or can be accurately measured in environmental samples using available technologies." Looking at the most frequently observed compounds and comparing them to commercially available ELISA kits, caffeine, triclosan and nonlyphenol rise to the top as three of the compounds identified most frequently (70.6%, 57.6% and 50.6% detection frequency) that are considered to be PPCPs or EDCs. The following table (Table 1) of relevant chemical properties for the proposed chemical indicators will be referred to often in the sections that follow:

Compound	pKa	Solubility (mg/L) at 20°C	logKow	logKoc
Caffeine	10.4	21600	-0.07	1.85-3.89
Estradiol	10.2	13	4.01	3.58-3.90
Triclosan	7.9	10	4.76	3.99-4.30
logKoc values from (Karnjanapiboowong 2010) and varied depending on soil composition				
pKa, solubility and logKow values from http://toxnet.nlm.nih.gov and (Ying et al. 2005)				

Table 1: Relevant Chemical Properties of Proposed Indicators

1.4.4.1 Proposed Indicator: Caffeine

Caffeine, while not specifically an EDC, is occasionally classified as a pharmaceutical and is ubiquitous in wastewater effluents. Indeed, since 2002 many studies have proposed the use of caffeine as an indicator of exposure to other organic waste water contaminants because it is so frequently detected (Buerge et al. 2003; Chen et al. 2002; Glassmeyer et al. 2005). Caffeine is highly soluble (Table 1) and has been shown in the higher tissues (xylem and fruit) of tomato and soybean plants (Dettenmaier et al. 2009) as well as apples leaves and alfalfa (Calderón-Preciado 2011). Additionally, it can be accurately measured in water samples at low concentrations (µg/L) with ELISA methods.

1.4.4.2 Proposed Indicator: Triclosan

Triclosan is a widely used antimicrobial found in soaps, toothpastes, and deodorants among other products (Sabaliunas et al. 2003). It is relatively hydrophobic (see Table 1) and has been found to sorb onto soils and waste water treatment plant sludges (Thomas et al. 2005; Wu et al. 2010). As a persistent antiseptic found in the environment, triclosan has been suspected for contributing to observed antibiotic resistance (Jones et al. 2004). While not yet reported in the crop tissues of any field grown crops, laboratory experiments have

shown uptake by soybeans (Wu et al. 2010) and pinto beans (Karnjanapiboonwong et al. 2011) with significant bioconcentration factors observed.

1.4.4.3 Proposed Indicator: Estradiol

17-β estradiol (E2) is a sex hormone endogenously produced by all mammalian species that has been detected in waters sources worldwide (Ying et al. 2002) especially near animal operations and agricultural fields that have been applied with biosolids (Peterson et al. 1998; Casey et al. 2003), an especially relevant point of consideration when considering E2 as an indicator since biosolid application may provide an addition means of exposure for uptake. E2 is the most potent steroid estrogen hormone and is, in fact, the compound against which all other steroids and EDCs are measured in terms of estrogenicity. Commercial ELISA kits for E2 are extremely sensitive with limits of detection for water samples at the low ng/L concentrations.

1.4.5 Potential for Crop Uptake and Translocation

There are many factors influencing whether, and how much, a chemical contaminant is likely to be removed from water and into plant tissues, and they are not well understood. Assuming the compounds are taken up by the plant, the rate of uptake appears to be influenced by transpiration rate, contaminant concentration in water and soil, soil composition, and uptake *efficiency*, a factor which varies by plant type, leaf area, nutrients, soil moisture, temperature, wind conditions and relative humidity (Kamath et al 2004).

Many studies have been attempted to develop models that predict whether compounds will be taken up by crops based on their chemical properties. For a long time the Briggs model (Briggs et al. 1982) has been used to make predictions based on a chemical's octanol-water partition coefficient (Kow) relating it to a plants transpiration stream

concentration factor (TSCF), an indirect measure of uptake efficiency (See Figure 1). TSCF measures the ratio between the concentration of a chemical in the xylem to that in the solution used by the roots, and is used to describe the relative ability of an organic chemical to be passively transported from root to shoot (Dettenmaeir et al. 2009). A TSCF value of one therefore indicates that the compound is taken up from the roots and into the xylem as passively as water, while a TSCF of zero indicates a complete lack of uptake. The Briggs model was specifically based on the TSCF values measured for a number of pesticides in nutrient solution for young barley plants. The model proposes a bell shaped relationship between Log Kow and TSCF, suggesting that moderately hydrophobic compounds are most likely to be uptaken and transported through the plant, strongly hydrophobic compounds may be sorbed strongly onto soils making them unavailable for uptake and hydrophilic compounds would not cross lipophilic root membranes efficiently.

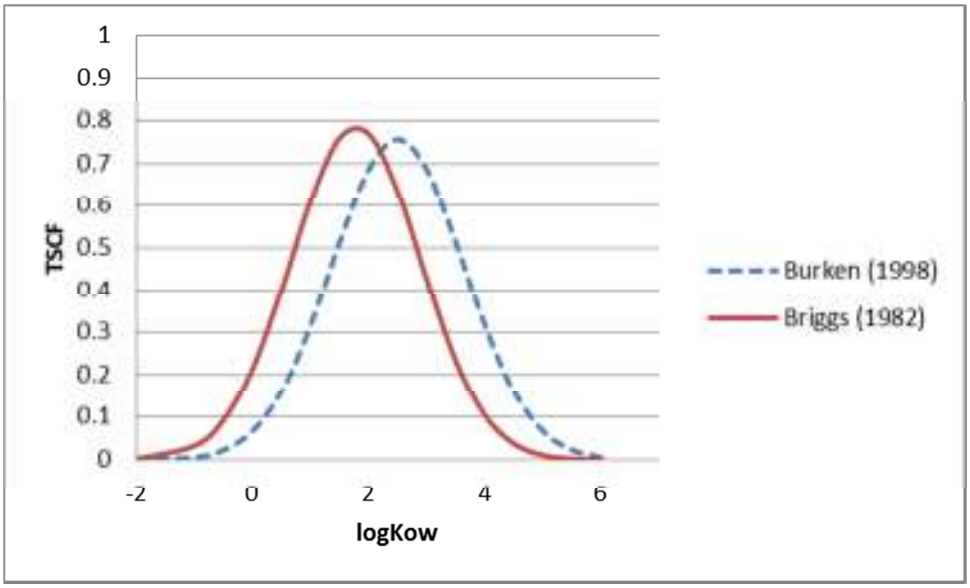

Figure 1: Briggs and Burken Bell-Shaped Models Comparing Log Kow vs. TSCF

In 1998 Burken et al. performed a similar experiment to Briggs et al. using a variety of 12 organic compounds rather than just pesticides, measuring the uptake by poplar trees and coming up with very similar results (Burken et al 1998). The Burken model actually

shifts the bell curve to the right slightly suggesting even less uptake of hydrophilic compounds than Briggs (Figure 1)

Many studies, however, have reported uptake of hydrophilic compounds (including caffeine) within plant tissues, and in 2009 Dettenmaier et al. proposed a drastically different model (see Figure 2) for nonionizable, polar, highly water soluble compounds based on uptake experiments within tomato and soybeans (Dettenmaier et al. 2009). Their sigmoidal model suggests that these compounds will actually be uptaken by plants more efficiently than any other compounds based on Log Kow, though the exact methods of how these compounds cross the membranes of the plants are not well understood.

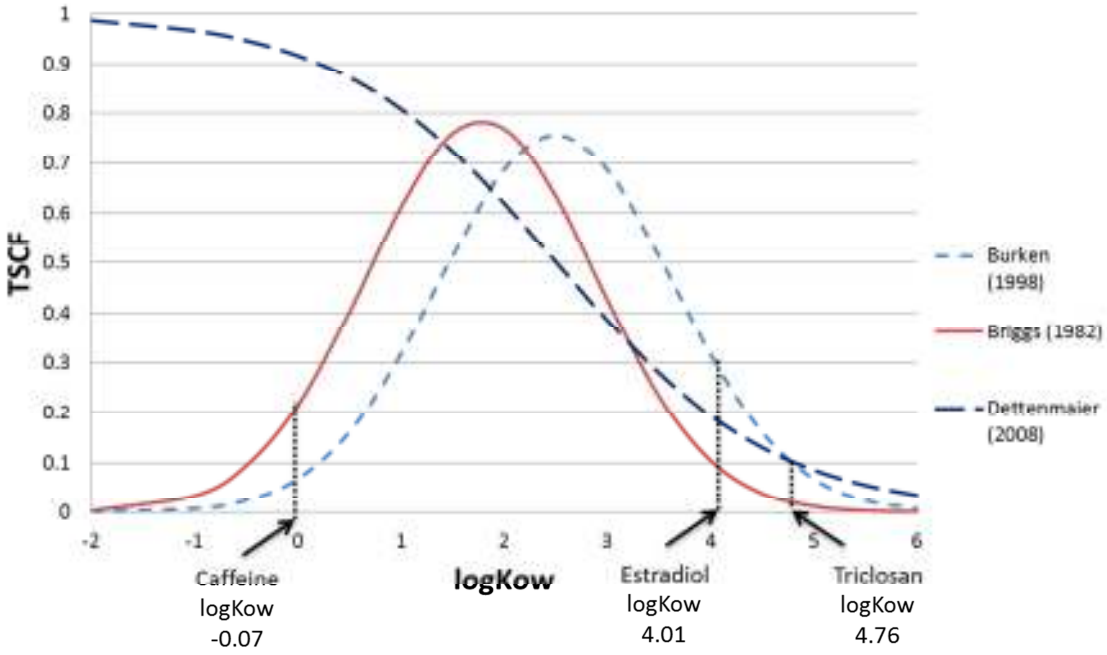

Figure 2: Dettenmaier Sigmoidal Model vs. Burken and Briggs Bell Shaped Models relating Log Kow and TSCF with proposed indicators Caffeine, Estradiol and Triclosan

While it may be tempting to scrutinize the cogency of each model, consider the following compilation of TSCF vs. Log Kow values from 30 publications (Figure 3) as seen in the Dettenmaier (2009) paper and recall all the factors (plant type, leaf area, nutrients etc.) that can influence TSCF:

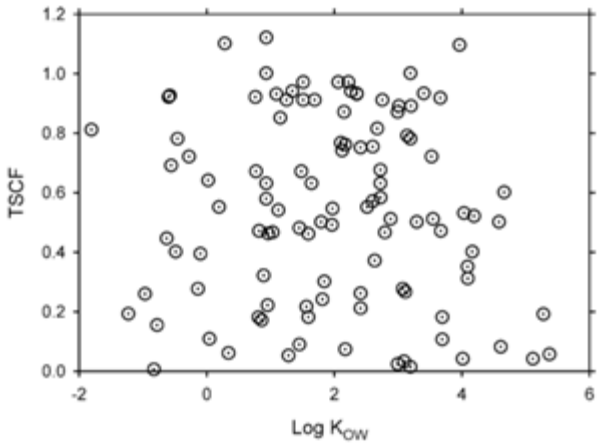

Figure 3: Compilation of Log Kow vs. TSCF from 30 publications reproduced from (Dettenmaier et al. 2009)

Adding further complexity to the study of compounds within crops, some chemicals can be actively transported (i.e. Nitrogen Phosphorus Potassium) and have TSCF values greater than one as seen in Figure 3 (Dettenmaier et al. 2009). Chemicals can also enter plants via transport across the lipophilic cell walls of leaves, fruits, stems, and seeds as well as their roots (Menn 1978). Indeed, in addition to efficiency considerations that can be associated with drip irrigation, it also circumvents direct transport across leaves and fruit as a potential route of exposure, especially for crops in which these tissues represent the edible portion.

As with uptake and entry of chemicals into plants, translocation of compounds within crop tissues also appears to lack consistency and varies from plant to plant, compound to compound (Mattina et al. 2000). Once inside a plant, several phytolytic and hydrolytic enzymes may act upon compounds causing them to degrade or transform (Menn 1978). If compounds survive enzymatic action, and even if they do not, they or their metabolites may be transported short intercellular distances through plasmadesmata, or long systemic distances through the vascular tissues (xylem and phloem). Transport through the living phloem is considered symplastic; through the nonliving xylem apoplastic. The variability of

both uptake and translocation of specific chemicals within individual crops indicate that analysis of the entire plant will be most informative. The potential for enzymatic and microbial action upon chemicals in soil/crop systems further complicates compound specific analysis, and indeed mass balance of non-radio-labeled compounds is unlikely. Thus there are clear benefits to selecting indicator compounds such as caffeine and triclosan which have an already established precedence for crop uptake, transport, and detection in their undegraded form.

1.4.6 Summary of Proposed Indicators for Crop Uptake of Waste Water Contaminants

Together, caffeine, triclosan, and estradiol span the range of relevant Log Kow values for crop uptake while representing a combination of synthetic and naturally occurring compounds commonly detected in waste water effluents. Additionally, and not insignificantly, commercially available ELISA kits have been developed for these three compounds with very good sensitivities. Indeed, if found to be compatible with QuEChERS extracts after a 10 fold dilution, the minimum expected for solvent compatibility, the sensitivities of commercially available ELISA kits could potentially allow for detection of indicator compounds within crop tissues at the ng/g and pg/g level.

1.5 Objectives of the Research

There are two main objectives within this project. The first is to investigate the pairing of the QuEChERS extraction method with commercially available ELISAs as a potential highly sensitive screening method of crop tissues for indicator species of waste water micro-contaminants including PPCPs and EDCs. The second is to irrigate select crops with reclaimed water from an on-site green-infrastructure waste water treatment system and

investigate the potential human health or bioremediation ramifications as measured by the uptake of certain indicator species. Analysis of individual crop tissues, in particular the edible portion, is expected to show either growth with or without significant contaminant uptake. If the latter is observed, irrigation of the crop with reclaimed water for human consumption may be recommended. If the former is observed, significant contaminant uptake, a remediative rather than consumptive endpoint may prove a more appropriate recommendation. Indeed, identification of a crop with significant contaminate uptake potential could be applied to future green-infrastructure system designs for more efficient treatment using natural media.

CHAPTER 2: EXPERIMENTAL SKETCH, MATERIAL AND METHODS

2.1 Field Study Experimental Sketch

With the aforementioned background and objectives in mind, the following experimental sketch was developed (with the specific elements described in detail in the sections that follow):

In a greenhouse, sweet potatoes and lettuce were grown using an automated irrigation system to deliver known volumes of water onto the crops daily. The automated system pumped water from reservoirs stocked with one of three characterized water sources: a) reclaimed water from a local green infrastructure waste water reclamation system; b) reclaimed water with the target analytes spiked in to elevate their concentrations; c) tap water. In addition to the various irrigation sources, crops were grown in both sand and soil. At the end of the growing season, the various tissues and growing matrices within each treatment group were analyzed for the target analytes using methods described in section 2.5.

2.1.1 The Two Crops

The crops chosen for this proposed experiment were Covington sweet potatoes and Paris Island lettuce. These crops were chosen because a) the edible component of sweet potatoes and lettuce are distinctly different plant tissues and b) both have significant prevalence in American and North Carolina agriculture. According to the Agricultural Marketing Resource Center (www.agmrc.org), in 2010, North Carolina was the top producer of sweet potatoes in

the country and lettuce was the leading crop in terms of production value in the United States.

2.1.2 The Three Waters

Because of the numerous environmental and social benefits water reclamation has the potential to positively influence, this field study was particularly keen on investigating the use of reclaimed waste water for crop irrigation. Fortunately, we had the support of a local North Carolina entrepreneur of on-site green infrastructure systems for waste water treatment and reclamation, Dr. Halford House, who assisted in providing reclaimed waste water from an onsite treatment system (described in section 2.3) for use in this experiment. This water was collected and stored within a plastic reservoir kept inside a modified refrigeration unit (see section 2.3.2.2). Two other reservoirs were also stored within the refrigeration unit. One was also filled with reclaimed water, however it was spiked with a cocktail of the target compounds designed to raise the concentration within the reservoir by approximately 10µg/L at the time the spike was delivered. The purpose of this reservoir is to guarantee that a certain concentration of the target analytes is being applied to some of the crops and to maximize the likelihood that the analytes will be observed at some point during the crop and soil analysis. The third reservoir was filled with tap water, an "applied world control" in the sense that it is the same water that home gardeners and irrigations from municipal systems would use to grow fruits and vegetables for personal consumption.

2.1.3 The Two Growing Matrices

Each individual plant was grown in one of two growing media: initially sterilized sand or sterilized soil. Both were available for use at the Method Road Greenhouse head houses. As

"soil" is a wildly variable, heterogeneous, globally, regionally and locally inconsistent product, it was determined that growing crops in any single "soil" could lead to an incomplete understanding of the research questions. Factors that vary from soil to soil, in particular the sorptive properties and organic matter content, could greatly affect the availability of the compounds for plant uptake. Sand represents a minimum organic matter matrix and could foster an environment with minimum sorption and greater compound availability for the crops. Nevertheless, as crops are invariably grown in soil of some constituency, it was determined that the sterilized soil made available would also be used in the experiment.

2.2 Materials

2.2.1 Laboratory Materials

2.2.1.1 Chemicals

Acetonitrile (ACN, HPLC grade), hexane (GC Resolv), sodium sulfate anhydrous (Na_2SO_4, granular), methanol (MeOH, Certified ACS), and nitric acid were purchased from Fisher Scientific (Pittsburgh, PA). N,O-bis(trimethylsilyl) trifluoroacetamide + trimethylchlorosilane (BSTFA + 1% TMCS), and hexachlorobenzene (HCB) neat standard were purchased from Supelco (Bellefonte, PA). Neat Standards of 17β-Estradiol (E2), 17α-ethinyl estradiol (EE2), Estriol (E3) were purchased from Sigma Aldrich (St. Louis, MO). Caffeine neat standard (anhydrous, 99%) was purchased from Fluka (St. Louis, MO). Technical grade nonylphenol (NP, mixture of isomers, 99% pure though the isomers are uncharacterized) was purchased from Acros Organics (Geel, Belgium). Triclosan neat standard (>96% purity) was purchased from TCI America (Portland, OR). Magnesium sulfate ($MgSO_4$, anhydrous 99.5%) was purchased from Alfa Aesar (Ward Hill, MA).

Primary Secondary Amine (PSA, Bondesil-PSA) was donated by Agilient Technologies (Santa Clara, CA). Laboratory Grade Water (LGW) was prepared in-house from a Pure Water Solutions system (Hillsborough, NC), which filters chloraminated tap water to 1μm, removes residual disinfectant, reduces total organic carbon to less than 0.2 mg/L as C with an activated carbon resin, and removes ions to 18 MΩ with mixed bed ion-exchange resins.

Stock solutions were prepared in ACN for caffeine, triclosan, E2, EE2 and E3 at 500mg/L by weighing the standards onto Fisherbrand plastic weigh boats using a Fisher Scientific Balance (accu124D dual range). The weigh boat was rinsed into a volumetric flask with ACN and filled to the mark. The nonylphenol stock solutions were prepared using a micropipette to deliver a predetermined volume of NP mixture into a volumetric flask of ACN and filling to the mark. Stock solutions were stored in amber vials for 4-6 months in a freezer set at -15°C. Working solutions of the standards were prepared by dilution of stock solutions and stored for 1-2 months in amber vials stored in the -15°C freezer. (Refer to Appendix A for the specific stock and working solutions created and referenced).

2.2.1.2 Other Laboratory Materials Used

- Waring Commercial Blender (Model) (Stamford, CT);
- Sieves: USA Standard Test Sieves No. 10 (2mm) and No. 18 (1mm) (Newark Wire Cloth Company);
- Micropipettes: Gilson Pipetman 10-100μL. Fisherbrand Finnpipete 100-1000μL. Lab Systems Finnpipette Digital Multichannel 50-300uL;
- Micropipette tips: Fisherbrand non-sterilized natural color specialized standardization pipet tips 1-250μL;

- Disposable volumetric pipettes: Fisherbrand 5mL borosilicate disposable pipettes with 1/10mL demarcations.
- Vortex: Thermolyne Maxi-Mix Type 16700 Mixer
- Centrifuge: Beckman Coulter Allegra 6 centrifuge with GH-3.8 Swining Bucket Rotor.
- Heating Block and Evaporating Unit: VWR Standard Heatblock and Pierce Chemical Company Model 18780 Reacti-Vap Evaporating Unit.
- Nitrogen gas (UHP) Airgas National Welders (Charlotte, NC).
- Amber GC Vials, Caps, Inserts: 2mL Amber GC vials (Laboratory Supply Distributors Corp, Millsville, NJ), 11mm seal caps with red faced silicone septa (Supelco), 250µL flat bottom inserts (Laboratory Supply Distributors Corp)
- Syringes and syringe filters: 10µL glass syringes #701 (Hamilton Co), and 4mm Nylon syringe filters 0.45µm (National Scientific)
- Furnace: Thermolyne 48000 Furnace (used to dry Na_2SO_4 and $MgSO_4$)
- Scales: Fisher Scientific Balance (accu124D dual range), Sartorius Basic and Sartorius MC210P
- Refrigerated Storage Units: Thermmax Walk in refrigerator set at 4°C (used to store greenhouse samples until processing) and GE Freezer set at -15°C (used to store extracts until analysis).
- Abraxis Kits LLC Microtiter Plate ELISA Kits: Caffeine, Triclosan, and 17-Beta Estradiol ELISA kits (Warminster, PA)
- Molecular Devices Emax Precision Microplate Reader and Softmax Pro 3.1.2 software (Sunnyvale, CA)

- GraphPad Prism 5 software package (La Jolla, CA)

2.2.1.3 Cleaning Procedures

All glassware was soaked in a bath of Alconox detergent (Research Products International Corp, Mt. Prospect, IL) for 24 hours, rinsed with LGW, soaked in a 10% nitric acid bath for at least 12 hours, and rinsed with LGW rinse (3x). Volumetric glassware was then rinsed a 3x wash with MeOH and set to dry for at least 12 hours. Non-volumetric glassware was placed in an oven at 110°C for at least 24 hours. Plastic caps and Teflon coated septa were soaked in a bath of Alconox detergent for 24 hours, rinsed with LGW (3x) and MeOH (3x) and set to dry for at least 12 hours. Metal implements were soaked in a bath of Alconox detergent for 24 hours, rinsed with LGW (3x) and placed in an oven at 110°C for at least 24 hours. The Warring Commercial Blender used for homogenization was filled with an Alconox solution and run on the high setting for 1-2 minutes, rinsed with LGW (3x) and MeOH (3x).

2.2.2 Field Materials

- 66L clear plastic Sterilite ® (Townsend, MA) bins, used as reservoirs, were purchased from Target.
- One Directional Check Valves (PVC Schedule 40 valves), and mechanical timer (Utilitech Indoor 2 outlet mechanical timer) were purchased from Lowe's Home Improvement.
- Two and give gallon high-density polyethylene (HDPE) buckets with Leaktite brank lids were purchased from Home Depot.
- Plastic Nursery Pots (2 and 3 gallon) from Grower's Solution (Cookeville, TN).

- Irrigation Lines (½ inch and ¼ inch black poly tubing); Pressure Regulated Emitters (5-gallon-per-hour PC Drip Emitter with Anti-Drip Mechanism); T-Connectors (¼ inch barbed tee); PVC/Mesh Filter Unit (Compression ¾ inch FHT Swivel Adapter with Screen) were purchased from The Drip Store (Vista, CA).
- Access Valves (¼ inch barbed in-line flow control valves Model DD-FCV250) were purchased from Irrigation Direct, Inc. (Livermore, CA).
- Dayton 1/6HP Submersible polypropylene dewatering and utility pumps (Model 3YU54) were purchased from Dayton Electric Manufacturing Co. (Niles, IL).
- Chest Freezer: Frigidaire Model FFC15C4CW0 with 15 cubic foot capacity (Augusta GA),
- ChronTrol® Table Top unit Model DC-4 FZBN (San Diego, CA), plastic hand syphon and tygon tubing (1/2 inch) to lengthen syphon, all available in-house.
- Phytotron Nutrient Solution provided by North Carolina State University (NCSU).
- Sterilized Sand/Soil (made available from NCSU headhouses).

2.3 Site Descriptions

2.3.1 Source of Reclaimed Waste Water: Jordan Lake Business Center on-site Integrated Water Strategies (IWS) Water Reclamation System in Chatham County, NC

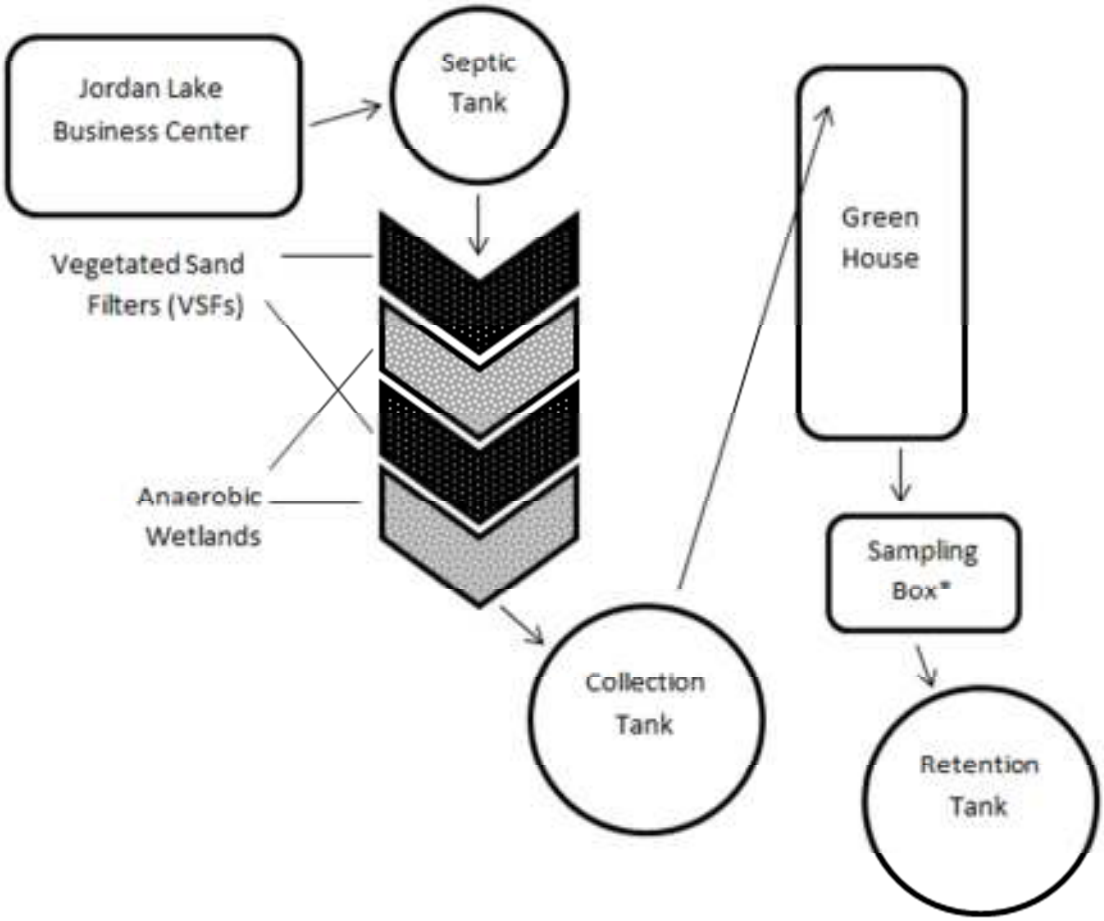

Figure 4: Jordan Lake Business Center IWS water reclamation system. * Indicates the sampling location.

A schematic of this system is shown in Figure 4, and the water collection site highlighted. Waste from the Business Center first enters a traditional septic tank where settling and anaerobic decomposition can take place. Effluent is then gravity fed to a pump tank which applies the water in timed batch loads to a series of aerobic vegetative sand filters (VSF) and anaerobic wetlands, exposing the water to multiple routes of treatment within the various conditions of each system component. Aerobic microbial treatment in the sand and

root zones of the VSF filters degrades or deactivates suspended solids, pathogenic bacteria and viruses while the constructed wetlands provide an environment for further anaerobic treatment and denitrification. After passing through the constructed wetland environments, water is held in a collection tank until it is pumped to an onsite greenhouse and additional planter boxes. After passing through the greenhouse, water is collected in a retention tank where it is chlorinated and held for reuse in the toilets of the Business Center. The system was designed for use by about 60 people or 900gallons per day (gpd). Usage was not closely monitored during the period of collection, however during previous studies performed on this system it was found that it was typically between 500-700gpd (Chalew 2006). As shown in Figure 4, water was collected from this system after it had passed through the greenhouse but before it was chlorinated. Collected water was transported in Alconox detergent washed, five gallon HDPE buckets with snap on Leaktite brand lids.

2.3.2 Method Road Greenhouse

Crops were grown in a greenhouse complex managed by North Carolina State University in Raleigh, NC. The following sections describe the physical set-up within the greenhouse and the experimental growing conditions.

2.3.2.1 Physical Layout and Treatment Group Blocking

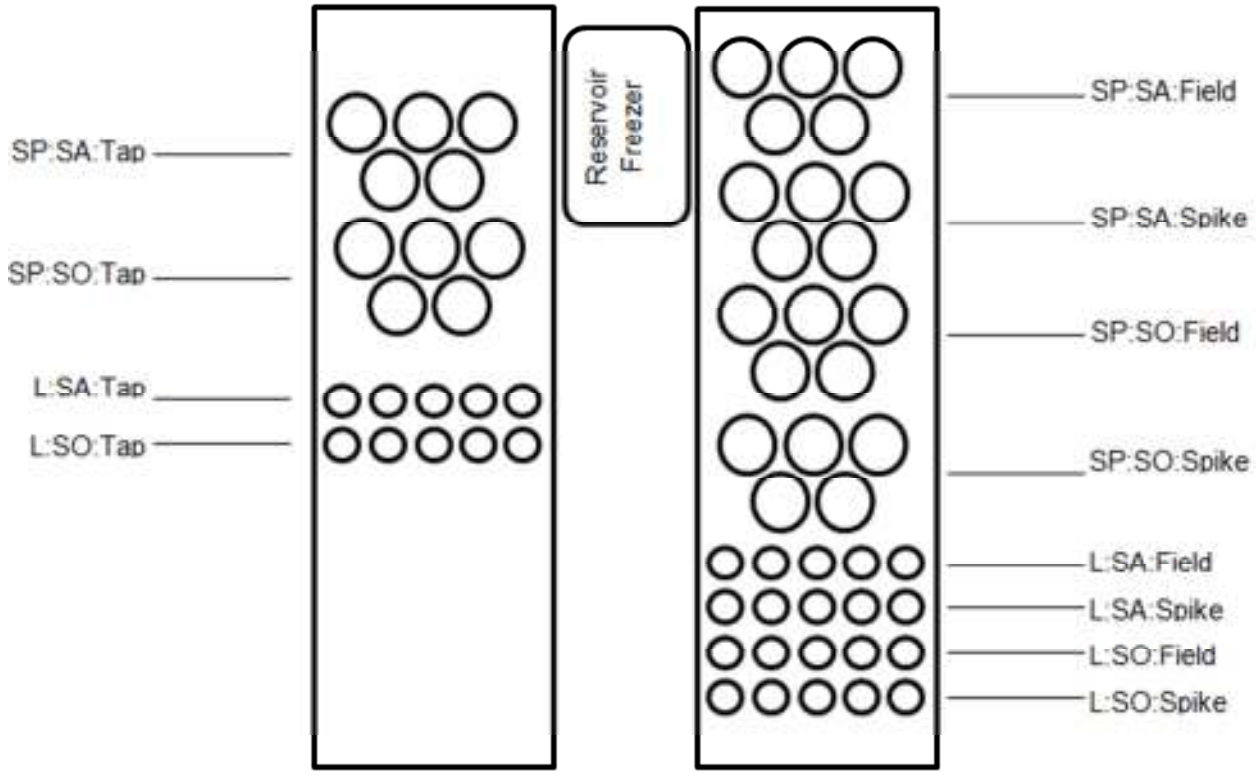

Figure 5: Physical layout of greenhouse experiment and blocking setup. SP = Sweet Potato. L = Lettuce. SA = Sand. SO= Soil. Tap = Irrigated from Tap Water reservoir. Field = Irrigated with IWS treated water as collected from field site. Spike = Irrigated with IWS water with additional spike of target analytes.

The allotted space within the greenhouse consisted of two growing tables (~4ft x 16ft). Plastic nursery pots (2 gallon for lettuce, 3 gallon for sweet potato) were arranged on the tables as seen in Figure 5. For practicality in creating an automated irrigation system (described below) treatments were blocked on the tables as seen in Figure 5. Pots were filled with either sterilized sand or sterilized soil, both of which were made available at the head houses of the Method Road Greenhouse complex. Pots were filled to approximately the same level, but effort was not taken to be exact. Irrigation was not going to result in the entire soil column being wetted; soil samples to be analyzed at the end of the growing season

were to be column samples nearest the plant and emitter (expected to have greatest exposure to irrigation water) and not a representative sample of the entire pot.

2.3.2.2 Reservoir Storage

A chest freezer was brought to the Method Road greenhouse in order to store the treatment reservoirs while keeping the water inside moderately temperature regulated and shielded from sunlight. A homemade scaffolding system was constructed from pressure-treated 2x4s in order to hold three covered 66L plastic Sterilite reservoirs within the freezer. The freezer could not be run constantly as even the most modest settings proved to be sufficient to turn water into blocks of ice. As such, the freezer was placed on a Utilitech mechanical plug-in basic timer to run only during the warmest hours of the day. Temperature readings, using a mercury thermometer, over the study period showed water temperatures ranging from 10C to 20C.

2.3.2.3 Automated Irrigation System

The following is a description of the automated system constructed for a single reservoir. Each of the three reservoirs within the chest freezer was outfitted with one of the following:

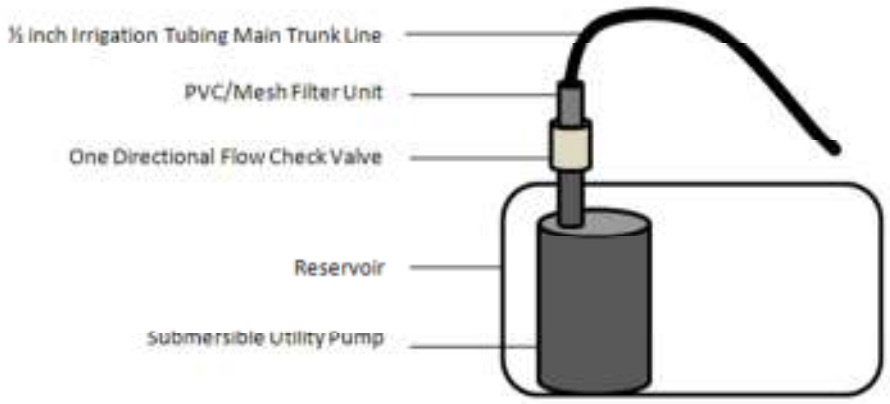

Figure 6: Reservoir pump system

As seen in Figure 6, a 1/6 HP Dayton Portable submersible utility pump was placed into each reservoir. A one way directional flow check valve was fitted to the outlet of each pump to protect the pump from any backpressure. A PVC mesh filter unit with a ½ inch pressure fit coupling was attached to each check valve. One end of an approximately 30 ft length of ½ inch black PVC irrigation tubing was fit into the pressure fitting of the filter unit and looped around the pots on an entire table as depicted in Figure 7. An adjustable PVC ball valve was attached to the end of the ½ inch irrigation tubing and placed back into the reservoir from which it originated, thus creating an adjustable pressurized loop for the main irrigation trunk line.

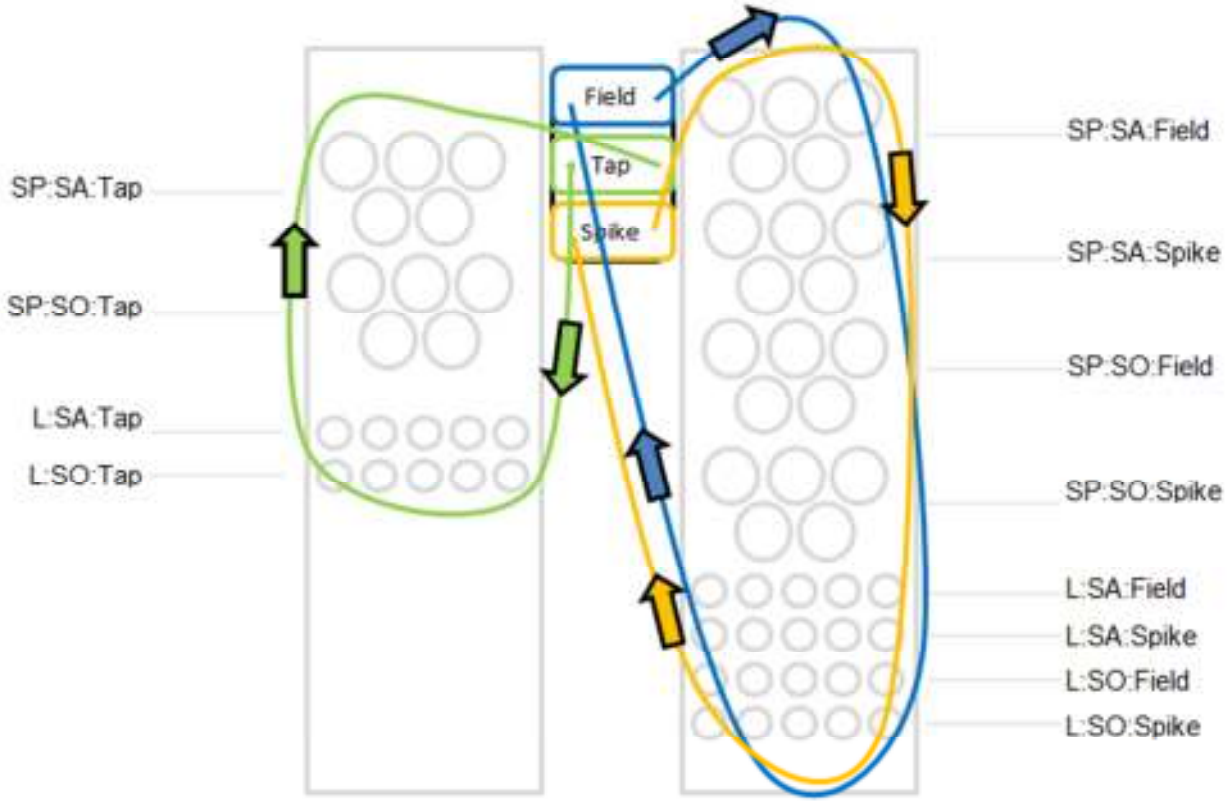

Figure 7: Main irrigation trunk lines: Pressurized loops originating from and returning to a single reservoir.

In order to access the water flowing through the trunkline (see Figure 8), ¼ inch barbed PVC irrigation ball valves were used (access valves). To these access valves, loops

of ¼ inch irrigation tubing were constructed (access loops), each with 5 barbed T-connectors within the loop in order to create 5 emitter lines from each access loop. The end of each emitter line was fit with an internally pressure regulated (0.5gph) emitter which was anchored to a treatment pot by a twist tie attached to a plastic anchor placed in the soil. The pumps were turned on automatically using a ChronTrol® unit capable of exact programming down to the second. Initial tests of the system were run to determine the rate of flow from the emitters(0.06 mL/sec), and that flow rate was used to determine the amount of time the pumps would be turned on to achieve the desired delivery volume. The flow rate from the emitters was tested monthly without any change.

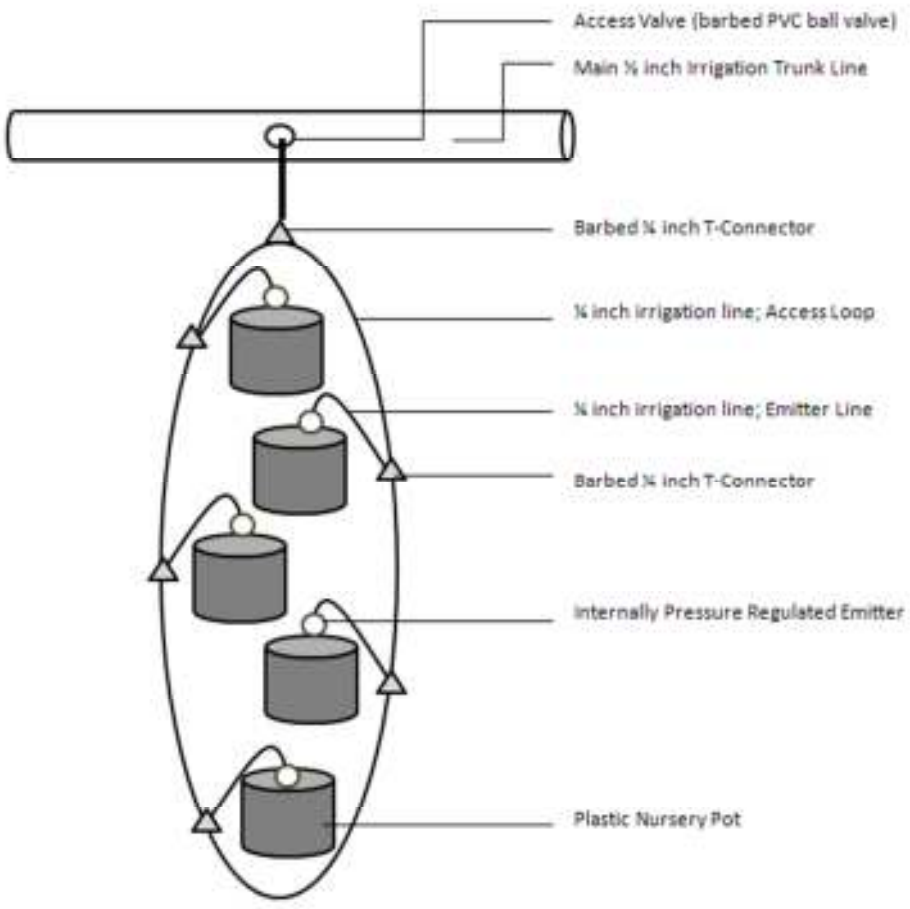

Figure 8: Irrigation access loops from main trunk line.

2.4 Field Methods

2.4.1 Collection of Water from Field Site

Refer to section 2.3.1 Source of Reclaimed Waste Water: Jordan Lake Business Center on-site Integrated Water Strategies (IWS) Water Reclamation System in Chatham County, NC

2.4.2 Filling the Reservoirs

An access hole was cut into the lid of each reservoir. When not being used, this access hold was covered by a plastic cap. To fill each reservoir, lengths of tygon tubing were connected to a hand syphon which was used to transfer treatment water from five gallon HDPE buckets (see section 2.3.1 Source of Reclaimed Waste Water: Jordan Lake Business Center on-site Integrated Water Strategies (IWS) Water Reclamation System in Chatham County, NC into the reservoirs within the freezer unit. Time between collection at the IWS site and transfer into the reservoirs was about an hour. The tap water reservoir and the IWS field reservoir were transferred non-volumetrically (the reservoirs were simply filled to near capacity). To fill the IWS spike reservoir, a 5 gallon HDPE bucket was marked at the 15L volume level. The bucket was filled to the mark, and then spiked with a premade "spike bomb" which had been created in the lab the day before. This "spike bomb" was made by delivering known volumes of the stock solutions made in ACN into a 40mL amber vial that was mostly filled with LGW. The volumes of stock solution added to the vial were designed such that the addition of the entire "spike bomb" would increase the concentration of each analyte within the newly added water (either 30L or 45L added each time) by 10µg/L. This "spike bomb" was added to the first 15L aliquot transferred. Subsequent 15L volumes were

measured into the same bucket before transfer into the reservoirs. The syphon was washed between the transfers of each treatment water into its reservoir by passing approximately 2 gallons of an Alconox+tap water solution through it, followed by approximately 2 gallons of tap water alone. All buckets were washed after each transfer with an Alconox+tap water solution followed by a rinsing with tap water.

2.4.3 Collecting Water Samples from Reservoirs

Prior to the addition of new water into the reservoirs, water samples were collected from each reservoir in 1L amber glassware and taken back to the UNC laboratory for various analyses. Samples were stored in a 4°C walk in refrigeration unit when not being analyzed. Reservoir samples were stored between a few days and a few weeks before being discarded.

2.4.4 Covington Sweet Potatoes: Planting, Irrigation Summary and Harvest

Covington sweet potato cuttings were generously provided by Jared Driscoll of NCSU and planted in 3 gallons pots on April 29th 2011. The plants were irrigated from an automated system (described in section) on a dynamic schedule that varied depending on the perceived needs of the plants. The amount of treatment water delivered throughout the growing period was tracked rigorously. When the sweet potatoes were harvested on October 3 2011, each plant had received 35.1L of water from their respective treatment reservoirs over the entire growing period. Plants were harvested in their entirety. Potatoes from each pot were weighed on site using a Sartorius Basic balance, wrapped in aluminum foil and stored in gallon sized Ziploc bags. Stems and leaves from each plant were coiled and weighed on site and stored in Ziploc bags. An approximately 400 gram column of field moist soil was taken from each pot, with efforts being taken to collect soil from all depths of

the soil column and nearest to the cavities left behind by the harvested potatoes. Moist soil samples were weighed on site, wrapped in foil and stored in Ziploc bags. All samples (Potatoes, Stems/Leaves, Sand/Soil) were taken to the UNC laboratory and stored in a 4°C walk in refrigerator until further processing.

2.4.5 Parris Island Lettuce: Planting, Irrigation Summary and Harvest

Parris Island Lettuce seeds obtained from Lowes were planted in 2 gallon pots (5 seeds per pot) of either sand or soil on October 4, 2011. The plants were irrigated from the automated system (described in detail in section 2.3.2.3) with each plant receiving 145mL of treatment water per day. When the lettuce plants were harvested on December 1, 2011, each plant had received 8.265L of treatment water. Plants were harvested in their entirety. Leaves and roots were weighed as a bundle on site, wrapped in foil and stored in Ziploc bags. An approximately 400g column of field moist soil was taken from each pot, with efforts being taken to collect soil from all depths of the soil column and nearest to the cavities left behind by the harvested roots. Moist soil samples were weighed on site, wrapped in foil and stored in Ziploc bags. All samples were taken to the UNC laboratory and stored in a 4°C walk in refrigerator until further processing.

2.5 Laboratory Methods

2.5.1 Homogenization and Sample Preparation for Extraction:

2.5.1.1 Sweet Potatoes

The following describes the homogenization/extraction performed on a single treatment group (e.g. Potato tissue, grown in sand, irrigated with tap water). Recall there were 5 pots per treatment group.

All sweet potatoes were rinsed thoroughly with LGW and set to dry for approximately 1 hour. The collective mass of the sweet potatoes from each pot were weighed and recorded. A knife was used to cut transverse samples of all the sweet potatoes from a single pot until 50g had been accumulated. This 50g sample was placed into a Waring Commercial Blender. This process was repeated for the sweet potatoes originating from each of the 5 pots in the treatment group, resulting in a total of 250g of sweet potato being placed into the blender. It was found that a 6:4 mass/mass ratio of sweet potato to LGW resulted in sufficient liquid for the blender to create homogenous slurry, and thus 166mL LGW was also added to the blender. After slurry was achieved, the blender was left to run on the highest setting for 1-2 minutes. 10 gram homogenated samples were then transferred from the blender into 7 clear 40mL glass vials using metal spatulas. The vials were sealed with plastic caps that had been fit with Teflon lined septum. Homogenates of 4 of the 7 sample vials were spiked with a working solution of the 6 target analytes (concentration of each analyte within working solution approximately 35-40ug/mL) made up in ACN for the purposes of running spike-recovery experiments (Figure 9). Two spike levels were performed in duplicate (A1, A2, and B1, B2) with the B spikes twice the concentration of the A spikes. The remaining 3 homogenates were left unspiked. All 7 vials were vortexed for 1 minutes using a Thermolyne Maxi-Mix, and left for 12-24 hours to allow the added analytes to incorporate into the homogenate.

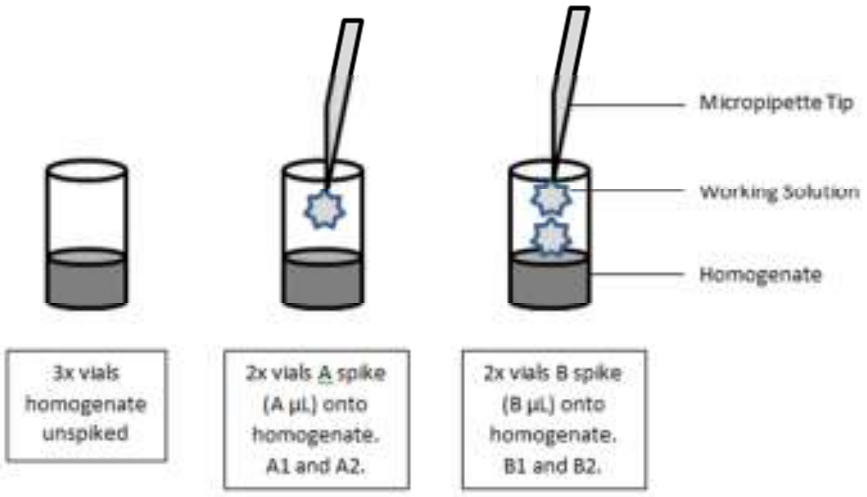

Figure 9: Preparation of homogenates for extractions and homogenate spikes.

2.5.1.2 Sweet Potato Leaves

The leaves from each plant in a treatment group were separated from the stems, rinsed thoroughly with LGW, shaken vigorously, pat down with Kimwipes and set to air dry for approximately 30 minutes. After this initial drying, leaves were weighed to determine their fresh weight. A single treatment (tap irrigated grown in sand) was processed fresh, with all leaves added to the Waring Commercial Blender. LGW was added until slurry was achieved, the volume added recorded. 10 gram samples were then transferred from the blender into 7 clear 40mL glass vials using metal spatulas, and the remaining preparation of these homogenates for extraction carried out in the same manner as described sweet potato samples in section 2.5.1.1 Sweet Potatoes

All remaining leaf treatments groups were dried more rigorously and processed dry rather than fresh. After rinsing and recording fresh weights, leaves were covered and left to air dry 2-5 days. Dry leaves were weighed and typically found to have lost approximately 80% of their mass. Dry leaves were added to the Waring Commercial Blender and pulverized into a fine dust. Depending on the mass available, 2-5g of dry leaf dust was

transferred from the blender into clear 40mL glass vials using metal spatulas. The remaining preparation of these homogenates for extraction was carried out in the same manner as described for the sweet potato samples in section 2.5.1.1.

2.5.1.3 Lettuce Leaves

It was determined that the mass of leaves from the treatment group grown in sand and irrigated with tap water was insufficient to be able to achieve a homogenate.

The leaves from each lettuce plant were separated from the roots, rinsed thoroughly with LGW and set to air dry for 30 minutes. After the initial drying, all leaves from all treatments were weighed to determine their fresh weights. Leaves were left to dry for 5-8 days and were found to have lost approximately 90% of their fresh weight mass. Dried and weighed leaves were added to the Waring Commercial Blender and pulverized to a fine dust. Depending on the mass available, 0.5-0.9g dry leaf dust was transferred from the blender into clear 40mL glass vials using metal spatulas. The remaining preparation of these homogenates for extraction was carried out in the same manner as described for the sweet potato samples in section 2.5.1.1 Sweet Potatoes

For two treatments (IWS field irrigated, sand and soil grown), it was determined that pulverization of the dry leaf material in the blender was unsatisfactory. LGW had to be added to these samples to create a slurry. The amount of LGW added to each was recorded. 10-12g of slurry was transferred from the blender into clear 40mL glass vials using metal spatulas. The remaining preparation of these homogenates for extraction was done in the same manner as described for the sweet potato samples in section 2.5.1.1 Sweet Potatoes

2.5.1.4 Sand and Soil Samples

The foil containing sand samples was opened and a metal spatula used to spread out the field moist samples. The samples were covered with a large Kimwipe and left to air dry overnight. Dried sand samples were sieved through a No 10. (2mm) sieve, and 100g sieved sand from each sample within the treatment group added to the Waring Commercial Blender (500g per treatment group). The blender was shaken vigorously and then run on high for 1-2 minutes. 10 gram samples were transferred from the blender into clear 40mL Pyrex vials using metal spatulas.

Soil was prepared in the same way as sand, but dried soil was passed through a stack of two sieves, first through a No. 10 (2mm) and then through No. 18 (1mm). The remaining preparation of both sand and soil homogenates for extraction was done in the same manner as described for in section 2.5.1.1 Sweet Potatoes

2.5.2 Sample Extractions, Fractions, Concentration and Storage

The extraction method used is based on the QuEChERS method developed by Michelangelo Anastassiades in (2002) for the analysis of pesticide residues in crops. Figure 10 below summarizes how samples were processed with detailed sections to follow.

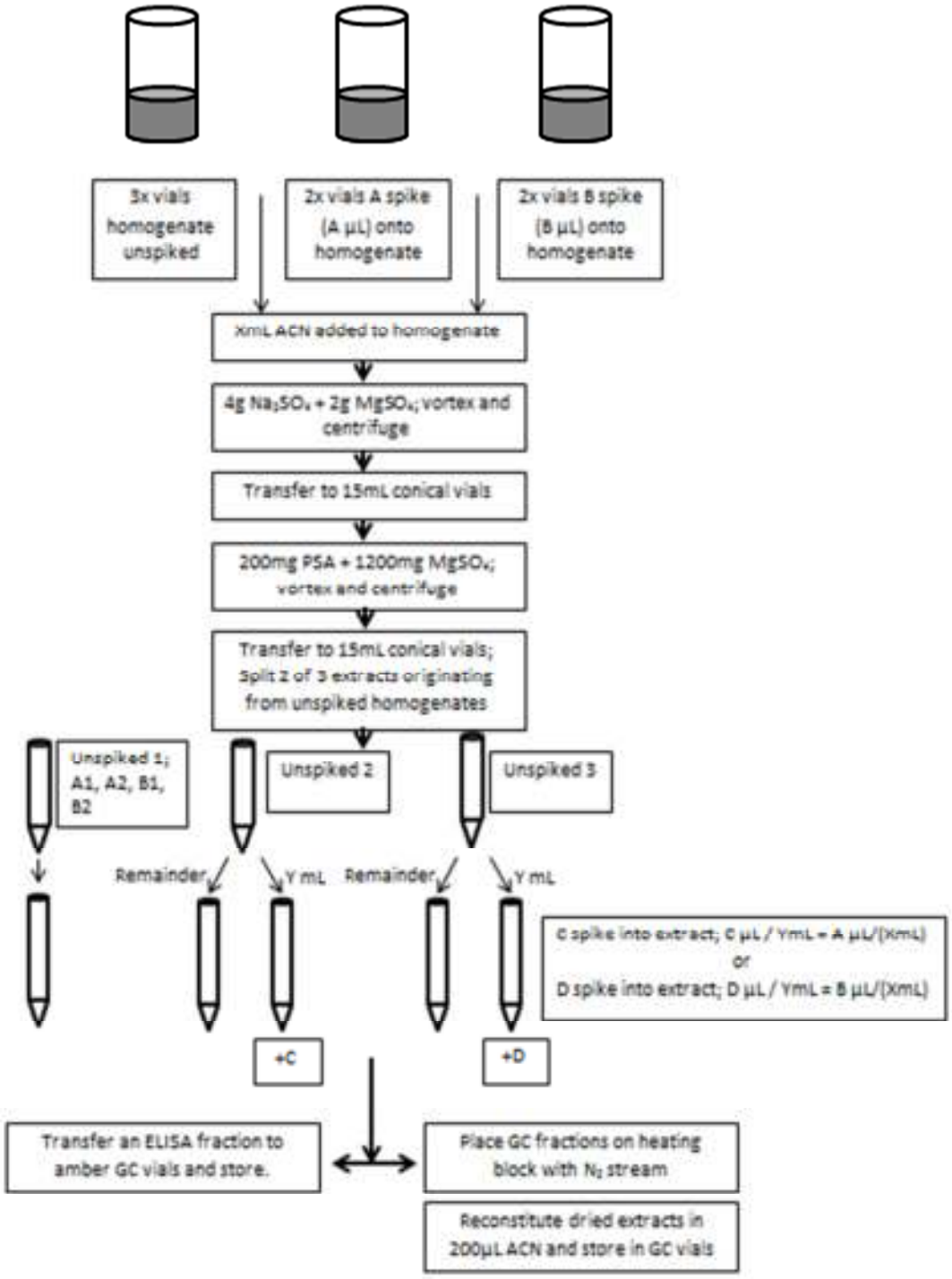

Figure 10: Extraction of sample homogenates; Homogenate and extract spikes for recovery experiments; ELISA fractions and concentration of GC fractions.

2.5.2.1 Sweet Potatoes

10 mL ACN was added to each vial of homogenate (note in Figure 10, this initial volume is referred to as "XmL"). The vials were vortexed approximately 30 seconds and left to sit 2-6 hours to allow the solvent time to percolate through the homogenate. 4g Na_2SO_4 (+/- %) and 2g $MgSO_4$ were added to each vial. After addition of the $MgSO_4$, the vials were noticeably warm to the touch. The vials were vortexed for 1 minute, and placed into a Beckman Coulter Allegra 6 centrifuge with GH-3.8 Swinger Bucket Rotor at 2000rpm for 10 minutes. A Pasteur pipette was used to transfer the upper solvent layer into a 15mL conical vial. It was found that of the 10mL ACN added to each vial, approximately 8mL were recovered at this junction, the remaining being inaccessible within the wetted homogenate tissue. 200mg PSA (+/-5%) and 1200mg $MgSO_4$ were added to the conical vials for a scaled up* dispersive solid phase extraction (dSPE) (*Note: A typical QuEChERS extraction only processes a 1mL sample with 25mg PSA and 125mg $MgSO_4$, but a larger volume of cleaned sample was desired in order to pursue subsequent sample concentration). The conical vials were vortexted for 30 seconds and placed into the centrifuge at 2000rpm for 10 minutes. The remaining solvent of the dSPE extracts was transferred to fresh 15 mL conical vials and the volume measured using disposable Fisherbrand 5mL borosilicate volumetric pipettes with 1/10mL demarcations. Of the three extracts corresponding to the unspiked homogenates, the volumes of two were split further into a 3mL fraction and a remainder fraction (in Figure 10, the "Y mL" and "Remainder"). The two 3mL fractions were spiked (labeled C and D "extract spike") with working solution of the analyte mix such that the C spike would match the concentration of the original A homogenate spike in 10mL ACN and the D spike would match the original B homogenate spike in 10mL ACN. A comparison of the A and C spiked

extracts (as well as a comparison of the B and D spiked extracts) should give an indication of the recovery efficiency of the extraction method. 0.5mL of each extract was transferred to labeled amber GC vials and stored in a freezer, this fraction being destined for analysis by the ELISA kits. The remaining volume of each extract (typically between 2-6mL) was placed on a heating block set to 50ºC, and a gentle stream of nitrogen gas was used to blow the samples down to dryness. After cooling to room temperature, 200µL of ACN was added to each conical vial and vortexed for 1 minute to reconstitute the sample. A Pasteur pipette was used to transfer the entire volume of each reconstituted sample into 250µL flat bottom glass inserts within an amber GC vial, this fraction being destined for analysis on the GC-ion trap-MS. For an example of the full workup of the homogenates from a single treatment group, including the volumes of the homogenate and extract spikes, refer to Table 2: Full workup of the 7 homogenates into the 9 final treatment extracts for of the treatment group P:S:SA:. The same extensive details for the sample preparation and extraction for the matrix samples from each group are available in the Z and Y series Supporting Materials (part of an electronic labbook on file at UNC, not included in thesis).

Working Solution Used:	#2									
Treatment Group: Sweet Potato Tissue, Spike IWS Irrigated,Sand Grown: **P:S:SA:P**										
Initial Sample Designation	Mass (g) Homogenate	Volume Working Solution Spiked (uL)	Volume ACN (mL) added	Post Extraction Sample Designation	Volume (mL) After Extraction	Volume Working Solution Spiked (uL)	Volume (mL) stored for ELISA	Volume (mL) remaining before blowdown	Final Volume (uL) after blowdown	Blowdown Concentration
P:S:SA:P1	10.03	0	10	P:S:SA:P1	2.35		0.5	1.85	200	9.25
			10	P:S:SA:P1+C	3.00	18	0.5	2.5	200	12.5
P:S:SA:P2	10.01	0	10	P:S:SA:P2	2.55		0.5	2.05	200	10.25
			10	P:S:SA:P2+D	3.00	36	0.5	2.5	200	12.5
P:S:SA:P3	10	0	10	P:S:SA:P3	6.60		0.5	6.1	200	30.5
P:S:SA:P+A1	10.03	60	10	P:S:SA:P+A1	5.30		0.5	4.8	200	24
P:S:SA:P+A2	10.08	60	10	P:S:SA:P+A2	5.25		0.5	4.75	200	23.75
P:S:SA:P+B1	10.04	120	10	P:S:SA:P+B1	5.15		0.5	4.65	200	23.25
P:S:SA:P+B2	10.01	120	10	P:S:SA:P+B2	5.60		0.5	5.1	200	25.5

Table 2: Full workup of the 7 homogenates into the 9 final treatment extracts for of the treatment group P:S:SA:P

Working Solution #2			
Analyte	Concentration in Working (ug/uL)	Concentration [ug/L] in A/C Samples	Concentration in B/D Samples
E2	0.0387	232	464
Caffeine	0.0357	214	428
EE2	0.0370	222	444
E3	0.0418	251	502
Triclosan	0.0360	216	432
Nonlyphenol	0.0327	196	393

Table 3: Working solution #2 and the resulting spike boost to the A,B,C,D samples of P:S:SA:P

2.5.2.2 Sweet Potato and Lettuce Leaves

The wet homogenates were extracted exactly as described in section 2.5.2.1 Sweet Potatoes

The dry homogenates were extracted exactly as described in section 2.5.2.1 Sweet Potatoeswith two exceptions: a) that the initial volume of ACN added (XmL in Figure 10) to the pulverized leaves was 15mL or 20mL depending on the amount of leaf mass and b) 500mg $MgSO_4$ was used during dSPE rather than 1200mg. The larger volumes was chosen in order to ensure sufficient volume would be available to achieve similar concentration factors in the GC fraction when blown down and reconstituted. The reduced $MgSO_4$ was due to the fact that the dried samples had less water within them. The initial homogenate spikes (A&B) and final extract spikes (C&D) were appropriately adjusted to reflect the initial ACN volume added.

2.5.2.3 Sand/Soil

The sand and soil homogenates were extracted exactly as described in 2.5.2.1 Sweet Potatoeswith two exceptions: a) that the initial volume of ACN added (XmL in Figure 10) to the sand and soil samples was 15mL and b) 500mg $MgSO_4$ was used during dSPE rather than 1200mg. The larger volume was chosen in order to ensure sufficient volume would be available to achieve similar concentration factors in the GC fraction when blown down and reconstituted. The reduced $MgSO_4$ was due to the fact that the sand/soil samples had less water within them than fresh crop samples. The initial homogenate spikes (A&B) and final extract spike (C&D) were appropriately adjusted to reflect the initial ACN volume added.

2.5.3 Analytical Instrumentation, Software and the Abraxis Enzyme Linked Immunosorbent Assay (ELISA) kits:

2.5.3.1 Gas Chromatography

Gas chromatographic analysis was performed on a Varian 3800 gas chromatograph with a Saturn 2000 ion trap mass spectrometer (Santa Clara, CA), and an HP5890 GC Series II (Palo Alto, CA) with an electron capture detector (ECD). The analytical column used on the Varian 3800 GC was a Zebron Phenomenex ZB5-ms, 30m x0.25mm with 0.25µm film thickness (Torrance, CA). The GC column used on the HP5890 was a Zebron Phenomenex ZB1-ms, 30m x 0.25mm with 1.0µm film thickness. The Helium (UHP) carrier gas, Nitrogen (UHP) and Carbon Dioxide (USP) used with these instruments were purchased from Airgas National Welders (Charlotte, NC).

2.5.3.1.1 Ion Trap Mass Spectrometry

Stored plant tissue and soil extracts (section 2.5.2 Sample Extractions, Fractions, Concentration and Storage) were filtered through a 0.45µm nylon syringe filter (4mm, National Scientific, Rockwood, TN). 1µL filtered extracts were injected manually through a Varian 1079 injection port fitted with a deactivated glass SPI liner (Restek, Bellefonte, PA) using a 10µL glass syringe (#701, Hamilton Co., Reno, NV). The injection port was held at 60ºC for 0.1 minute and then brought up to 250ºC at a rate of 100ºC/min for the remainder of the sample run. The carrier gas was UHP Helium set at a constant flow rate of 1.5mL/min.

The oven program started at 90ºC, was held for 1 minute, then ramped up to 150ºC at a rate of 15ºC/min, held for 15 minutes, then ramped up to 200ºC at a rate of 5ºC/min, held 5 minutes, then ramped up to 290ºC at a rate of 15ºC, held for 6 minutes. Total run time was 47 minutes.

The ion trap filament was turned on and off multiple times during the 47 minute sample run in order to protect the filament. When the ion trap was on, it was set to run in EI mode with axial modulation voltage set at 4.0v, emission current at 10μamps, and automatic gain control (AGC) on. After a 7 minute solvent delay (filament off), the filament was turned on (caffeine scan) and set to scan a range from mass-to-charge ratio (m/z) of 40 to 300 until minute 24. From minute 24 to minute 28, the filament was turned off. At minute 28 filament was turned on (triclosan scan) and set to scan a range from m/z of 100 to 515 until minute 30.50. From minute 30.50 to minute 38 filament was turned off. At minute 38 filament was turned on (hormone scan) and set to scan a range from m/z 70 to 515 until minute 43. From minute 43 to minute 47, the filament was turned off. Data were analyzed using Varian MS Workstation software v. 6.4.1.

2.5.3.1.2 Electron Capture Detection

A Hewlett Packard 7673 autosampler injected 1μL samples of extract through an injector port fitted with split/splitless injector sleeve, containing deactivated glass wool of 4mm inner diameter. The injector port of the HP5890 GC was held at a constant temperature of 250ºC. The carrier gas was UHP Helium set at a flow rate of 1.0-1.5mL/min.

The oven program started at 90ºC, was held for 1 minute, then ramped up to 150ºC at a rate of 15ºC/min, held for 15 minutes, then ramped up to 200ºC at a rate of 5ºC/min, held 5 minutes, then ramped up to 290ºC at a rate of 15ºC, held for 6 minutes. Total run time was 47 minutes. The ECD detector was on turned on after a 5 minute solvent delay and left on during the entire sample run. The ECD was set at a constant temperature of 300ºC.

2.5.3.2 Abraxis Enzyme Linked Immunosorbent Assay (ELISA) Method

Direct competitive ELISA kits for caffeine, triclosan and estradiol were donated by Abraxis Kits LLC (Warminster, PA). Samples were analyzed according to the instructions included in the test kits. Briefly, 50µL of standard or extract (diluted with LGW as needed) were placed into the wells of a disposable microtiter plate which are coated by immobilized (second) antibodies. 50µL of a primary antibody solution (engineered with binding sites specific to the compound of interest) were added to the wells and the samples given 30 minutes for any target analytes present to bind to the antibodies. 50µL of an analyte+enzyme conjugate solution were then added to each well. The conjugate used is horseradish peroxidase (HRP) that has been chemically bound to the target analyte. Since this conjugate contains the analyte of interest it will also attach to the binding sites of the primary antibody solution. The samples and conjugate were left to incubate at room temperature for a specified time during which they compete for the analyte specific binding sites of the primary antibody solution. The greater the concentration of analyte in the sample, the fewer sites available for the HRP-conjugate. During this incubation period, the primary antibodies are also bound to the immobilized (second) antibodies on the walls of the wells. The wells were then washed and a patented color solution added. The color solution reacts with any HRP that was immobilized on the wells during the competitive incubation period, resulting in a blue color development proportional to the amount of HRP present. Therefore, greater color development corresponds to more HRP-conjugate on the wells, which correlates to less analyte being present in the sample of interest. A stopping solution of dilute sulfuric acid was added after a specified time to quench the color development reaction and change the complex from blue to yellow. The quenched wells were analyzed within 15 minutes at

450nm using a Molecular Devices Emax Precision Microplate Reader and Softmax Pro 3.1.2 software (Sunnyvale, CA). The reported detection limit of the Abraxis ELISA kits in water samples are 0.15 µg/L (caffeine), 0.02 µg/L (triclosan), and 2.7 ng/L (estradiol).

2.5.3.3 Additional Software

Absorbance values from the standards and samples were input to the GraphPad Prism 5 software package (La Jolla, CA) for analysis using the Sigmoidal Dose Respose Variable Slope regression fit as described by Abraxis in the instructions included in the test kits. Whenever possible, 99% confidence intervals of the interpolated sample values were extracted from the GraphPad Prism 5 software.

2.6 Supplemental Methods Section

The following describe additional methods that were used during the investigation that ultimately were not included in the final extraction and concentration of crop tissues and growing matrices from the greenhouse experiment. They are presented here for reference as they are referred to within the Results and Discussion section.

2.6.1 Glassware Silinization Method: Used to prepare conical vials prior to derivatization

Using a Pasteur pipette to coat the side walls, glass conical vials were rinsed with 5% dimethyldichlorosilane in toluene. The vials were then rinsed successively with toluene (three times), methanol (two times), and lab grade water (two times). The tops of the vials were then covered in foil and placed in an oven at 150°C until dry. Vials were allowed to cool prior to placing sample extracts within for blow-down and derivatization.

2.6.2 Derivatization Method A

Dry extracts were reconstituted with 50µL of BSTFA (+1% TMCS) plus 50µL pyridine. The vials containing reconstituted extracts were capped with PTFE-lined septum, vortexed for 30 seconds to reconstitute residual sample from the sides of the vials, and heated for 35 minutes at 65°C on a VWR Standard Heatblock. The samples were allowed to cool, and then quenched with 100µL LGW, followed by 100-200µL Hexane. The samples were again capped and vortexed, and allowed 30 minutes to rest prior to transferring the topmost (hexane) layer into amber GC vials for storage and analysis. During some initial method development investigations, it was observed that neither caffeine nor nonylphenol were being detected in the final hexane extract upon derivatization with this method. As a result, additional derivatization methods were investigated, though this method was used for some of the initial compatibility and recovery experiments described in the Results and Discussion Chapter 3.

2.6.3 Derivatization Method B: Acetonitrile Reconstitution

Dry extracts were reconstituted first in 200µL of ACN, followed by 50µL of BSTFA (+1% TMCS) plus 50µL pyridine. The vials containing reconstituted extracts were capped with PTFE-lined septum, vortexed for 30 seconds to reconstitute residual sample from the sides of the vials, and heated for 35 minutes at 65°C on a VWR Standard Heatblock. The samples were allowed to cool, and then quenched with 100µL LGW, followed by 100-200µL Hexane. The samples were again capped and vortexed, and allowed 30 minutes to rest prior to transferring the topmost (hexane) layer into amber GC vials for storage and analysis. Using this method, nonylphenol was observed within the final hexane extract on the GC-MS,

however the concentration required within the extract for detection appeared be between 3-10mg/L. Caffeine continued to be absent in the final hexane extract.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Method Compatibility and Extraction Efficiency

One of the primary objectives of the project was to investigate the use of commercial ELISA kits in the analysis of complicated, solvent based, crop extracts obtained by the QuEChERS extraction method. A number of investigations were performed to determine a) the ability of the ELISA kits to analyze the complex extracts from each matrix (Compatibility) and b) the ability of the extraction method to recover each analyte from the various matrices of interest (Extraction Efficiency).

3.1.1 Strategies Utilized

The most rigorous method compatibility investigations were primarily performed by spiking the analytes of interest into finished QuEChERS extracts (standard addition or "extract-spiked samples") and comparing the ELISA kit responses for these extract-spiked samples to the responses observed for unspiked-homogenate extracts (see sections 3.2.2 and 3.3.3). All ELISA responses were quantified using a standard calibration curve run on the day of analysis. If the response of the extract-spiked sample was found to be elevated above the response of the unspiked-homogenate extract by the magnitude expected from the spike delivered, the conclusion could be made that the ELISA kit was capable of detecting and quantifying the analyte of interest within the complex extract. If the response observed for the extract-spiked samples above the unspiked-homogenate extract did not correspond to the expected analyte concentration from the spike delivered, (i.e. the ELISA could not

differentiate between unspiked-homogenate extracts and extract-spiked samples), then the determination was made that, as currently prepared and tested, the extracts were not compatible with the ELISA kits.

Extraction efficiency investigations were performed by spiking the analytes of interest directly onto the homogenates of plant tissues or growing matrices prior to extraction and quantifying the difference in responses, based on the ELISA calibration curve, between the homogenate-spiked and unspiked-homogenate extracts. The difference observed was compared to the difference expected, based on the spike delivered, and the ratio of these values (observed/expected) was deemed the recovery or extraction efficiency. In some of the initial investigations described in the sections that follow, homogenate-spiked extracts were also used as a surrogate compatibility investigation: so long as the ELISA was capable of differentiating between unspiked-homogenate extracts and homogenate-spiked extracts, the conclusion was made that on some basic level, the ELISA was capable of analyzing the crop tissue extracts.

The final technique utilized to provide additional merit to the ELISA responses observed was the practice of performing serial dilutions; the concept being that the response observed for an extract 10 fold dilute should indicate a concentration twice that of the same extract 20 fold dilute. If an extract, dilute at multiple levels, gave responses consistent with the dilution level, then, baring other compatibility issues, the responses were considered to be verified. Responses that were not consistent with their dilution level led to the conclusion that the analysis was experiencing compatibility complications as performed.

3.2 A Chronological Evolution Investigating Method Compatibility

3.2.1 Phase 1 Investigation: Grocery Store Sweet Potato Tissue

The first investigation of method compatibility and extraction efficiency was performed on sweet potatoes obtained from the local grocery store. The sweet potatoes were rinsed thoroughly with lab grade water (LGW), and then 255.18g potato tissue was homogenized with 25mL LGW using a Waring commercial blender, and split into nine 5g samples. Onto these homogenates, analytically large concentration spikes (between 0 and 20µg/g) were applied using mixtures of stock and working solutions of the target compounds dissolved in acetonitrile (Table 4). While few studies have reported observing caffeine in crops within the food distribution system, Calderón-Preciado et al. (2011) observed caffeine in alfalfa and apple tree leaves, (irrigated under field conditions with a variable river-water and reclaimed waste water mixture) at concentrations between 21-55ng/g and 0.1-110ng/g respectively. Thus most of the spikes delivered onto the sweet potato homogenates during this investigation were orders of magnitude higher than expected in environmental samples.

	Concentration from spike (µg/g) per sample		
Sample Letter	Triclosan	Caffeine	Estradiol
A	20.01	20.02	19.99
B	10.01	10.00	10.01
C	4.01	4.00	4.01
D	2.01	2.01	2.01
E	1.00	1.01	0.99
F	0.50	0.50	0.50
G	0.10	0.10	0.10
H	2.50E-02	4.99E-03	2.50E-02
K	0	0	0

Table 4: Concentration (µg/g) of spikes delivered onto sweet potato homogenates

The benefit of using these high spike levels is that large dilution factors of the final extracts were required to drop the concentration of the analytes within the extract into the quantitation range of the ELISA kits (see Table 5). For this first investigation, large dilutions were desired as they were expected to provide extracts that represented the "best case scenario". These highly dilute extracts would contain the potentially confounding matrix components, but at a minimum concentration. Also during this initial investigation, extracts of sweet potato homogenate were placed onto the GC-ECD and GC-MS to obtain complimentary analysis to the ELISA findings.

ELISA Kit Analyte	Quantitation Range
Caffeine	0.175-5.0 µg/L
Triclosan	0.05-2.5 µg/L
Estradiol	2.7-25 ng/L

Table 5: Quantitation ranges for each ELISA kit investigated

3.2.1.1 ELISA Analysis of Grocery Store Sweet Potato Extracts

The QuEChERS extraction procedure was performed outlined in section 2.5.2.1, with the caveat that since 5g samples were being extracted instead of 10g, all volumes and masses were appropriately scaled down. Thus, the 5g samples were extracted with 5mL acetonitrile, and a 10µg/g analyte spike (as was performed for sample B) would be expected to result in an extract with concentration 10mg/L if full analyte recovery was observed.

Concentration values reported in the tables that follow (and in Appendix B.2) were interpolated from standard curves run on the day of analysis using the software package GraphPad Prism5 to create a variable slope calibration curve, as per ELISA kit protocol, based on the absorbance responses given by 5 or 6 standard calibration solutions run in duplicate (calibration solutions provided with the ELISA kits). Figure 11demonstrates the calibration curve created in this manner for triclosan during this Phase 1 investigation.

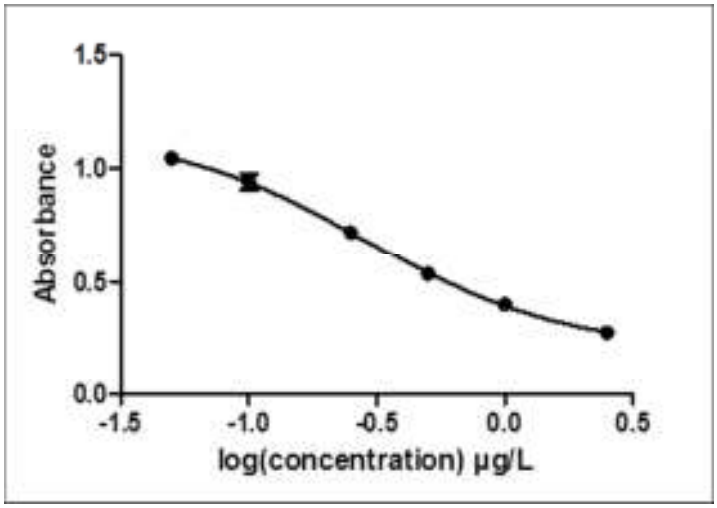

Figure 11: Triclosan calibration curve for phase 1 experiment

After creating the variable slope calibration curve, the software package was further utilized to interpolate the concentration of the sweet potato homogenate-extracts, from their absorbance responses, based on the calibration curve. The software package was also used to obtain 99% confidence intervals (CI) for the concentration of extracts. Table 6 demonstrates the utilization of the 99% confidence interval interpolation of extract concentrations using the responses of the standard calibration solutions used to make the triclosan calibration curve.

Standard	Curve Fit (µg/L)	Upper 99% CI Bound (µg/L)	Lower 99% CI Bound (µg/L)	Expected concentration (µg/L) from calibration solution
Std0				0
Std1	0.051	0.068		0.050
std2	0.099	0.124	0.079	0.100
std3	0.249	0.290	0.210	0.250
std4	0.512	0.613	0.438	0.500
std5	0.961	1.218	0.768	1.000
Std6	2.583		1.692	2.500

Table 6: Interpolated concentration of triclosan standards based on their response on the calibration curve with upper and lower bounds for the 99% Confidence Intervals (CI). Empty plots indicate that the value could not be reported within the quantitation range of the ELISA kit (see Table 5)

Table 7 shows the responses of the triclosan ELISA kit to extracts of sweet potato homogenate (refer to Appendix B.2 for caffeine and estradiol). Referring to Table 4, recall the (K) sample is an extract from an unspiked-homogenate. The (A) and (B) extracts had

triclosan spiked onto the homogenate at concentrations of approximately 20µg/g and 10µg/g respectively.

Sample	Spike (µg/g) onto homogneate	Dilution	Triclosan in dilute extract (µg/L) from ELISA calibration curve			Triclosan concentration recovered from homogenate (µg/g)			Spike Recovery (%)		
			Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
K	0	10	0.00	0.00	0.00	0	0	0			
B	10.01	25000	0.35	0.37	0.34	8.86	9.31	8.42	89%	93%	84%
B	10.01	10000	0.62	0.66	0.58	6.15	6.57	5.78	62%	66%	58%
A	20.01	8000	1.94	2.43	1.67	15.48	19.46	13.40	77%	97%	67%

Table 7: ELISA response to extracted grocery store sweet potato homogenates for triclosan

There are a number of noteworthy items to outline within Table 7. First, at 10 fold dilution, the minimum extract dilution required since the ELISA kits have a reported tolerance for acetonitrile of 10%, the unspiked-homogenate extract (K) gave a response below the limit of detection (LOD) of the ELISA kit. This suggests that at 10 fold dilutions and greater, the matrix components within the QuEChERS extract of sweet potato tissue will not result in false positive responses for the triclosan ELISA kit. Thus any responses observed within the quantitation range in other samples would be indicative of triclosan present and not confounding matrix components.

Also seen in Table 7, the responses of the ELISA to two different dilutions of the extract from homogenate spiked with10µg/g (B) indicate that the concentration recovered from (B) (when considering the upper and lower 99% CI) was between 5.78 and 9.31µg/g. This suggests recovery of the triclosan spike between 58% and 93% (extraction efficiency), while also demonstrating the ability of the ELISA kit to detect triclosan within highly dilute QuEChERS extracts (compatibility). Analysis of the (A) extract demonstrates similar method compatibility and suggests extraction efficiency between 67% and 97%. Notice was taken that the more diluted (B) extract gave a response suggesting a greater concentration within the original extract than was suggested by the less diluted (B) extract. Potential

homogenate-spiked extracts suggested extraction efficiency rates between 60% and 220% for caffeine, 53% and 96% for estradiol.

The responses of the caffeine ELISA kit to the 10 fold dilute extract of unspiked-homogenate sample (K) suggested that the concentration of caffeine within the extract was between 0.55 and 0.72µg/L (Appendix B.2). As such, it was expected that a 50 fold dilution, as was used for confirmatory analysis, would drop the extract concentration concerns that the matrix components may be inhibiting the ELISA, resulting in muted or false negative responses, will be investigated by spiking triclosan directly into finished extracts (standard addition, see strategies section 3.1.1) in the analyses presented in the Phase 2 and Phase 3 compatibility sections (3.2.2 and 3.2.3).

Similar ELISA analysis was performed on extracts of homogenates spiked with caffeine and estradiol (Appendix B.2). Responses of the ELISAs to highly diluted extracts also demonstrated a basic degree of method compatibility as the ELISA kits responses to below the range of detection (0.175µg/L; refer to Table 5). Indeed, a 50 fold confirmatory dilution of the (K) sample gave a response indicating that the concentration of caffeine within the extract was below the LOD. At the time of Phase 1 analysis it was determined that further investigation would need to be designed near the 10 fold dilution range prior to being able to propose conclusions about the concentration of caffeine within the sweet potato tissue. Upon completing the Phase 2 and Phase 3 investigations (sections 3.2.2 and 3.2.3) these results were re-evaluated with the knowledge that sweet potato extracts diluted 10 fold can be analyzed with the caffeine ELISA with minimum concern for confounding matrix effects, and that recovery of caffeine from sweet potato homogenates appears to be greater than 90%. Thus, though only a single analysis, without a duplicate or confirmatory second

dilution within the quantitation range of the ELISA kit, was performed on the extract of unspiked-sweet potato homogenate (K), the response observed indicates a caffeine concentration within the sweet potato tissue between 5.5 and 7.2ng/g (Appendix B.2).

While few studies have reported observing caffeine in crops within the food distribution system, Calderón-Preciado et al. (2011) observed caffeine in alfalfa and apple tree leaves, (irrigated under field conditions with a variable river-water and reclaimed waste water mixture) at concentrations between 21-55ng/g and 0.1-110ng/g respectively. As discussed in section 1.4.4.1, Dettenmaier et al (2009) demonstrated that caffeine was efficiently uptaken and translocated within the xylem and fruit of tomato and soy bean plants. And while the origin and growing conditions of the grocery store sweet potato are unknown, given the prevalence of caffeine in surface water (refer to section 1.4.4) it is clear that the crop exposure to caffeine through irrigation should not be unexpected.

Also seen in Appendix B.2, 10 fold and 30 fold dilutions of the extract of unspiked-homogenate (K) gave responses within the quantitation range of the estradiol ELISA kit that were consistent with their dilution level. This finding, in tandem with the 53 to 97% recovery of the extract of the homogenate spiked with 0.100µg/g estradiol (Appendix B.2) supported the initial conclusion that there may be estradiol present within the sweet potato tissue. As with caffeine, it was determined that further investigation would need to be designed near the 10 fold extract dilution range to investigate the performance of the estradiol ELISA kit when analyzing more concentrated extracts. Upon completion of the Phase 2 and Phase 3 compatibility experiments (sections 3.2.2 and 3.2.3) it was determined that the response of the estradiol ELISA kit to sweet potato tissue extracts, when diluted

between 10 to 80 fold, could not be conclusively attributed to estradiol as the matrix components within the extracts appear to be confounding.

3.2.1.2 GC-ECD Analysis of Grocery Store Sweet Potato Extracts

The same acetonitrile QuEChERS extracts of spiked sweet potato homogenates (Table 4) were injected, undilute, onto the GC-ECD and analyzed using the temperature program described in section 2.5.3.1.2 in order to perform complementary analysis for triclosan. The GC-ECD instrument response to triclosan in the extracts (area under the chromatographic peaks) increased with good linearity when plotted against the homogenate spike level, indicating that QuEChERS was extracting the triclosan spiked onto the homogenates, and doing so with consistency relative to the spike level (Figure 12 and Appendix B.3). Only triclosan was analyzed with the GC-ECD as neither caffeine nor estradiol possess sufficient electronegativity to be analyzed with the ECD detector without derivatization.

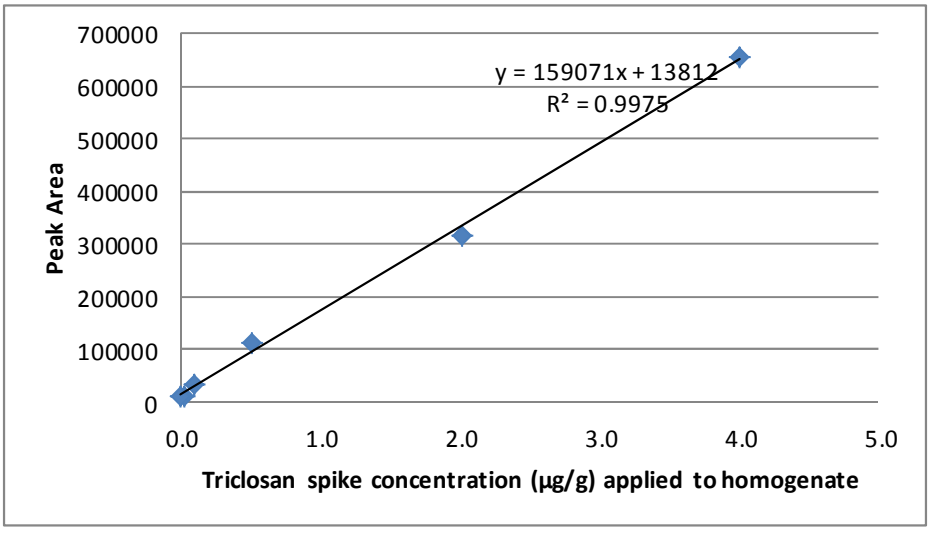

Figure 12: GC-ECD triclosan peak response (Peak Area) vs. homogenate spike concentration (µg/g)

The ECD could detect significant differences between the extract of unspiked-homogenate (K) and all extracts of homogenate-spiked samples with at least 100µg/g

triclosan (A-G). The response for the extract of unspiked-homogenate (K) was actually observed to be slightly greater than response for the extract of the homogenate spiked with 5ng/g (H). This indicated that the method was not sufficiently sensitive (or that extract matrix components were sufficiently confounding) to differentiate between the extract of an unspiked sample and an extract expected to have additional concentration of approximately 5µg/L (Appenidix P1.3). As the ELISA kit indicated that the 10 fold dilute extract of unspiked-homogenate (K) was below the LOD of 0.05µg/L (0.5µg/L in the original extract), it does not appear that the GC-ECD has the sensitivity to confirm or refute the ELISA results.

Because the calibration curve was built from homogenate-spiked samples only (i.e. no standard addition spikes into finished extracts), the extraction efficiency could not be determined, however the linearity observed indicated consistent efficiency.

3.2.1.3 Ion Trap GC-MS Analysis of Grocery Store Sweet Potato Extracts

The same acetonitrile QuEChERS extracts of the unspiked homogenate (K) and greatest spiked homogenate, 20µg/g (A) were injected directly onto the GC-Ion Trap-MS, in electron impact mode, for analysis using the program described in section 2.5.3.1.1. This continued investigation was of particular interest since the initial ELISA tests were suggestive of the presence of caffeine and estradiol in the sweet potato. Additionally, the below detection response for triclosan in the extract of unspiked-homogenate, using both the ELISA and GC-ECD, merited further investigation.

First however, a neat mixture of caffeine and triclosan in acetonitrile as well as a neat solution of derivatized estradiol (Mix A and DN_E2; see Appendix A.2 for sample preparation and section 2.6.2 for derivatization Method A) were analyzed to determine the

compound elution times and instrument detection limits (see Figure 13, Figure 14, and Figure 15).

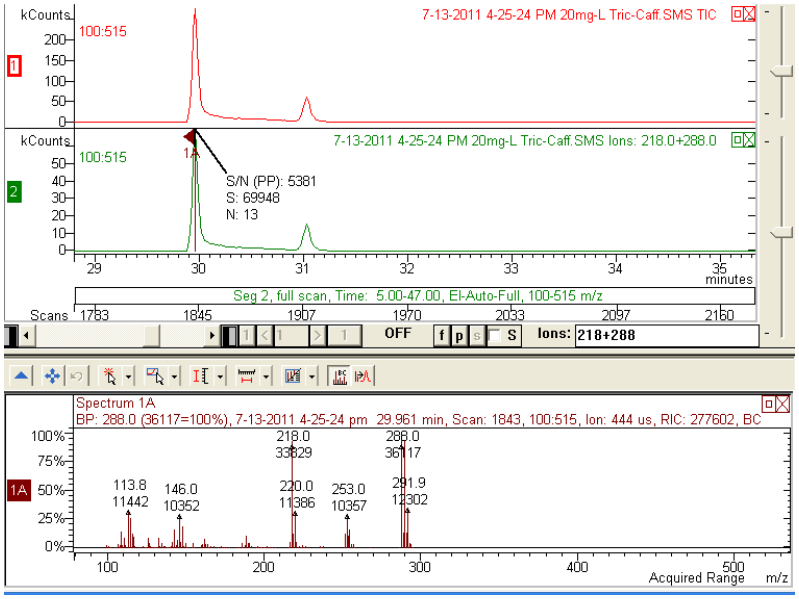

Figure 13: Triclosan chromatogram of neat solution (Mix A) in acetonitrile

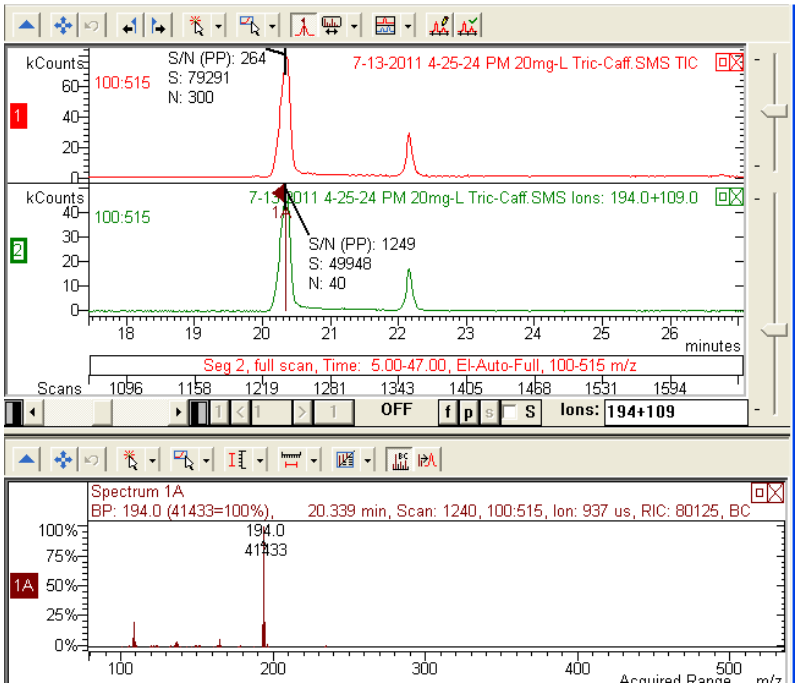

Figure 14: Caffeine chromatogram of neat solution (Mix A) in acetonitrile

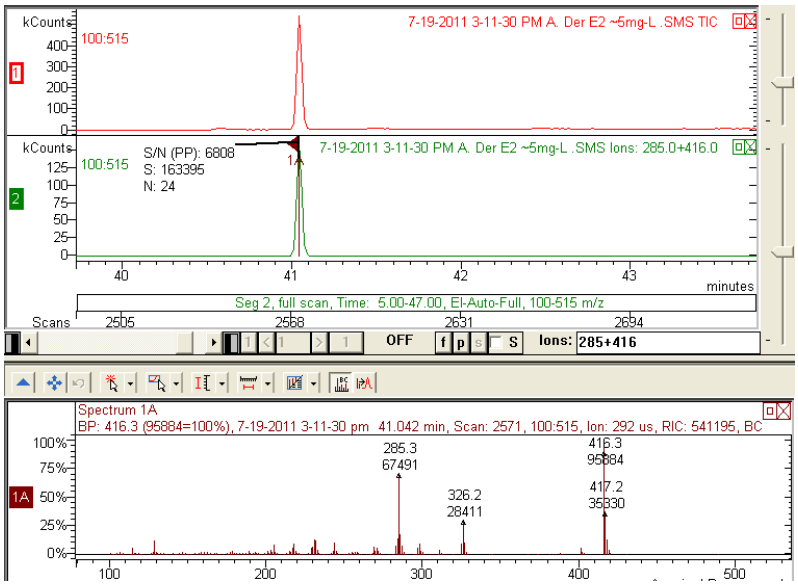

Figure 15: Derivatized estradiol chromatogram of neat solution (DN_E2) in hexane

The concentration of the target analytes in each sample and the signal to noise ratio (S/N) observed under the analyte peaks are reported in Table 8. The apparent Instrument Detection Limit (IDL) was then determined by setting the detection limit criteria as S/N = 10, and assuming a linear relationship between concentration and S/N:

$$10/IDL = (S/N)/(\text{concentration of neat sample})$$

For example, to determine the IDL for caffeine based on the neat Mix A sample, solve:

$$10/IDL = (1249)/(17.952 mg/L)$$

$$IDL = 0.144 mg/L$$

Sample*	Compound	Derivatized (Y/N)	Concentration (mg/L)	S/N	Instrument Dection Limit IDL (mg/L)
Mix A	Caffeine	N	17.952	1249	0.144
Mix A	Triclosan	N	20.336	5381	0.038
DN_E2	Estradiol	Y	4.344	6808	0.006
*See Appendix G2					

Table 8: GC-Ion Trap-MS responses and apparent Instrument Detection Limit (IDL) to neat solutions of caffeine and triclosan in acetonitrile and derivatized estradiol in hexane.

The remaining volume of the same (K) and (A) sweet potato extracts, the same that had been previously analyzed using the ELISA kits and GC-ECD, were filtered through a

0.45µm nylon syringe filter, and 1µL injected onto the GC-Ion Trap-MS using the same program conditions (refer to section 2.5.3.1.1). No chromatographic peaks were observed at the target elution times for triclosan or caffeine in the (K) sample. The signal to noise response for the caffeine and estradiol peaks of the (A) extract are given in the first two rows Table 9. The apparent Practical Detection Limit (PDL) for reported in Table 9 was determined by setting the detection limit criteria as S/N=10, and assuming a linear relationship between concentration and S/N. The PDL represents the minimum concentration (µg/g) that must be present within the sweet potato in order to give a quantifiable signal

$$10/PDL = (S/N)/(\text{concentration of homogenate spike})$$

For example, to determine the PDL for caffeine based on the (A) extract sample, solve:

$$10/PDL = (195)/(20.02\mu g/g)$$

$$PDL=1.026\mu g/g$$

Sweet Potato Extract	Compound	Homogenate Spike Delivered (µg/g)	Derivatized (Y/N)	Concentration After Derivatization (µg/g)	S/N	Practical Detection Limit (µg/g)
A	Caffeine	20.02	N	N/A	195	1.026
A	Triclosan	20.01	N	N/A	3167	0.063
A	Triclosan	20.01	Y	33.351	2507	0.133
A	Estradiol	19.99	Y	33.318	2250	0.148
C	Triclosan	4.01	Y	4.674	701	0.067
C	Estradiol	4.01	Y	4.683	396	0.118

Table 9: GC-Ion Trap-MS responses to filtered acetonitrile QuEChERS extracts of sweet potato and derivatized extracts with apparent Practical Detection Limit (PDL) reported for each target compound

Using this criterion, it appeared that the PDL was about 1µg/g for caffeine and 60ng/g for triclosan (Table 9). The suggested concentration of caffeine in the sweet potato from ELISA analysis was approximately 6-8 ng/g (Appendix B.2), which is between 2 and 3 orders of magnitude below the PDL for the GC-Ion-Trap-MS. Recall that the quantitation

range for the triclosan ELISA (which indicated that the unspiked-homogenate extract was below the LOD) is 0.05-2.5µg/L (Table 5). Taking into account the 10 fold minimum dilution required for ELISA analysis, the concentration within an original sweet potato extract would need to be between 0.5-25µg/L. As a consequence of the ratio of solvent to homogenate used during extraction (1mL acetonitrile per gram homogenate) the required triclosan concentration within the potato tissue of 0.5-25ng/g. The minimum value of this range, 0.5ng/g, is 2 orders of magnitude below the PDL for the GC-Ion-Trap-MS. As such, it was determined that the extracts would require a greater degree of cleanup and/or concentration in order to confirm or refute the results of the ELISA analysis for both caffeine and triclosan within the (K) sample.

During the early stages of this project, it was anticipated that the extracts would require derivatization in order to observe the steroid hormones on the Ion Trap. As such, 500µL of the extract of the homogenate spiked with ~20µg/g (A), and 350µL of the extract of homogenate spiked with ~4µg/g (C) (refer to Table 4) were derivatized as described in section 2.6.2 (Method A) into a final volume of 300µL hexane. Thus each sample was concentrated, as indicated in the fifth column of Table 9. The derivatized extracts were filtered through a 0.45µm nylon syringe filter and analyzed with the GC-Ion-Trap-MS. The responses observed and apparent PDL for each compound are reported in Table 9.

Caffeine was not observed in the derivatized samples. The PDL for triclosan was approximately the same in the derivatized and underivatized samples and continues to demonstrate the need for greater clean-up and/or concentration to confirm or refute ELISA analysis. The apparent PDL observed for estradiol (118-148ng/g) is between three and four orders of magnitude less sensitive than the potential quantitation range of the estradiol

ELISA kit as the quantitation range of 2.7-25ng/L (Table 5) translates to a concentration required concentration within the potato tissue between 27-250pg/g (after taking into consideration the same solvent/homogenate ratio and the 10 fold minimum dilution previously described).

3.2.1.4 Conclusions of Initial Analysis of Grocery Sweet Potato Extracts

Caffeine was the only compound observed in the extract of unspiked grocery sweet potato homogenate, at a concentration between 6-8ng/g. While the estradiol ELISA indicated analyte recovery and method compatibility for highly dilute samples, analysis of crop tissue extracts 10 fold dilute were ultimately determined to be confounding for the estradiol ELISA kit (section 3.2.3), and thus the responses for the extract of the unspiked homogenate were deemed unreliable. Given the practical detection limits calculated for all compounds using the GC-Ion-Trap-MS, significant extract clean-up and/or concentration (between two and three orders of magnitude) would be required to achieve the same sensitivity that appears possible for ELISA kits.

3.2.2 Phase 2 Investigation: Analysis of Extracts of Sweet Potato Leaf Tissue from an Agricultural Field Site

Having observed the ELISA kits compatibility in detecting the target analytes within highly dilute sweet potato tissue extracts (section 3.2.1), the purpose of this investigation was to test ELISA compatibility with extracts from another matrix of interest; sweet potato leaves. Additionally, it was determined that the performance of the ELISA kits needed to be further investigated near the 10 fold minimum required dilution, as it is expected that environmental levels of the target analytes will.

Sweet potato leaves were obtained from an agricultural field site (courtesy Extension agents at North Carolina State University),homogenized with equal weight LGW water

(determined necessary to achieve uniform slurry), and split into ten 5 gram samples (see Table 10) each of which were spiked with triclosan, caffeine, and estradiol in acetonitrile (see Appendix C.1 for detailed spike information). The homogenates were vortexed, centrifuged and allowed to sit for 36 hours as described in section 2.5.1.1. The QuEChERS extraction method was then performed as described in section 2.5.2.1 using 5mL acetonitrile with 2g Na_2SO_4 and 1g $MgSO_4$. For the even numbered samples (refer to Table 10), 1mL of extract was further cleaned by dispersive solid phase extraction (dSPE)using 25mg PSA and 150mg $MgSO_4$. As dSPE is largely designed for the removal of fatty acids within crop extracts, co-extractants that can interfere with ionization efficiency and the ability of analytical detectors to detect target compounds, the utility of this additional step for ELISA analysis was not well known. Therefore an additional investigation within this experiment was to analyze the odd numbered extracts without dSPE cleanup to investigate whether coextracted matrix components impaired analyte response by the ELISA kits. All extracts were dilute volumetrically (150µL in 2mL), and analyzed with the ELISA kits. Ultimately it was determined that dSPE was of benefit for ELISA analysis (reasons outlined below).

	Concentration spiked onto homogenate		
	Caffeine (ng/g)	Triclosan (ng/g)	Estradiol (pg/g)
Sample 2	0	0	0
Sample 4	5.01E+01	1.26E+01	2.50E+02
Sample 6	2.51E+01	1.26E+01	2.50E+02
Sample 8	5.01E+01	1.26E+01	1.26E+02
Sample 10	2.51E+01	1.26E+01	1.26E+02

	Concentration spiked onto homogenate		
	Caffeine (ng/g)	Triclosan (ng/g)	Estradiol (pg/g)
Sample 1	0	0	0
Sample 3	5.01E+01	2.50E+01	2.50E+02
Sample 5	2.51E+01	2.50E+01	2.50E+02
Sample 7	5.01E+01	2.50E+01	1.26E+02
Sample 9	2.51E+01	2.50E+01	1.26E+02

Table 10: Phase 2 sample designations and concentration of compounds spiked onto homogenates: Even numbered extracts were cleaned with dSPE, odd numbered were not.

Four replicate dilutions (150µL in 2mL) of the final extract of the unspiked-leaf homogenate processed without dSPE (Sample 1) were prepared (Table 11). One of these

replicates was analyzed without further preparation. The other three replicates were spiked using working solutions B1.2 (refer to Appendix A.3) of the target analytes in acetonitrile, the method of standard addition, to investigate whether coextracted matrix components present in un-cleaned extracts (no dSPE) impaired ELISA response. Having not undergone dSPE, Sample 1 represented a worst case (most complex matrix) extract composition and was expected to provide the greatest challenge to method compatibility.

	Volume(µL) Working Solutions (B1.2) spiked into 2 mL dilute extract*			Concentration Contribution Delivered from Spike		
Sample 1	Caffeine	Triclosan	Estradiol	Caffeine (µg/L)	Triclosan (µg/L)	Estradiol (ng/L)
+A	10	15	30	5.33E+01	1.61E+00	1.73E+01
+B	40	10	10	2.13E+02	1.07E+00	5.77E+00
+C	20	20	20	1.07E+02	2.15E+00	1.15E+01
	*Refer to Appendix G3					

Table 11: Standard addition spikes delivered into the extract of unspiked-leaf homogenate (Sample 1)

3.2.2.1 Standard Addition: Caffeine Analysis

As seen in Table 12, the ELISA appears capable of detecting caffeine that has been spiked directly into the dilute extract of unspiked sweet potato leaf homogenate, even within the complex Sample 1 extract that had not been processed with additional dSPE cleanup. While the responses above the unspiked sample (fit) were a little lower than expected from the spike delivered (73-85%), the 99% CI range encompassed the value of the expected spike.

Spike Delivered into Extract (µg/L)	Caffeine Concentration (µg/L) from ELISA Calibration Curve			Concentration (µg/L) above Background			% Response Expected Above Background		
	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	3.25	3.69	2.82						
1.07	4.17	4.95	3.65	0.91	1.25	0.83	86%	117%	78%
1.07	4.04	4.73	3.54	0.79	1.03	0.72	74%	97%	67%
2.13	4.81		4.19	1.56		1.37	73%		64%
4.26	High								
4.26	High								

Table 12: ELISA responses for caffeine in spiked extracts of sweet potato leaves processed without dSPE

It is also worth noting that the working solutions used to deliver the spikes was not independently tested in LGW for quality control. The working solutions used (Appendix A.3) to deliver spikes into the extract are not the same as the standard calibration solutions provided with the ELISA kits used to create the calibration curve. Without verifying the concentration of the working solution, it is difficult to assess whether there is some matrix interference preventing the ELISA from observing the full standard addition spike delivered, or if perhaps the working solution used was somewhat more dilute than anticipated. It is recommend that the working solutions used to make spikes, either onto homogenate or into extracts, be dilute in LGW and analyzed on the ELISA with the rest of the samples, as done in Phase 3 analysis (Section 3.2.3).

3.2.2.2 Standard Addition: Triclosan Analysis

The responses of the Triclosan ELISA kit to standard addition spikes directly into dilute extracts of unspiked-sweet potato leaf homogenate worked up without dSPE were 118% to 149% the expected response (Appendix C.2). This demonstrating the ability of the ELISA kit to detect triclosan within the complex matrix. As mentioned prior (section 3.2.2.1), it is worth noting that the working solution used to make the spikes was not independently tested in LGW samples for quality control.

3.2.2.3 Standard Addition: Estradiol Analysis

The response of the estradiol ELISA kit for the extract of unspiked-sweet potato homogenate without dSPE cleanup was above the ELISA kit upper limit of quantification of 25ng/L. As a result, it was expected, and observed, that all extract-spikes would be out of range for analysis (Appendix C.2).

3.2.2.4 Homogenate Spike Recovery from Sweet Potato Leaves

The ELISA analysis of the sweet potato leaf extracts with homogenate spikes continued to demonstrate that a) the QuEChERS extract method was capable of recovering the target analytes from sweet potato leaf homogenates and b) the ELISA kits were capable of analyzing and differentiating concentration differences in QuEChERS extracts dilute near the 10 fold minimum (150µL in 2mL).. For the samples extracted using the full QuEChERS extraction method (with dSPE), comparison of the responses of the extract from the unspiked-homogenate to the responses of the extracts from spiked-homogenates reveals recovery of the homogenate spikes of was approximately 20% for triclosan (Appendix C.3), 40-50% for caffeine (Table 13), and 50-90% for estradiol (Appendix C.3).

Caffeine concentration (ng/g) spiked onto homogenate	Caffeine Concentration (µg/L) in Diluted Extract from Calibration Curve			Dilution Factor	Caffeine recovered from homogenate (ng/g)			Caffeine recovered from homogenate (ng/g) above unspiked sample			% Recovery		
	Fit	Upper	Lower		Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	0.66	0.77	0.56	13.33	8.8E+00	1.0E+01	7.4E+00						
5.01E+01	2.26	2.60	1.96	13.33	3.0E+01	3.5E+01	2.6E+01	2.1E+01	2.4E+01	1.9E+01	42%	49%	37%
2.51E+01	1.61	1.85	1.41	13.33	2.1E+01	2.5E+01	1.9E+01	1.3E+01	1.4E+01	1.1E+01	50%	57%	46%
5.01E+01	2.19	2.53	1.90	13.33	2.9E+01	3.4E+01	2.5E+01	2.0E+01	2.3E+01	1.8E+01	41%	47%	36%
2.51E+01	1.48	1.70	1.31	13.33	2.0E+01	2.3E+01	1.7E+01	1.1E+01	1.2E+01	1.0E+01	44%	49%	40%

Table 13: ELISA analysis of caffeine spiked sweet potato leaf homogenates with dSPE

For the samples extracted without dSPE, comparison of the responses of the extract from the unspiked-homogenate to the responses of the extracts from spiked-homogenates

reveals recovery of the homogenate spikes was approximately 12-35% for triclosan (Appendix C.4), and 22%-49% for caffeine (Table 14).

Caffeine concentration (ng/g) spiked onto homogenate	Caffeine Concentration (µg/L) in Diluted Extract from Calibration Curve			Dilution Factor	Caffeine recovered from homogenate (ng/g)			Caffeine recovered from homogenate (ng/g) above unspiked sample			% Recovery		
	Fit	Upper	Lower		Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	3.25	3.69	2.82	13.33	4.3E+01	4.9E+01	3.8E+01						
5.01E+01	4.68		4.09	13.33	6.2E+01		5.5E+01	1.9E+01		1.7E+01	38%		34%
2.51E+01	3.99	4.65	3.49	13.33	5.3E+01	6.2E+01	4.7E+01	9.8E+00	1.3E+01	9.0E+00	39%	50%	36%
5.01E+01	4.08	4.80	3.58	13.33	5.4E+01	6.4E+01	4.8E+01	1.1E+01	1.5E+01	1.0E+01	22%	29%	20%
2.51E+01	4.17	4.96	3.66	13.33	5.6E+01	6.6E+01	4.9E+01	1.2E+01	1.7E+01	1.1E+01	49%	67%	45%

Table 14: ELISA analysis of caffeine spiked sweet potato leaf homogenates without dSPE.

Thus the recovery of analytes spiked onto leaf homogenate were not significantly different for extracts that underwent dSPE and those that did not: using the student t-test to compare the (fit) recovery rates observed for compounds with and without dSPE (null hypothesis is that the mean recovery rate is the same), the p-values returned $p = 0.29$ for caffeine; $p=0.14$ for triclosan (Appendix C.5). The weakness of this analysis is the number of samples in each group available for comparison; however it does provide some input beyond the eye test to indicate that neither triclosan nor caffeine appears to be appreciably lost during dSPE.

The recovery rate for estradiol without dSPE could not be calculated in the same manner due to the high response observed in the unspiked extract (Appendix C.4). However, as the homogenate-spiked extracts without dSPE cleanup gave responses within the quantitation range of the estradiol ELISA, it appears that the response observed in the unspiked sample may be an artifact. Instead, the matrix contribution for all the non-dSPE cleaned samples was estimated using a paired-t-test to obtain the mean and 99%CI bounds for the response elevation of the homogenate-spiked extracts (no-dSPE) above the

homogenate-spiked extracts with dSPE (Appendix C.5). Using this estimate for the background contribution, the recovery rate (fit) for estradiol was found to range from 56-97% (Appendix C.4) and was not found to be significantly different than extracts cleaned with dSPE (p=0.88) (Appendix C.5).

3.2.2.5 Extraction with and without dSPE and Determination of Compound Concentration within Sweet Potato Leaves

Section 3.2.2.4 demonstrated that the recovery of analyte spikes onto sweet potato leaf homogenate were not significantly lower for the extracts cleaned via dSPE than those that were not (i.e. compounds are not lost during dSPE). Since the target compounds are not lost during dSPE, the extracts of identically spiked homogenates should give the same ELISA response with or without dSPE; if they do not (as was observed), then the matrix components within the uncleaned extracts must be assumed to be confounding.

no-dSPE Sample	dSPE Sample	Caffeine concentration (ng/g) spiked onto homogenate	Caffeine "Recovered" from homogenate (ng/g) no-dSPE	Caffeine Recovered from homogenate (ng/g) with dSPE	Response Difference (ng/g) (no-dSPE less dSPE)
Sample 1	Sample 2	0	4.3E+01	8.8E+00	3.5E+01
Sample 3	Sample 4	5.01E+01	6.2E+01	3.0E+01	3.2E+01
Sample 5	Sample 6	2.51E+01	5.3E+01	2.1E+01	3.2E+01
Sample 7	Sample 8	5.01E+01	5.4E+01	2.9E+01	2.5E+01
Sample 9	Sample 10	2.51E+01	5.6E+01	2.0E+01	3.6E+01
			Average Response Difference		3.1E+01
			Difference Unspiked Samples		3.5E+01

Table 15: Determining the difference in caffeine ELISA response for extracts without dSPE cleanup vs. those with dSPE

Table 15 shows the caffeine ELISA responses of identically spiked homogenates with and without dSPE. The response (fit) of the spiked extracts that did not undergo dSPE are significantly greater (p=0.0007 when performing a paired t-test) than those that did with a mean difference 31.3 +/- 7.0 (Appendix C.5). Since the recovery rate (response above the

appropriate unspiked sample) with and without dSPE are not significantly different (section 3.2.2.4) this observed difference is expected to arise from confounding matrix components in the uncleaned extract (i.e. caffeine ELISA responses for the extracts without dSPE are giving, on average, a false response indicative of 32ng/g). The difference between the unspiked samples (35ng/g) confirms these findings and the concentration of 8.8ng/g observed in the unspiked sample with dSPE is expected to be the actual concentration within the homogenate. Given the 1:1 ratio of leaves to LGW in the homogenate, this represents 17.6ng/g within the leaves. As mentioned in section 3.2.1.1, while few studies have reported observing caffeine in crops within the food distribution system, Calderón-Preciado et al. (2011) observed caffeine in alfalfa and apple tree leaves, (irrigated under field conditions with a variable river-water and reclaimed waste water mixture) at concentrations between 21-55ng/g and 0.1-110ng/g respectively.

The same observation (similar recovery rates, with significantly elevated responses for the samples without dSPE) can be made for extracts analyzed for triclosan (Appendix C.5). Indeed, the response (fit) of the spiked extracts that did not undergo dSPE are significantly greater ($p=0.006$ for unpaired t-test) than those that did with a mean difference of 4.2ng/g +/- 2.2 (Appendix C.5). Given that the response (fit) of the unspiked sample for the extract that did not undergo dSPE was 5.5ng/g (Appendix C.4) it was determined that the response was likely an artifact of confounding matrix components and that the below measureable detection response observed for the sample cleaned with dSPE is more likely.

For estradiol, the response (fit) of the spiked extracts that did not undergo dSPE are significantly greater ($p= 0.005$ for paired t-test) than those that did with mean difference and 95% confidence 85pg/g +/- 38 (Appendix C.5). The response of the unspiked sample that

did undergo dSPE was below the LOD for the estradiol ELISA and expected to be a more accurate reflection of the estradiol concentration within the leaves.

In summary, the recovery rate observed (dSPE vs. no-dSPE) was not significantly different ($p>0.05$) for any of compounds (section 3.2.2.4), indicating that the analytes are not significantly lost during dSPE. At the same time, the response of the homogenate-spiked samples for the extracts that did not undergo dSPE were all significantly higher than the response than those that did. This indicates the presence of confounding matrix components within the uncleaned samples, and that samples without dSPE cleanup are likely to give an inaccurately large estimation of the concentration of compounds within any sample. Thus it was determined that dSPE should be utilized in the preparation of extracts for ELISA analysis.

3.2.3 Phase 3: Analysis of All Greenhouse Experimental Matrices Near Environmental Concentrations

In the months following the Phase 1 and Phase 2 initial investigations, numerous ELISAs were run on a variety of samples and many lessons learned. These lessons influenced the design of the final method compatibility and subsequent matrix extraction efficiency experiments. The following summarize some of the lessons learned and their design implications for the final compatibility and efficiency experiments:

- It was determined that serial dilutions of environmental samples should be at tight intervals (10 fold 20 fold 40 fold) rather than wide (10 fold 100 fold 1000 fold) since the apparent concentration of the unspiked homogenate extracts, once dilute 10 fold, do not appear to be an order of magnitude above the lower LOD for the ELISA kits. (i.e. 100 fold dilutions almost always drop the concentration out of range and confirmatory analysis is not accomplished).

- It was determined that the spikes onto the homogenates and into the extracts should be designed at a level such that 10 fold and 20 fold serial dilutions would likewise be in range of the ELISA quantitation range (Table 5). Spikes requiring greater sample dilution eliminate the ability to determine the analyte concentration in the original sample behind the spikes delivered, and make it impossible to compare spiked samples to unspiked samples. Additionally large dilutions greatly magnify the effect of the 99% confidence intervals (CI) when using the requisite dilution correction factors to determine the concentration range of the undilute samples (i.e. if the 99% CI range for the dilute sample analyzed is 0.5 to 0.6 µg/L, this translates to a still relatively narrow 5-6 µg/L in the undilute extract for a designed 10 fold dilution, but a wide 500-600µg/L in the undilute extract for a designed 1000 fold dilution).
- It was determined that the working solutions used to spike onto the homogenates and into the final extracts should be diluted in LGW and analyzed with the ELISAs as a measure of quality control.
- It was observed that the calibration curves not only shift up and down but also change shape from day to day, kit to kit. Hence the greatest amount of data is achieved when running full duplicate standard curves on the day of analysis. While trends can be investigated between samples run on the same day using partial standard curves, inter-day comparisons of sample responses can only be satisfactorily complete for samples analyzed in tandem with full calibration curves.

3.2.3.1 Sample Preparation and Extraction

With these lessons in mind, fresh extractions were performed on duplicate samples of four previously homogenized and stored lettuce leaf and sweet potato tissue homogenates as

well as "virgin" (non-irrigated) sand (VSA) and soil (VSO) samples from the sterilized bins at the Method Road Greenhouse. The sweet potato and lettuce homogenates analyzed were irrigated during the greenhouse experiment, with the four homogenates analyzed being a) lettuce grown in soil irrigated with tap water (L:G:SO:L), b) lettuce grown in soil irrigated with spiked-reclaimed water (L:S:SO:L), c) sweet potato grown in soil irrigate with tap water (P:G:SO:P) and d) sweet potato grown in soil irrigated with spiked-reclaimed water (P:S:SO:P). Refer to section 3.4 for information on the growing conditions, irrigation, estimated analyte exposure and ELISA analysis for all greenhouse samples. Though some analysis will be provided on the mass of analytes present in the four unspiked homogenates, the current section (3.2.3) will focus primarily on the ability of the ELISA kits to accurately quantify analytes spiked directly into the extract, as well as the recovery of analytes spiked onto homogenates.

	Homogenate mass (g) of each duplicate sample	Volume (µL) working #5 applied to homogenate-spiked sample	Volume (mL) of acetonitrile used for extraction
VSO	10.00	75	15
VSA	10.00	75	15
L:G:SO:L	0.82	50	10
L:S:SO:L	0.99	50	10
P:G:SO:P	10.00	50	10
P:S:SO:P	10.00	50	10

Table 16: Homogenate masses, volume of working solution spiked onto homogenates, and volume of acetonitrile used to perform each Phase 3 extraction

The mass of each duplicate homogenate extracted is shown in Table 16. One homogenate from the duplicate samples was spiked with a given volume of working solution #5 in acetonitrile designed such that there were 5µL applied for every 1mL ACN used to extract the homogenate (Table 16). The homogenates were vortexed, and allowed to sit for

36 hours to allow the spikes to incorporate and for the solvent to evaporate. Acetonitrile was added to each homogenate and the homogenate-solvent mixture vortexed and allowed to sit for 4 hours to allow the solvent to act upon the homogenate matrices. 4g of Na_2SO_4 and 2g $MgSO_4$ were added to each sample, which were subsequently vortexed and centrifuged as described in section 2.5.2. Dispersive solid phase extraction (dSPE) was then performed on 4mL of each extract with 100mg PSA and either 250mg or 600mg $MgSO_4$ (depending on whether the homogenate was a wet or dry sample). This is consistent with the method described in section 2.5.2, however since less total volume was needed since samples were only being prepared for ELISA analysis, only 4mL (as opposed to 8mL) were cleaned via dSPE and thus the mass of PSA and $MgSO_4$ used are half that described in the 2.5.2 At this junction, approximately 1mL of the unspiked and homogenate spiked extracts were transferred into amber GC vials and stored. Exactly 2mL of the each unspiked homogenate extract were measured into fresh conical vials, and 10µL of working solution #5 were spiked into each. These standard addition extract-spiked samples were likewise transferred to amber GC vials and stored.

3.2.3.2 Analysis of Working Solution #5

As a quality control measure, working solution #5 in acetonitrile (Appendix A.4), the solution used to spike into the extracts and onto the homogenates, was spiked into lab grade water (LGW) and serially diluted (Appendix D.3) in order to confirm the concentration of each analyte within the solution. The responses of the serial dilutions were multiplied by their dilution factor to determine the concentration in the working solution, and the average undiluted extract values (fit) were identified as demonstrated in Table 17.

Sample	Dilution	Expected Caffeine Concenration in Working Solution (Appendix G1.2) (µg/L)	Caffeine Concentration (µg/L) and 99% CI for Original Working Solution #5			Concentration (µg/L) (fit) expected in 2 mL extract with 10µL spiked in
			Fit	Upper	Lower	
Working Solution # 5 in LGW	2000	5858	4660	6418	3344	23.3
Working Solution # 5 in LGW	4000	5858	4655	6375	3542	23.3
Working Solution # 5 in LGW	8000	5858	5638	7514	3905	28.2
Average (fit) Caffeine Concentration (µg/L) of working solution used for spiking					4985	
Concentration (µg/L) expected from 10µL spiked into 2mL extracts					24.9	+/-7.0

Table 17: ELISA analysis of caffeine in Working Solution #5 (solution used to deliver homogenate-spikes and extract-spikes)

As the standard addition spikes into extracts of all homogenates was performed by delivering 10µL into 2mL of the extract of unspiked-homogenate, the expected concentration from this delivered spike was calculated for each dilution (last column in Table 17). The variability between the responses expected and responses observed in during the analysis of working solution #5 emphasizes the importance of the practice of analyzing the neat working solution used to deliver homogenate and extract spikes. Analysis of working solution #5 for all compounds can be seen in Appendix D.3.

3.2.3.3 Caffeine ELISA Compatibility and Recovery Analysis of QuEChERS Crop and Soil Extracts

Standard Addition Compatibility Analysis

The caffeine ELISA responses to serial dilutions of the extracts of a) unspiked sweet potato homogenate and b) the same homogenate extract with a standard addition spike delivered into a known volume ("Extract Spiked") are provided in Table 18.

Matrix and sample prepartion	Dilution	Expected caffeine concentration (μg/L) from 10μL spike into 2mL original extract	Concentration (μg/L) and 99% CI range of original extract			Concentration (μg/L) and 99% CI range of original extract LESS average unspiked resposne		
			Fit	Upper	Lower	Fit	Upper	Lower
P:G:SO:P (Fresh Extraction)								
Extract Spiked	10	24.9 +/-7.0	28.8	40.6	20.0	24.3	33.5	16.7
Extract Spiked	20	24.9 +/-7.0	31.3	43.9	23.7	26.8	36.8	20.4
Extract Spiked	40	24.9 +/-7.0	44.5	60.5	33.7	39.9	53.4	30.3
Extract Spiked	80	24.9 +/-7.0	34.4	50.3	21.6	29.9	43.2	18.2
Unspiked Homogeante Extract	10	0.0	5.1	7.2	3.4			
Unspiked Homogeante Extract	20	0.0	3.9	7.0	No Detect			

Table 18: Caffeine ELISA analysis of extract-spiked and unspiked homogenate extracts of sweet potato tissue (P:G:SO:P) compared to expected responses from spike delivered

The concentration increase observed for extracts with standard addition -spikes above the extracts of unspiked sweet potato homogenate appear in the last three columns Table 18. Comparing the (fit) values for these standard addition concentration responses to the expected concentration from the standard addition spike, the responses observed appear to be indistinguishable from the responses expected (i.e. the ELISA is capable of detecting known concentrations of caffeine within the complex sweet potato extract). A t-test was run in R to compare the responses (fit) for the concentration observed in the standard addition sample above the unspiked extract (Table 18) with the values calculated as the expected value for 10μL working solution #5 within a 2mL extract in section 3.2.3.1 (i.e. last column in Table 17) . The p-value obtained of 0.23 (Figure **16**) suggests that the responses observed in the sweet potato extract are not significantly different than the responses expected after analysis of working solution #5 in LGW.

```
> neat.caff
[1] 23.3 23.3 28.2   NA
> pgp.caff
[1] 24.3 26.8 39.9 29.9
> t.test(neat.caff,pgp.caff)

        Welch Two Sample t-test

data:  neat.caff and pgp.caff
t = -1.3954, df = 4.196, p-value = 0.2322
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -15.629299   5.045966
sample estimates:
mean of x mean of y
 24.93333  30.22500
```

Figure 16**: Student t-test comparing the concentration responses of the extract-spiked P:G:SO:P above background(refer to Table 18) to the expected responses from the analysis of working solution #5 (refer to Table 17)**

The weakness of this analysis is the small number of samples available for comparative analysis; however it does provide some insight beyond the eye test. The same analysis was done on standard addition soil extracts and lettuce leaf extracts (Appendix D.4) with p-values 0.60 and 0.11 respectively. Conclusion: The matrix components within QuEChERS extracts of sweet potato, lettuce, and soil, dilute 10 fold and beyond, do not significantly impair the caffeine ELISA kits ability to detect and accurately quantify caffeine within the extracts.

Homogenate Spike Recovery Analysis

The responses of the homogenate-spiked samples above unspiked homogenate extracts indicated 90-104% recovery of caffeine from the sweet potato homogenate (Table 19 and Appendix D.7). For lettuce samples, this analysis suggested 19-42% recovery (Appendix D.7). Recovery from soil was approximately 31%, while recovery from sand was between 63-75% (Appendix D.7). While recovery efficiency appears to be variable

depending on the matrix, extraction optimization was not pursued and improved efficiency is expected to be possible.

P:G:SO:P (Sweet Potato) matrix		Caffeine Concentration and 99%CI (µg/L) within Original Extract			Mass Recovered (ng) within extract			% Recovery *			Concentration (ng/g) within potato matrix (% Recovery Adjusted)			Mass (ng) per plant within treatment group		
mass (ng) spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
	10	5.1	7.2	3.4	51	72	34				8.8	8.6	8.0	807	795	736
	20	3.9	7.0		39	70					6.7	8.4		619	776	
249	10	30.5	45.8	21.1	305	458	211	104%	155%	71%						
249	20	27.0	37.6	20.7	270	376	207	90%	122%	69%						

Table 19: Spike recovery analysis and concentration of caffeine within sweet potato tissue irrigate with tap water (P:G:SO:P).

Unspiked Homogenate Extract Analysis

No measureable caffeine was observed in the newly extracted virgin sand, soil or in lettuce irrigated with either tap water or spiked reclaimed water (Appendix D.7). These results are important as they indicate that the matrix components do not yield false positives, and detectable responses that might be observed in other samples are likely to be due to presence of caffeine. Caffeine was observed in sweet potato samples (Table 19 and Appendix D.7), with serial dilutions giving responses consistent with their dilution level and indicating a concentration of approximately 6.7-8.8ng/g within the homogenate of tap water irrigated potato tissue or approximately 600-800ng (Table 19) within the total potato tissue of each plant within the treatment group (after taking into account the potato:LGW ratio within the homogenate and the average sweet potato mass of the plants within the P:G:SO:P treatment group). This compares similarly to the analysis of grocery store potato from Phase 1 (5.0-6.6ng/g) (Section 3.2.1.1). The concentration observed in potato tissue irrigated with spiked-reclaimed water irrigated was between 5.2-10.6ng/g, indicating the presence of

approximately 1050 and 2150ng within the total potato tissue of each plant within the treatment group (Appendix D.7). Further analysis of the caffeine concentration within the crop tissues and growing matrices from the greenhouse experiment are provided in section 3.4.

3.2.3.4 Triclosan ELISA Compatibility and Recovery Analysis of QuEChERS Crop and Soil Extracts

Standard Addition Compatibility Analysis

The triclosan ELISA responses to serial dilutions of the extracts of a) unspiked sweet potato homogenate and b) the same homogenate extract with a standard addition spike delivered into a known volume ("Extract Spiked") are provided in Table 20.

Matrix and sample prepartion	Dilution	Expected triclosan concentration (μg/L) from 10μL spike into 2mL original extract	Concentration (μg/L) and 99% CI range of original extract		
			Fit	Upper	Lower
P:G:SO:P (Fresh Extraction)					
Extract Spiked	10	15.7 +/- 5.7	21.7		16.6
Extract Spiked	20	15.7 +/- 5.7	20.1	26.0	15.7
Extract Spiked	40	15.7 +/- 5.7	22.2	28.1	18.1
Extract Spiked	80	15.7 +/- 5.7	17.3	21.6	13.2
Homogenate Only	10	0.0	No Detect	No Detect	No Detect
Homogenate Only	20	0.0	No Detect	No Detect	No Detect

Table 20: Triclosan ELISA analysis of extract-spiked and unspiked homogenate extracts of sweet potato tissue (P:G:SO:P) compared to expected responses from spike delivered

The triclosan concentration observed in the standard addition samples above the concentration observed for extracts of unspiked sweet potato homogenate (last three columns Table 20) appear similar to, albeit not completely indistinguishable from, the expected responses from spiking a 2mL sample with 10μL working solution #5 based on the analysis of the working solution described in section 3.2.3.1 (Appendix D.3). A t-test was run in R to compare the four responses (fit) for the concentration observed in the standard addition samples (Table 20) with the three expected concentration values calculated for spiking 10μL

working solution #5 within a 2mL extract (Appendix D.3). The p-value obtained of 0.052 (Appendix D.5) suggest that the responses observed in the sweet potato extract may be slightly greater than expected after analysis of working solution #5 in LGW (perhaps some slight matrix enhancement). However given the overlap of confidence interval ranges for the concentrations observed and expected (Table 20), and the small number of samples available for comparison, perhaps that the most suitable conclusion is that the ELISA is clearly capable of detecting triclosan within the complex QuEChERS extract of sweet potato homogenate, and that quantification using confidence intervals is the most appropriate strategy.

The same analysis was performed for the responses of extract-spiked soil samples and extract spiked lettuce samples (Appendix D.5) with p-values 0.13 and 0.31 respectively. The results observed for soil were indicative of a possible matrix inhibition. However, as was the case for the sweet potato homogenate extracts, due to the overlap of confidence intervals for the concentrations observed and expected, as well as the limited sample size, the most appropriate conclusion is that the ELISA is clearly capable of detecting triclosan within the complex QuEChERS extracts of soil and lettuce leaf homogenates, and that quantification using confidence intervals is the most suitable strategy.

Homogenate Spike Recovery Analysis

The responses of the homogenate-spiked samples above unspiked homogenate extracts indicated 83-103% recovery of triclosan from the sweet potato homogenate (Table 21 and Appendix D.8). For lettuce samples, this analysis indicated 32-47% recovery (Appendix D.8). Recovery from soil was approximately 27%, while recovery from sand was approximately 35% (Appendix D.8).

P:G:SO:P (Sweet Potato) matrix		Triclosan Concentration and 99%CI (µg/L) within Original Extract			Mass Recovered (ng) within extract			% Recovery		
mass (ng) spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
	10									
	20									
157	10	16.2	22.0	12.4	162	220	124	103%	140%	79%
157	20	13.1	16.8	10.6	131	168	106	83%	107%	67%

Table 21: Spike recovery analysis and concentration of triclosan within sweet potato tissue irrigate with tap water (P:G:SO:P).

Unspiked Homogenate Extract Analysis

No measureable triclosan was observed in the newly extracted virgin sand, virgin soil, in lettuce irrigated with either tap water or spiked reclaimed water, or in sweet potato tissue irrigated with tap water (Appendix D.8). These results are important as they indicate that the matrix components do not yield false positives, and detectable responses that might be observed in other samples are likely to be due to presence of triclosan. Triclosan was observed in sweet potato samples irrigate with spiked-reclaimed water (Appendix D.8), with serial dilutions giving responses consistent with their dilution level and indicating a concentration of approximately 3.3-3.9ng/g within the sweet potato tissue resulting in a total mass between 650-800ng (Appendix D.8) within the total potato tissue of each plant within the treatment group (after taking into account the average sweet potato mass of the plants within the P:S:SO:P treatment group). During the Phase 1 analysis (Section 3.2.1.1), no measureable triclosan was observed in the grocery store sweet potato. Further analysis of the triclosan concentration within the crop tissues and growing matrices from the greenhouse experiment are provided in section 3.4.

3.2.3.5 Estradiol ELISA Compatibility and Recovery Analysis of QuEChERS Crop and Soil Extracts

Sweet Potato Tissue and Lettuce Leaf Tissue

Significant responses were observed in the unspiked homogenate extracts of both lettuce and sweet potato, regardless the irrigation source, for the estradiol ELISA kit. The extract-spiked sample of sweet potato homogenate were "significantly" larger than the unspiked homogenate extracts ($p=0.03$) however the elevation observed was only 10-20% the expected value from the spike delivered (Table 22).

Matrix and sample prepartion	Dilution	Expected estradiol concentration (ng/L) from 10μL spike into 2mL original extract	Concentration (ng/L) and 99% CI range of original extract		
			Fit	Upper	Lower
P:G:SO:P (Fresh Extraction)					
Extract Spiked	10	421 +/- 95	153		127
Extract Spiked	20	421 +/- 95	156	195	125
Extract Spiked	40	421 +/- 95	196	252	
Extract Spiked	80	421 +/- 95	No Detect	No Detect	No Detect
Homogenate Only	10	0.0	84	105	68
Homogenate Only	20	0.0	107	137	70

Table 22: Estradiol ELISA analysis of extract-spiked sweet potato homogenate extracts and unspiked-homogenate extracts, demonstrating the much lower than expected responses within the spiked extracts.

The responses of the extract-spiked samples of lettuce homogenate were indistinguishable from the responses of the unspiked homogenate extracts (Table 23).

Matrix and sample prepartion	Dilution	Expected estradiol concentration (ng/L) from 10μL spike into 2mL original extract	Concentration (ng/L) and 99% CI range of original extract		
			Fit	Upper	Lower
L:G:SO:L (Fresh Extraction)					
Extract Spiked	10	421 +/- 95	336		
Extract Spiked	20	421 +/- 95	303	356	251
Extract Spiked	40	421 +/- 95	425	520	348
Extract Spiked	80	421 +/- 95	314	423	
Homogenate Only	10	0.0	300		245
Homogenate Only	20	0.0	297	349	246

Table 23: Estradiol ELISA analysis of extract-spiked lettuce leaf homogenate extracts and unspiked-homogenate extracts demonstrating the indistinguishable responses.

The much lower than expected response of the extract-spiked samples appeared to indicate that the ELISA was not well suited for differentiating the presence of estradiol within the extract matrix as prepared, and the responses observed in any extracts including the original unspiked sample are suspect without further analysis.

Homogenate-spiked extracts of sweet potato gave similar responses as the extract-spiked samples (10-20% of the expected response above unspiked homogenate extracts) (Appendix D.9) seeming to indicate that the ELISA may be capable of extracting estradiol from potato homogenate, however until the confounding matrix effects can be resolved nothing further can be concluded. Homogenate-spiked extracts of lettuce leaf tissue were again indistinguishable from the unspiked extract (Appendix D.9).

It was determined that further method investigation would need to be complete prior to utilizing the estradiol ELISA kit to analyze extracts from crop tissues. While estradiol analysis was run on a number of unspiked crop extracts, at this time no conclusions will be asserted concerning the estradiol concentration within crop extracts from the greenhouse experiment. Considering that some of the analysis from the Phase 1 and 2 investigations with more dilute extracts did not indicate such clear incompatibility, it may be that further dilution or sample clean-up is required to be able to use the estradiol ELISA kit to analyze crop extracts.

Sand and Soil

No measureable estradiol was detected in the virgin sand or soil extracts. Standard addition was performed into a finished soil extract and ELISA analysis performed (Table 24).

Matrix and sample prepartion	Dilution	Expected estradiol concentration (ng/L) from 10μL spike into 2mL original extract	Concentration (ng/L) and 99% CI range of original extract		
			Fit	Upper	Lower
Virgin Soil (VSO)					
Extract Spiked	10	421 +/- 95	134	159	110
Extract Spiked	20	421 +/- 95	No Detect	No Detect	No Detect
Extract Spiked	40	421 +/- 95	429	524	351
Extract Spiked	80	421 +/- 95	506	638	385
Homogenate Only	10	0.0	No Detect	No Detect	No Detect
Homogenate Only	20	0.0	No Detect	No Detect	No Detect

Table 24: Estradiol ELISA analysis of extract-spiked soil homogenate extracts and unspiked-homogenate extracts

The ELISA responses to standard addition spiked extracts sample dilute 40 fold and 80 fold were consistent with the expected response from the spike delivered. When 10 fold and 20 fold dilute, the response of the ELISA to the standard addition spiked extracts seemed to give a muted response, perhaps indicating that the estradiol ELISA requires a dilution above 20 fold to accurately quantify QuEChERS extracts of soil. Additional soil extracts (from greenhouse experiment samples) were spiked with working solution, dilute 10 fold, and analyzed on the same day of analysis (Appendix D.12) and the results showed similar muted responses (20-45% the expected from the spike delivered) at 10 fold dilution. Analysis of a homogenate spiked sample 10 fold dilute indicated approximately 50% recovery (Appendix D.9), though given the apparent muted response at 10 fold dilution, this recovery rate is likely to be conservative.

3.3 Implications of Method Compatibility Investigations for the Analysis of Greenhouse Irrigated Samples:

3.3.1 Estradiol

As prepared, none of the crop tissue extracts can be satisfactorily analyzed with the estradiol ELISA kits with 10 fold and 20 fold dilutions (section 3.2.3.4). The kits are incapable of adequately distinguishing between unspiked homogenate extracts and extract

spiked samples at these dilutions. Future investigation might consider dilutions of greater magnitude or further sample clean up prior to analysis. Sand and soil extracts dilute 10 fold may give muted responses (perhaps larger dilution required for the estradiol ELISA kit), however, there is little concern about false positive responses at this time.

3.3.2 Caffeine and Triclosan

Sweet Potato Tissue

Recovery of caffeine and triclosan from sweet potato homogenates appears to be almost complete (90% and better). Analysis of extracts with the caffeine and triclosan ELISA kits demonstrate little complication at dilutions between 10 fold and 100 fold. Extracts of sweet potato tissue irrigated with tap water did not give measurable responses during triclosan analysis, while extracts of sweet potato irrigated with spiked-reclaimed water indicated a triclosan concentration of 3.3-3.9ng/g within the potato tissue. Extracts of the same potato tissues indicated a caffeine concentration between 6.7-8.8ng/g for tap irrigated tissue, 5.2-10.6ng/g for spiked-reclaimed water irrigated tissue.

Lettuce Leaves

Experiments demonstrate recovery of caffeine to be between 19 and 42% (see section 3.2.3.2), recovery of triclosan between 32-47% (section 3.2.3.3). Analysis of extracts with the caffeine and triclosan ELISA kits demonstrate little complication at dilutions between 10 fold and 100 fold with no detectable responses observed for unspiked homogenate samples, minimizing the concern of false positive responses. Therefore the responses of samples are likely to underestimate the amount of caffeine and triclosan present in lettuce leaves. While

responses within the quantitation range of the ELISA may be adjusted to reflect the recovery rate, the practical limit of detection is less sensitive due to the recovery observed.

Sweet Potato Leaves

Recovery of caffeine appears to be between 40-50% (section 3.2.2.4). Recovery of triclosan appears to be at best 20%. Analysis of extracts with the caffeine and triclosan ELISA kits demonstrate little complication at dilutions between 10 fold and 100 fold, with no measurable levels of analyte present in the leaves, minimizing the concern of false positive responses. As with lettuce leaves, it is likely then that the response of the ELISA is likely to underestimate the amount of both caffeine and triclosan present and the practical detection limit will be negatively influenced Extracts within quantitation range could be adjusted to reflect the recovery observed.

Sand

Recovery of caffeine was observed between 63% and 75% (section 3.2.3.2). Recovery of triclosan was between approximately 35% (section 3.2.3.3). Analysis of extracts with the caffeine and triclosan ELISA kits demonstrate little complication at dilutions between 10 fold and 100 fold, with no responses observed for unspiked “virgin” sand samples minimizing the concern of false positive responses. It is likely then that the response of the ELISA is likely to underestimate the amount of both caffeine and triclosan present and the practical detection limit will be negatively influenced. Extracts within quantitation range could be adjusted to reflect the recovery observed.

Soil

Recovery of caffeine was approximately 31% (section 3.2.3.2), while recovery of triclosan about 27% (section 3.2.3.2). Analysis of extracts with the caffeine and triclosan ELISA kits demonstrate little complication at dilutions between 10 fold and 100 fold with nil responses observed for background only samples minimizing the concern of false positive responses. It is likely then that the response of the ELISA is likely to underestimate the amount of both caffeine and triclosan present and the practical detection limit will be negatively influenced. Extracts within quantitation range could be adjusted to reflect the recovery observed.

3.4 Analysis of Greenhouse Samples

3.4.1 Reservoir Analysis

The contents of the reservoirs were analyzed on a number of occasions (See Appendix E). As expected, there was variability in the constituency of the reclaimed water collected form the reclaimed water from the Integrated Water Strategies system at the Jordan Lake Business Center. Unfortunately the contents of the reservoirs could not be analyzed as regularly as would have been ideal and, thus while the volume of water applied to each plant is known (35.1L for each sweet potato plant, 8.3L for each lettuce plant; Supporting Material Z1, part of an electronic labbook available on file at UNC), the mass of each analyte applied within these volumes cannot be well estimated. It was observed, however, that samples collected from the spiked-reclaimed water reservoir one to two weeks after the spike was delivered, still had elevated levels of each compound as compared to the unspiked reservoir. The amount of elevation observed also varied and was not always as large as expected from the spike administered (designed to increase the concentration of each analyte within the

reservoir by 10µg/L). This observation is not entirely surprising, however, for even though the reservoirs were shielded from light and moderately temperature-controlled within the refrigeration unit, they were not expected to provide a highly stable, degradation-resistant environment. Despite these limitations, an extremely crude estimate for the mass of each analyte applied to the sweet potato and lettuce plants is provided in Table 25. These estimates are based on the average responses for the reservoir samples shown in Appendix E.

	Estimated Mass Delivered to Sweet Potatoes (35.1L)			Estimated Mass Delivered to Lettuce (8.3L)		
Irrigation Source	Caffeine (µg)	Triclosan (µg)	Estradiol (ng)	Caffeine (µg)	Triclosan (µg)	Estradiol (ng)
Tap Water	3.6E+00	3.1E+00	0.0E+00	8.5E-01	7.4E-01	0.0E+00
IWS Reclaimed	1.7E+01	2.8E+01	2.4E+02	4.0E+00	6.7E+00	5.7E+01
Spiked IWS Reclaimed	1.5E+02	1.2E+02	2.7E+04	3.5E+01	2.8E+01	6.5E+03

Table 25: Estimated mass of target analytes delivered to sweet potato and lettuce based on limited reservoir analysis (Appendix E) and volume applied.

Additionally, with crop and soil samples only being taken at the end of the growing seasons, there was considerable time and opportunity for degradation processes to occur within the plant-soil system prior to harvest and before extraction. As a result of these limitations, mass balance was not pursued as a goal in this study. Rather, the extracts from the crop tissues and growing matrices were compared across treatment groups using the methods developed to determine whether significant differences in the matrix concentration and total analyte mass accounted for within each matrix were observable.

3.4.2 Crop Tissue and Growing Matrices Analysis

Extraction of crop and growing matrices, and storage of matrix extracts, were performed in the months following the crop harvests in the fall of 2011 (refer to Supporting Materials series X and Y for all details available, part of electronic labbook on file at UNC, including schedule, for the extraction of each matrix). Analysis of stored extracts was

performed on multiple occasions during the fall of 2011 and spring of 2012, with the strengths, weaknesses and results from all analysis presented below.

3.4.2.1 Fall 2011 Trend and "Concentration" Approach

Having limited supplies, and not knowing what to expect for analyte concentrations within each matrix, it was determined that preliminary ELISA analysis would be run without full duplicate standard curves in order to investigate appropriate dilution factors and determine trends between extracts (i.e. is extract X more or less concentrated than extract Y). Therefore, only the nil, highest and lowest analyte calibration solutions were analyzed by the ELISA kits in tandem with the experimental samples; and the responses of the experimental extracts judged on the basis of being between/above/below the quantification range of each ELISA kit. As a result of this, extracts of matrix homogenates processed for ELISA analysis can only be compared to other samples processed by the ELISA kit on the same day (i.e. without the full calibration curve we cannot make observations that definitely compare samples from one day to the next).

ELISA analysis was complete in this manner (three calibration points rather than a full calibration curve) on three days in the fall of 2011: November 28, November 29, and December 23. All "concentrations" associated with the fall 2011 findings (described in sections 3.5 and 3.6, and shown in and in the Z Appendices) were approximated by comparing the nil, high and low standards analyzed to the suite of full calibration curves run during the entire research period, and plugging the absorbance responses for the samples into the curve that most closely matched. These "concentrations" then are not presented as hard values, but provide numerical approximations to give a slightly more thorough scrutiny to the trends observed.

To emphasize of the limitations of this strategy, consider the following example from the analysis of triclosan run on November 29, 2011. The response of the nil, low and high calibration points on this day were equally well matched to either the full calibration curve from an analysis run on June 6, 2011 or the calibration curve run on March 8, 2012 (Figure 17).

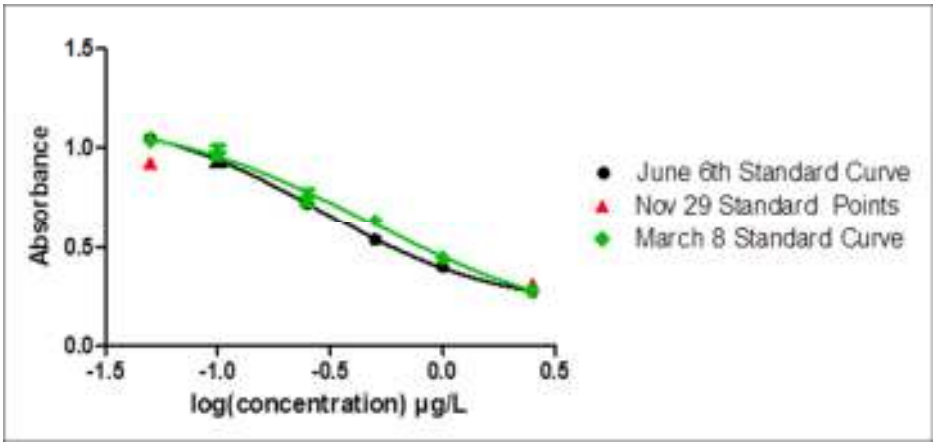

Figure 17: Triclosan ELISA full calibration curves from June 6, 2011 and Mach 8, 2012 and high/low calibration points from November 29, 2011.

Using the three calibration point strategy, one or both of these curves would have been used to estimate the concentration of triclosan within samples run on November 29, 2011. Figure 17 demonstrates how the calibration curves from different ELISA kits, run on different days, even with nearly identical absorbance values for the highest and lowest calibration points, will exhibit different shapes within the quantitation range. As a consequence of the different shapes, concentration estimates returned by the two curves for a sample within the quantitation range can vary significantly. For example, the absorbance value for the calibration solution #4 used to make the full calibration curve on June 6, 2011 (with known concentration 0.5µg/L) was 0.53. A sample with this absorbance on March 8, 2012, based on the calibration curve, would be expected to have a concentration of approximately 0.75µg/L, a relative percent difference of 40%. Also seen in Figure 17, the

lowest concentration calibration solution (furthest most left) on November 29, 2011, had a lower absorbance value than either best matching curve; yet for the highest concentration calibration solution (furthest most right) on November 29, 2011 had the greatest absorbance value of all three curves. Thus it would be expected that had a full calibration curve been created on November 29, 2011, it would cross over the calibration curves from June 6, 2011 and March 8, 2012 at some unknown absorbance values "X." Thus, the June and March calibration curves will overestimate the concentration within a sample from November with absorbance values greater than "X," and will underestimate the concentration from a November sample with absorbance values less than "X." Ultimately, without knowing the shape of the kit-specific calibration curve between the high and low calibration points, concentration estimates using "best matching" curves are made very tentatively.

3.4.2.2 Spring 2012 Trend Approach

In spring 2012, as method validation studies were being further investigated (see Phase 3 investigations, section 3.2.3) stored extracts from the greenhouse experiment (extractions executed in the fall/winter of 2011) were also analyzed. These extracts have the benefit of having been run in tandem with full duplicate standard curves, however by the time the extracts were analyzed, many had been in storage for 3-5 months and the stability of the extracts is not well known. Some samples were run both in the fall and in the spring, and while the concentration values cannot truly be compared apples to apples (recall the fall samples were not run in tandem with full calibration curves) it appears that most of these extracts gave responses of similar magnitude during both fall and spring analysis. For a few of these extracts, however, when analyzed in the fall of 2011 the analyte concentration appeared to be within the quantitation range of some ELISA kits (albeit very near the LOD),

but when analyzed in the spring of 2012 the same extracts gave responses below the LOD, indicating that stability may be an issue.

All of the tables referring to the analysis of experimental crops grown in the greenhouse use a 4 unit coding separated by colons to refer to the samples. Use of the key in Table 26 will facilitate an understanding of the origin of the samples being referenced in the subsequent text:

Crop	Irrigation Source	Growing Matrix	Matrix Extracted and Replicate #
(P) Sweet Potato	(G) Greenhouse tap	(SA) Sand	(P#) Potato
(L) Lettuce	(F) Field composition reclaimed IWS water	(SO) Soil	(L#) Leaves
	(S) Spiked-reclaimed IWS water		(S#) Sand/Soil

Table 26: Coding used to identify sample extracts from the greenhouse experiments.

Example: P:G:SO:P1 => Sweet Potato Crop, Greenhouse Tap Irrigated, Grown in Soil, Potato Tissue Extracted, Replicate #1. Recall that during extraction, replicate extracts were prepared from each homogenate. (Refer to Z and Y Series Appendices)

3.5 Fate and Transport of Caffeine

3.5.1 Fall 2011 Trend and "Concentration" Analysis: Caffeine

3.5.1.1 November 28, 2011

On November 28, 2011, three potato tissue extracts were analyzed by the caffeine ELISA kit after 10 fold dilution, with results from this analysis presented in Table 27. The extracts analyzed were of sweet potato tissue a) grown in soil, irrigated with spiked-reclaimed water; P:S:SO:P, b) grown in sand, irrigated with spiked-reclaimed water;

P:S:SA:P, and c) grown in sand, irrigated with tap water; P:G:SA:P. No replicate samples were run for any of the extracts. All samples gave responses in range of the ELISA (responses between the high and low standard run in tandem). The responses of the nil, low and high standard were best matched to the full calibration curve from an analysis run on July 13, 2011, in order to estimate the concentration within the extracts.

Sample*	Best Fit "concentration" estimate [µg/L]Ψ in extract	Average recovery rate from matrix during	Caffeine concentration (ng/g) in original matrix (% Recovery Adjusted)	Total mass of caffeine per plant (µg/plant) within the designated matrix (concentration x average matrix mass of treatment group*)
Nov 28 2011				
P:S:SO:P1	6	95%	10.4	2.1
P:S:SA:P1	13	95%	22.7	4.9
P:G:SA:P1**	18	95%	31.5	1.4
Nov 29 2011				
P:G:SA:P1**	8.1	95%	14.2	0.6
P:G:SA:L1***	17.1	45%	148.4	0.5
P:G:SA:S1	45	70%	95.5	29
Decemeber 23 2011				
P:S:SO:P1	3.9	95%	6.8	1.4
P:S:SO:L1	13.0	45%	23.4	1.2
P:S:SO:S1	1.3	30%	6.7	2.7
P:F:SO:P1	5.4	95%	9.3	1.4
P:F:SO:L1	5.1	45%	11.6	0.3
P:F:SO:S1	1.3	30%	6.7	2.7
P:G:SO:P1	1.0	95%	1.7	0.2
P:G:SO:L1	7.6	45%	19.8	0.4
P:G:SO:S1	1.7	30%	8.5	3.4

Ψ Note that full standard curve not run on day of analysis, all values in the table are estimated from other standard curves based on matching the high and low standard calibration solutions analyzed

* Refer to X and Y Series Appendices

**Did NOT undergo dSPE cleanup

***Only sweet potato leaf sample homogenized wet

Table 27: Fall 2011 ELISA trend and "concentration" analysis of caffeine within greenhouse grown sweet potato tissues and growing matrices.

Somewhat unexpectedly, the highest concentration sample was observed for the tap water irrigated sample (Table 27). However, upon review of the lab book, it was realized that these (P:G:SA:P) samples, (the first extractions executed from the greenhouse samples after harvest), mistakenly did not undergo dSPE while all other extracts did. The Phase 2 Investigation (section 3.2.2) demonstrated that dSPE was critical for analysis using the

ELISA kits, and that extracts of identically spiked-homogenates without dSPE cleanup consistently gave higher responses than those with dSPE, despite having indistinguishable recovery rates. Thus the high concentration observed for the tap irrigated sample is likely an artifact of the lack of dSPE cleanup.

The caffeine "concentration" estimated for the sweet potatoes irrigated with spiked-reclaimed water were between 10 and 23ng/g, with a greater concentration observed within the tissue of sweet potatoes grown in sand than in soil (Table 27). Note that all matrix concentration values were adjusted to reflect the average recovery rate observed during compatibility investigations (~95% for caffeine from sweet potato homogenate). These apparent "concentrations" are two to four fold higher than the grocery store sweet potato tissue analyzed during the Phase 1 Investigations (section 3.2.1) which was between 6 and 8ng/g. When taking into account the average potato mass per plant within each treatment group (average mass of potatoes per plant in P:S:SA:P treatment group ~215g; average mass of potatoes per plant in P:S:SO:P treatment group ~202g, refer to the X series Appendices), the total mass of caffeine accounted for, per plant, within the potato tissues irrigated with spiked-reclaimed water was between 2.1 and 4.9µg (last column in Table 27).

3.5.1.2 November 29, 2011

On November 29, 2011, the "full system," (potato, leaf, growing matrix) of the sweet potato treatment group grown in sand and irrigated with tap water was analyzed with the caffeine ELISA kit. No replicate samples were run for any of the extracts. All extracts gave responses in range of the ELISA kit (responses between the high and low standard run in tandem). The response of the nil, low and high standard were best matched to the full

calibration curve from an analysis run on June 6, 2011 in order to estimate the concentration within the extracts. Results from this day are presented in Table 27.

As previously reported (section 3.5.1.1), the potato tissue from this treatment group did not undergo dSPE and the magnitude of the response is expected to be an artifact of matrix components within the uncleaned extract. Despite this expected overestimation for caffeine within this potato tissue extract, the approximate total mass of caffeine accounted for within the leaf tissue (~0.5µg) compared to total mass within potato tissue (0.6µg), indicates significant translocation of caffeine through the sweet potato plant. This is consistent with Dettenmaier et al (2009) which assigned caffeine an average transpiration stream concentration factor (refer to section 1.4.5) of 0.83, indicating a high potential for uptake and translocation. In total, no more than 1µg caffeine appears to accounted for in the combined plant tissues of the tap irrigated plants.

The response of the sand sample was unexpectedly high, indicating the presence of more caffeine within the sand sample taken (29µg estimated) than was estimated to have been delivered in aggregate for this treatment group in section 3.4.1 (less than 5µg expected). Indeed, considering that 35.1L irrigation water was delivered to each sweet potato plant, the average caffeine concentration within the tap water would have had to have been greater than 0.8µg/L to deliver 29µg over the entire growing period (suspending degradation). Unfortunately, serial dilution and/or duplicate samples were not run on this extract to provide confirmation analysis of this response.

3.5.1.3 December 23, 2011

On December 23, 2011, the "full systems" (potato, leaf, growing matrix) of all treatment groups grown in soil were analyzed with the caffeine ELISA kits. Serial dilutions

(10 fold, 100 fold, 1000 fold) were run for all samples, however the only samples that gave responses in range of the ELISA kit were those 10 fold dilute. Indeed, this observation was the influence for designing the Phase 3 Investigation (section 3.2.3) using the serial dilution strategy of 10 fold, 20 fold, 40 fold. The response of the nil, low and high calibration solutions were best matched to the full calibration curve from an analysis run on September 8, 2011 in order to estimate the concentration within the extracts. As previously observed, the leaves were the most concentrated tissue (Table 27), indicating significant translocation potential. The total mass of caffeine per gram edible tissue appears to be 3 to 5 times greater in the samples irrigated with reclaimed waters than with tap water. Even so, no more than 2µg caffeine appears to be present within the edible tissues of any plant regardless the irrigation source.

The responses of soil extracts 10 fold dilute were all very near the LOD with no differentiation observed between extracts of samples from different irrigation source. The most likely explanation for the lack of differentiation is that the time lag between collecting the soil samples during the sweet potato harvest and extracting from them was approximately 4 weeks, allowing significant time for degradation. Indeed, Bradley et al. (2006) observed that 50-90% of radio-labeled caffeine within stream sediments (under oxic conditions) had been mineralized within 2 days. Considering their proximity to the LOD and the time lag between sample collection and extraction, it seems probable that these positive responses were artifacts.

3.5.2 Spring 2012 Trend and Concentration Analysis

Caffeine was not observed within any of the soil extracts analyzed, regardless the irrigation treatment (Table 28). This finding gives further merit to the hypotheses presented

in section 3.5.1.3 that the positive responses near the LOD observed on December 23, 2011, may have been artifacts and that the caffeine applied to the soil samples from the irrigation sources appears to have degraded prior to extraction. .The extracts of sweet potato leaf continue to be of greater concentration than the extracts of sweet potato, continuing to indicate translocation of caffeine through the sweet potato plant. The concentration within the sweet potato leaves were approximately 34ng/g, 38ng/g, and 42 ng/g for the plants irrigate with tap, reclaimed water and spiked-reclaimed water respectively. Taking into consideration the aggregate leaf mass within each treatment group, these concentrations indicate a total caffeine mass accounting, per plant, of 0.6µg, 1.1µg and 2.1µg within the leaves (Table 28).

Sample*	Best (fit) concentration [µg/L] in extract based on ELISA calibration curve	Average recovery rate from matrix during compatability investigations	Caffeine concentration (ng/g) in original matrix (% Recovery Adjusted)	Total mass of caffeine per plant (µg/plant) within the designated matrix (concentration x average matrix mass of treatment group*)
January				
P:S:SO:P1	9	95%	16	3.2
P:S:SO:L2	23	45%	42	2.1
P:S:SO:S2	0	30%	0	0
P:F:SO:P2	12	95%	21	3.2
P:F:SO:L1	17	45%	38	1.1
P:G:SO:P1	0	95%	0	0
P:G:SO:L1	13	45%	34	0.6
L:S:SO:L2	0	30%	0	0
February				
P:S:SO:P4	4.0	95%	7	1.4
P:S:SO:S3	0	30%	0	0
P:F:SO:S2	0	30%	0	0
P:G:SO:P4	5.0	95%	9	0.8
P:G:SO:S2	0	30%	0	0
L:S:SO:L1	0	30%	0	0
L:S:SO:L4	0	30%	0	0
L:F:SO:L1	2.0	30%	11	0.4
L:F:SO:S1	0	30%	0	0
L:G:SO:L4	0	30%	0	0
L:G:SO:S2	0	30%	0	0

*(Refer to X and Y series Appendices)

Table 28: Spring 2012 ELISA trend and concentration analysis of caffeine within greenhouse grown sweet potato tissue, lettuce leaves, and soil

Combining the mass recovered within the potato and leaf tissues, the total caffeine mass accounted for, per plant, is approximately 0.6-1.4µg/plant for the tap irrigated, 4-5µg/plant for the reclaimed water irrigated, and 5-6µg/plant for the spiked-reclaimed water irrigated. Recalling the estimates from section 3.4.1, it was expected that the sweet potato plants within the treatment groups were exposed to approximately 3.6, 17 and 150µg of caffeine; nearly an order of magnitude increase in caffeine exposure per group. Thus the sweet potato plants irrigated with reclaimed water may have as much as 30% of the estimated caffeine applied within their plant tissues. The spiked-reclaimed water irrigated sweet potato plants appeared to account for less than 5% the estimated caffeine applied within their plant tissues. Thus while the caffeine concentration was clearly higher in the plants irrigated with reclaimed water sources than tap water, it appears that the practical uptake of caffeine by sweet potatoes is limited given the similar mass accounting within the sweet potato plants irrigated with reclaimed water and spiked-reclaimed water.

It appears the uptake of caffeine into the leaves of lettuce is minimal (Table 28). The ELISA responses suggested the presence of caffeine in only one lettuce sample, namely that grown with reclaimed water. The response of this extract when 10 fold dilute (0.2µg/L) was very near the LOD (0.175µg/L), and though a 20 fold dilution of this extract was analyzed, it could not confirm the response as the extract predictably fell below the quantitation range. A duplicate analysis of this sample could not be run. Adding further suspicion to this response is that caffeine was not detected in the extract of lettuce irrigated with spiked-reclaimed water.

3.6 Fate and transport of triclosan

3.6.1 Fall Trend and "Concentration" Analysis: Triclosan

3.6.1.1 November 28, 2011

On November 28, 2011, three potato tissue extracts were analyzed by the triclosan ELISA kit after 10 fold dilution (Table 29). The extracts were from sweet potato plants a) grown in soil, irrigated with spiked-reclaimed water; P:S:SO:P, b) grown in sand, irrigated with spiked-reclaimed water; P:S:SA:P, and c) grown in sand, irrigated with tap water; P:G:SA:P No replicate samples were run for any of the extracts. The responses of the nil, low and high standard were best matched to the full calibration curve from an analysis run on July 13, 2011, in order to estimate the concentration within the extracts.

Sample*	Best Fit "concentration" estimate [µg/L]Ψ in extract	Average recovery rate from matrix during compatability investigations	Triclosan concentration (ng/g) in original matrix (% Recovery Adjusted)	Total mass of triclosan per plant (µg/plant) within the designated matrix (concentration x average matrix mass of treatment group*)
Nov 28th				
P:S:SO:P1	0.65	90%	1.19	0.2
P:S:SA:P1	0	90%	0.0	0.0
P:G:SA:P1	0	90%	0.00	0
Nov 29th				
P:G:SA:P1	0	90%	0.00	0
P:G:SA:L1	0	20%	0.00	0
P:G:SA:S1	0	35%	0.00	0
Decemeber 23				
P:S:SO:P1	4.2	90%	7.7	1.6
P:S:SO:L1	0	20%	0.00	0
P:S:SO:S1	12.7	30%	63	2.5E+01
P:F:SO:P1	0	90%	0.00	0
P:F:SO:L1	0	20%	0.00	0
P:F:SO:S1	0	30%	0	0
*(Refer to X and Y series Appendices)				
Ψ Note that full standard curve not run on day of analysis, all values in the table are estimated from other standard curves based on matching the high and low standard calibration solutions analyzed				

Table 29: Fall 2011 ELISA trend and "concentration" analysis of triclosan within greenhouse grown sweet potato tissues and growing matrices.

The only extract that gave a response in range of the ELISA (response between the high and low calibration point run on the day of analysis) was of the sample irrigated with spiked-reclaimed water and grown in soil. Therefore it appears that greater uptake of triclosan is achieved for potatoes grown in soil than in sand.. The triclosan concentration within the potato tissue was estimated to be about 1ng/g, which would give an accounting of approximately 0.2µg total triclosan within the aggregate potato tissue of each plant within the treatment group. This is less than1% of the estimated 120µg applied (section 3.4.1) to each plant in the treatment group. During the Phase 1 investigation of grocery store sweet potato (section 3.2.1) no triclosan was observed within the potato tissue. The Phase 3 investigation (section 3.2.3) also observed triclosan within the potato tissue irrigated with spiked-reclaimed water at a concentration between 3.3 and 3.9ng/g.

3.6.1.2 November 29, 2011

On November 29, 2011, the "full system," (potato, leaf, growing matrix) of the sweet potato treatment group grown in sand and irrigated with tap water was analyzed with the triclosan ELISA kit. No replicate samples were run for any of the extracts. The responses of the nil, low and high standard were best matched to the full calibration curves from analyses run on either June 6, 2011, or March 8, 2012 (see Figure 17 in section 3.4.2.1) in order to estimate the concentration within the extracts. None of extracts gave responses within the quantitation range of the ELISA kit (responses between the high and low standard run in tandem). Given the low mass estimated to have been delivered from the tap water reservoir (section 3.4.1), the below detection responses were expected for the extracts of this treatment group and, importantly, suggest that any positive responses observed in other samples are indicative of triclosan present and are not a result of confounding matrix influences.

3.6.1.3 December 23, 2011

On December 23, 2011, the "full systems" (potato, leaf, growing matrix) of all treatment groups grown in soil were analyzed with the triclosan ELISA kits. Serial dilutions (10 fold, 100 fold, 1000 fold) were run for all samples. The responses of the nil, low and high standard were best matched to the full calibration curves from an analysis run on June 6, 2011, in order to estimate the concentration within the extracts. The only samples that gave responses within the quantitation range of the ELISA kit were of the extracts of sweet potato tissue and soil irrigated with spiked-reclaimed water (Table 29). The concentration estimated within the potato tissue (7ng/g) was consistent with the Phase 3 investigation of 3.3-3.9ng/g (section 3.2.3) and indicated a total mass accounting of less than 2µg per plant within the treatment group. This represents approximately 1% of the 120µg estimated to have been applied to each plant within the treatment group (section 3.4.1).

No triclosan was observed in the extracts of sweet potato leaf homogenate, indicating that triclosan may not be translocated significantly within the sweet potato plant. Triclosan was estimated to be present at a concentration of 63ng/g within the soil that was irrigated with spiked-reclaimed water (after a 30% recovery rate adjustment). This concentration was applied to the 400g soil samples collected from each pot during the sweet potato harvest in order to arrive at the 25µg total mass accounted for in Table 29. This represents approximately 20% the total estimated mass applied to each plant within the treatment group. Overall, these results suggest significant sorption of triclosan onto soil with limited uptake by the sweet potato plants.

3.6.2 Spring 2012 Trend and Concentration Analysis: Triclosan

Sand and Soil

The only soil samples which gave a positive response for triclosan were those irrigated with spiked-reclaimed water (Table 30 and Table 31). The triclosan concentration within the soils ranged from 7.5-99ng/g, with the concentration observed falling during each consecutive ELISA analysis, potentially indicating stability issues during extract storage. Nevertheless, from these concentrations, it was determined that the mass accounted for within the soil of each plant irrigate with spiked-reclaimed water was between 5% and 25% the total mass estimated to have been applied (section 3.4.1). None of the sand samples indicate the presence of triclosan. Given that the recovery of triclosan for sand and soil was found to be similar (section 3.2.3), it appears that triclosan applied to sand was no longer present for extraction. Within soil however, triclosan appears to have greater stability, perhaps as a result of sorption to available organic matter, and remains available for extraction.

Sample*	Best (fit) concentration [µg/L] in extract based on ELISA calibration curve	Average recovery rate from matrix during compatability investigations	Triclosan concentration (ng/g) in original matrix (% Recovery Adjusted)	Total mass of triclosan per plant (µg/plant) within the designated matrix (concentration x average matrix mass of treatment group*)
January				
P:S:SO:P1	5.8	90%	11	2.1
P:S:SO:L2	1.8	20%	7.3	0.4
P:S:SO:S2	20	30%	99	40
P:F:SO:P2	0	90%	0	0
P:F:SO:L1	0	20%	0	0
P:G:SO:P1	0	90%	0	0
P:G:SO:L1	0	20%	0	0
L:S:SO:L2	0	40%	0	0
February				
P:S:SO:P4	2.2	90%	4.1	0.8
P:S:SO:S3	4.5	30%	22	8.9
P:F:SO:S2	0	30%	0	0
P:G:SO:P4	0	90%	0	0
P:G:SO:S2	0	30%	0	0
L:S:SO:L1	0	40%	0	0
L:S:SO:L4	0	40%	0	0
L:F:SO:L1	0	40%	0	0
L:F:SO:S1	0	30%	0	0
L:G:SO:L4	0	40%	0	0
L:G:SO:S2	0	30%	0	0

*(Refer to X and Y series Appendices)

Table 30: Spring 2012 ELISA trend and concentration analysis of triclosan within greenhouse grown sweet potato tissues, lettuce leaves, and growing matrices.

Sweet Potato Tissue

None of the extracts of sweet potato irrigated with tap water gave ELISA responses indicating the presence of triclosan (Table 30 and Table 31). Extracts from sweet potato tissue grown with spiked-reclaimed water consistently gave responses indicating that the concentration within the tissue was between 4 and 11ng/g, accounting for approximately 1-2µg total triclosan per plant within the potato tissue. A single sample of sweet potato tissue irrigate with unspiked-reclaimed water gave response indicating the presence of triclosan near the LOD (P:F:SO:P3 in Table 31). The concentration indicated for this sample was approximately 2ng/g, and accounted for less than 0.5µg triclosan per plant within the edible tissue.

Sample*	Best (fit) concentration [µg/L] in extract based on ELISA calibration curve	Average recovery rate from matrix during compatability investigations	Triclosan concentration (ng/g) in original matrix (% Recovery Adjusted)	Total mass of triclosan per plant (µg/plant) within the designated matrix (concentration x average matrix mass of treatment group*)
March				
P:S:SO:L3	0		0	0
P:F:SO:P3	1.0	90%	1.8	0.3
P:F:SO:L3	0		0	0
P:G:SO:P2	0		0	0
P:S:SA:P2	2.1	90%	3.9	0.8
P:S:SA:L1	0		0	0
P:S:SA:S1	0		0	0
P:F:SA:P1	0		0	0
P:F:SA:L1	0		0	0
P:F:SA:S1	0		0	0
L:S:SO:S2	1.5	30%	7.5	3.0
L:S:SA:L1	0		0	0
L:S:SA:S1	0		0	0
L:F:SA:L2	0		0	0
L:F:SA:S1	0		0	0
*(Refer to X and Y series Appendices)				

Table 31: Spring 2012 ELISA trend and concentration analysis of triclosan within greenhouse grown sweet potato tissues, lettuce leaves, and growing matrices (Continued)

Sweet Potato Leaves

Triclosan was observed in a single sweet potato leaf extract, namely that from the treatment group irrigated with spiked-reclaimed water (Table 30). The indicated concentration within the leaf tissue was approximately 7ng/g. The total triclosan mass accounted for per plant within the leaf tissue is less than 0.5µg; less than 1% the estimated mass applied to each plant within the treatment group (section 3.4.1). A replicate extract from the leaves of this treatment group (Table 29) did not corroborate these findings.

Lettuce Leaves

Triclosan was not observed in any of the lettuce extracts regardless of irrigation source (Table 30 and Table 31). It appears therefore that uptake of triclosan by lettuce is minimal.

3.7 Fate and Transport of Estradiol

Crop Tissues

Analysis of 10 fold and 20 fold dilute extracts of sweet potato tissue, sweet potato leaf tissue, and lettuce leaf tissue, frequently gave responses within the quantitation range of estradiol ELISA kit over the entire study period. However, because of the complications described in in the Phase 3 investigation sections 3.2.3.4 and 3.5.1, (namely significant matrix confounding observed during the analysis of unspiked-homogenate extracts into which working solution was then spiked directly), it was determined that further method investigation would need to be complete prior to asserting any conclusions for the analysis of crop matrix samples from the greenhouse experiment.

Sand and Soil

Estradiol ELISA analysis of soil extracts appeared to have fewer complications than plant tissue extracts. Analysis of virgin sand and soils during method compatibility analysis (section 3.2.3), indicated that false positive responses due to matrix components within the soil extracts did not appear to be an issue. Indeed, muted responses for soil extracts 10 fold and 20 fold dilute appeared to be more a more likely concern than false positive responses. Analysis of a single, homogenate-spiked soil sample, diluted at the potentially muted 10 fold level, indicated approximately 50% recovery (section 3.2.3.4).

Table 32 summarizing the estradiol ELISA analyses of soil samples from the greenhouse experiment. Only two greenhouse soil extracts gave responses indicating the presence of estradiol; the soil from the sweet potato treatment group irrigated with spiked-

reclaimed water, and the soil from the lettuce treatment group irrigated with unspiked reclaimed water (Table 32). All other samples gave responses below detection.

Sample*	Best (fit) concentration [ng/L] in extract based on ELISA calibration curve	Average recovery rate from matrix during compatability investigations	Estradiol concentration (pg/g) in original matrix (% Recovery Adjusted)	Total mass of estradiol per plant (ng/plant) within the designated matrix (concentration x average matrix mass of treatment group*)
February				
P:S:SO:S3	454	50%	1342	537
P:F:SO:S2	0	50%	0	0
P:G:SO:S2	0	50%	0	0
L:F:SO:S1	41.5	50%	124	50
L:G:SO:S2	0	50%	0	0
March				
P:S:SA:S1	0	40%	0	0
P:F:SA:S1	0	40%	0	0
P:G:SA:S2	0	40%	0	0
L:S:SO:S2	0	40%	0	0

*(Refer to X and Y series Appendices)

Table 32: Spring 2012 ELISA trend and concentration analysis of estradiol within greenhouse irrigated growing matrices

The response of the sample irrigated with spiked-reclaimed water was approximately 10 fold larger than the sample irrigated with unspiked-reclaimed water, indicating a concentration of approximately 1300pg/g and 120pg/g respectively. Applying these concentrations to the 400g soil samples collected at the time of the crop harvests, this accounts for 500ng and 50ng estradiol; this mass accounts for less than 2% the estimated mass applied from spiked irrigation source section 3.4.1.

CHAPTER 4: CONCLUSIONS AND RECOMMENDATIONS

4.1 Method Compatibility

The Phase 1-3 investigations (section 3.2) provided progressively more rigorous insight into the methodological compatibility of analyzing acetonitrile-based QuEChERS extracts of crop tissues and growing matrices using proprietary ELISA kits. In Phase 1 (section 3.2.1), analytically large spikes of the target analytes were applied to homogenate of grocery store sweet potato, allowing for large dilutions of the final crop extracts, thereby minimizing the effect of any potentially confounding matrix interferences on the assay. The results from Phase 1 demonstrated that a) the QuEChERS method was capable of extracting the target compounds and b) the ELISA kits for all compounds tested were capable of detecting their respective compounds within the highly dilute extracts. The former was confirmed by both ELISA and GC analysis. Practical detection limits (the lowest analyte concentration in the homogenate from which a linear calibration can be obtained) for using the GC-Ion-Trap-MS to analyze the QuEChERS extracts as prepared, without any additional clean up, were calculated for each compound as shown in Table 33. It was determined in later investigations that caffeine and triclosan were recovered from sweet potato homogenate with better than 90% efficiency, and thus additional extract clean-up and/or concentration would be required to improve upon these values.

Compound	Practical Detection Limit (µg/g)
Caffeine	1.026
Triclosan	0.063
Estradiol	0.118

Table 33: Practical Detection Limits for GC-Ion-Trap-MS

Comparing the practical detection limits calculated for the GC-MS to those using the ELISA kits (Table 34) it was clear that the ELISA approach is far more sensitive with less sample preparation. Even though the values shown in Table 34 assume 100% analyte recovery from the homogenate, these values are 5-6 orders of magnitude lower and demonstrate the viable application of ELISAs to measuring trace chemical uptake in crops. A demonstration of this was the finding of 6-8ng/g caffeine in store-purchased sweet potato. This would not have been detected using the GC/MS method.

Compound	ELISA Quantitation Range	Potential Detection Limit
Caffeine	0.175-5.0 µg/L	1.75 ng/g
Triclosan	0.05-2.5 µg/L	0.5 ng/g
Estradiol	2.7-25 ng/L	27 pg/g

Table 34: Potential Detection Limits of ELISA kits

The Phase 2 analysis (section 3.2.2) considered sweet potato leaf extracts at a more environmentally relevant dilution level (i.e. one that would assure that the extracted analytes would be detectable by the assay). Results from this analysis demonstrated the benefit of using dispersive solid phase extraction (dSPE) for preparing extracts for ELISA analysis. Extracts from identically spiked homogenates were prepared with and without dSPE, with similar recoveries observed. This demonstrated that the compounds were not appreciably lost during dSPE. Direct comparison of the extracts prepared with and without dSPE, however, consistently showed that the samples prepared without dSPE reported a significantly higher concentration. As compounds are not lost during dSPE, it was clear that the elevated responses in the extracts prepared without dSPE were the result of confounding matrix components within the uncleaned extract.

During the Phase 2 investigation analytes were spiked into extracts of an unspiked homogenate prepared without dSPE and clearly demonstrated that the caffeine and triclosan

ELISA kits were capable of detecting and quantifying the added compound concentration within the complex matrix. Estradiol concentrations were outside the detection range and due to limited availability of the kits the methodology could not be re-evaluated during this phase of the study.

The Phase 3 analysis (section 3.2.3) was the most rigorous method compatibility analysis and was performed using standard addition, spike recovery, and serial dilution of extracts from all matrices of interest alongside those of the various standards employed in the study. The caffeine and triclosan ELISA kits were found to be highly compatible with 10 fold and 20 fold diluted QuEChERS extracts from all matrices tested. As a concluding example of this, consider a final standard addition caffeine ELISA analysis, this time for extracts of the virgin (non-irrigated) soil.

Matrix and sample preparation	Dilution (x-fold)	Expected caffeine concentration (μg/L) from 10μL spike into 2mL extract	Concentration (μg/L) and 99% CI range of original extract		
			Fit	Upper	Lower
Unspiked Homogenate Extract	10	0	No Detect	No Detect	No Detect
Unspiked Homogenate Extract	20	0	No Detect	No Detect	No Detect
Extract Spiked	10	24.9 +/-7.0	23.2	32.0	16.7
Extract Spiked	20	24.9 +/-7.0	21.6	29.3	16.3
Extract Spiked	40	24.9 +/-7.0	26.3	35.3	18.0
Extract Spiked	80	24.9 +/-7.0	37.3	53.5	24.1

Table 35: Caffeine ELISA analysis of extract-spiked and unspiked homogenate extracts of virgin (non-irrigated) soil (VSO) compared to expected responses from spike delivered

Table 35 shows the ELISA analysis of the extract of unspiked homogenate, unmodified (white) and with 10μL of working solution (~4.99mg/L) spiked into 2mL of the finished extract (grey). These two samples were then serially diluted as shown in Table 35, resulting in a total of six independent ELISA outputs. The resulting concentration for each dilute extract (not shown) was multiplied by the sample dilution factor (column 2) in order to arrive at the best fit concentration and 99% confidence interval (Upper/Lower), within the

original extract. As seen in Table 35 no caffeine was detected within the extract of the unspiked soil. The concentration observed within the spiked extract was consistent with the expected concentration due to the spike delivered. Indeed, though the number of samples available for comparative analysis is small, a t-test comparing the four (fit) responses in Table 35 to the 3 (fit) responses from the analysis of the working solution (Appendix D.3) returns a p-value of 0.60 (Appendix D.4), indicating that the expected and observed responses for the soil extracts are indistinguishable.

During the Phase 3 investigations, analysis of spiked extracts from crop tissue using the estradiol ELISA kit proved confounded at extract dilutions between 10 and 80 fold (section 3.2.3.4). While the concentrations reported for the sweet potato spiked extracts were elevated compared to those unspiked, the increase observed was only 10-20% of the expected value. For extracts of lettuce leaves, the spiked extracts were completely indistinguishable from those unspiked. It was determined that further investigation would be required for the estradiol ELISA kit and that interpretation would have to be suspended for all 10-80 fold dilutions of crop tissue extracts (such as seen in Phase 1). Analysis of soils using the estradiol ELISA kit proved more successful.

Extraction efficiency of the compounds was found to be variable depending on the matrix, and future work would benefit from an optimization of the matrix homogenization and QuEChERS extraction parameters. In particular, the homogenization of leaf tissue was inconsistently executed during this experiment as unforeseen complications challenged the anticipated homogenization technique and improvisations had to be made in real time. Complications encountered included a) the effects of storage on the leaf tissue prior to sample homogenization (wilting and drying) and b) the limited aggregate leaf mass available

for some treatment groups. Standardizing and optimizing the homogenization of crop tissues should be thoroughly vetted with these considerations in mind prior to processing experimental samples. The best extraction efficiencies (greater than 90%) were observed for both caffeine and triclosan from sweet potato tissue homogenates, and this may a function of the more satisfactorily complete homogenization obtained with this matrix.

4.2 Fate and Transport

Caffeine

As discussed in section 1.4.5, crop uptake and translocation of organic compounds is complex and prediction based on physiochemical properties is difficult at present. During pressure chamber experiments, Dettenmaier et al. (2009) found that caffeine was uptaken and translocated by soybean and tomato plants. In fact these findings led, in part, to the development of a new model relating the Log Kow for certain compounds to the transpiration stream concentration factor (Figure 2).

Analyses of sweet potato tissues in the current experiment appears to demonstrate that uptake of caffeine does occur, though is perhaps limited, as crops irrigated with reclaimed water and spiked-reclaimed water had a very similar total mass of caffeine within their aggregate tissues (4-6µg) despite the estimation that the crops irrigated with the spiked water source had been exposed to an order of magnitude greater caffeine mass (~17µg and ~150µg estimated application). Sweet potatoes irrigated with tap water as well as those purchased from the local grocery store also indicated the presence of caffeine at low ng/g concentrations (~0-8ng/g) within the edible potato tissue. While those irrigated with reclaimed water sources indicated higher caffeine concentrations (~16-21ng/g), the total masses indicated by these concentrations represent less than 20% (unmodified composition) and less than 2%

(spiked composition) of the total aggregate mass estimated to have been applied during irrigation, indicating limited uptake when greater concentrations are applied. While the concentration differences observed within tissues of plants irrigated with different water sources are not especially stark, the fact that differentiation was observed is encouraging for the application of the analytical method. In terms of crop growth, as compared to irrigating with a tap water source, it does not appear that irrigating with wastewater treated through engineered wetlands processes will result in a significant increase in caffeine uptake (and perhaps other compounds with similar uptake tendencies) within the edible portion of sweet potato plants.

Analysis of sweet potato leaves from an independent agricultural field site with unknown irrigation practices (Phase 2) did not indicate the presence of measurable caffeine within the leaf tissue. Note that there was a significant mass of fresh leaf tissue available for homogenization during this investigation, and the wet slurry created with a 1:1 ratio of lab grade water was more satisfying than for than many of the greenhouse samples.

Analysis of sweet potato leaves from the greenhouse investigation suggested caffeine within the leaf tissue from all treatment groups. Indeed, the concentration reported within the leaf tissue was greater than that in the potato tissue, perhaps indicating the potential for translocation through the plant once uptaken. This would be consistent with the pressure chamber experiments performed by Dettenmaier et al. (2009) which demonstrated uptake and translocation of caffeine in both soybean and tomato plants. While the leaves irrigated with spiked reclaimed water did appear to be the most concentrated of any treatment group, the differentiation between the three groups was not especially pronounced. Additionally, the aggregate mass accounted for within the leaf tissue of the plants irrigated with the spiked

water source (~ 2μg) continued to demonstrate limited uptake of caffeine. As previously mentioned, there is some concern over the homogenization improvisation that was required for leaf samples.

Caffeine was not found in any of the soil extracts of unmodified samples taken during either the sweet potato or lettuce harvests. The most likely explanation is that the time between ceasing irrigation, collecting the soil samples, and performing the extractions (approximately 4 weeks with storage in a 4°C refrigerator) was sufficient to allow microbial degradation. Indeed, Bradley et al. (2006) observed that 50-90% of radio-labeled caffeine within stream sediments (under oxic conditions) had been mineralized within two days by indigenous microbial populations. Topp et al (2005) also observed rapid indigenous microbial degradation of caffeine in agricultural soils. Both research groups ultimately questioned the utility of caffeine as a marker of long term contamination within soils because of its rapid biodegradation.

Triclosan

Triclosan was not found in either the grocery store sweet potato or the sweet potato leaves from the independent agricultural field site (Phase 1 and 2, section 3.2.1 and 3.2.2). From the greenhouse experiment, the only sweet potato tissue extracts with triclosan present (between 4 and 11ng/g) were those irrigated with spiked reclaimed water (section 3.6) representing less than 2% of the aggregate mass estimated to have been applied. Triclosan was observed in a single sweet potato leaf extract (irrigated with spiked-reclaimed water) near the LOD. Analysis of a replicate extract of the same homogenate was below the LOD and could not confirm this finding. Triclosan was not observed in any lettuce samples. Though the homogenization concerns previously mentioned for leaves should not be

overlooked, the uptake and translocation of triclosan appears to be limited for both sweet potato and lettuce.

The only soil extract found to have triclosan present were from those irrigated with spiked-reclaimed water at concentration between 7.5 and 99ng/g, accounting for between 5% and 25% of the estimated mass applied. Given the reported Log Koc values (Table 1) between 3.99 and 4.30 for various soil compositions (Karnjanapiboowong 2010) and Log Kow value of 4.76, it was anticipated that triclosan would preferentially adsorb to soil and have limited bioavailability to the crops, and the analyses of crop tissue and soil extracts in this experiment correlated with these expectations.

Estradiol

As prepared, crop tissue extracts were found to be confounded at the dilution range investigated (section 3.2.3). As a consequence, only the extracts of sand and soil were considered for drawing conclusions. The soil extracts found to have estradiol present were those where crops had been irrigated with reclaimed (120pg/g) and spiked (~1300pg/g) reclaimed water sources. This indicated recovery of less than 2% the mass estimated to have been applied to the spiked irrigation source. With reported Log Koc values (Table 1) between 3.58 and 3.90 for various soil compositions (Karnjanapiboowong 2011) and Log Kow of 4.01 it was expected that estradiol would preferentially adsorb onto soils. Thus, the low concentrations observed are likely due to microbial degradation prior to extraction. Indeed, Ying et al. (2005) observed that estradiol had a half-life of approximately 3 days in aerobic soils and that both estradiol and its primary metabolite estrone were greater than 90% degraded within non sterile soils after 15 days.

4.3 Final Comments

This research demonstrated compatibility between proprietary ELISA kits and complex acetonitrile based extracts of crop tissues and soils. While extraction efficiency improvements can be expected, the demonstrated compatibility suggests that ELISAs are well suited for providing high throughput screening analysis of chemicals within crops and soils at low ng/g concentration levels. Uptake of caffeine and triclosan, two prominent indicators of wastewater contamination, appears to be limited for sweet potato and lettuce, suggesting that irrigation of edible crops with reclaimed water sources is unlikely to result in significantly elevated public exposure if such crops are consumed. Future work would benefit from expanding the net of target chemicals investigated as well as interdisciplinary research to more thoroughly vet the most appropriate choices of indicator compounds for efficient screening analyses.

APPENDIX A:

STOCK AND WORKING SOLUTIONS

A.1: Stock solutions in acetonitrile (used to make working solutions)

Date Made	Compound	Mass Weighed (g)	Volume Acetonitrile (mL)	Concnetration (µg/mL)
2/10/2011	E2	0.01629	25	651.6
2/18/2011	Caffeine	0.01122	25	448.8
5/2/2011	EE2	0.00903	25	361.2
2/18/2011	E3	0.01234	25	493.6
2/18/2011	Triclosan	0.01271	25	508.4
5/2/2011	Nonlyphenol	13µL of 0.940g/mL	25	488.8

Table 36: Stock solutions (Batch "A") of target compounds in acetonitrile

Date Made	Compound	Mass Weighed (g)	Volume Acetonitrile (mL)	Concnetration (µg/mL)
8/5/2011	E2	0.02885	50	577
8/5/2011	Caffeine	0.02663	50	532.6
8/5/2011	EE2	0.02763	50	552.6
8/5/2011	E3	0.031209	100	312.09
8/5/2011	Triclosan	0.02687	50	537.4
8/5/2011	Nonlyphenol	26µL of 0.940g/mL	50	488.8

Table 37: Stock solutions (Batch "B") in acetonitrile

A.2: Working solutions used for Phase 1 investigation

	Concentration	Dilution Strategy	Dilution Execution
Triclosan			
Stock A: (µg/µL)	0.5084		
Working TricA: (µg/µL)	2.034E-02	1:25 of stock A	200µL in 5mL
Working TricB:(µg/µL)	2.034E-03	1:10 of working A	1mL in 10mL
Caffeine			
Stock A: (µg/µL)	0.4488		
Working CaffA:(µg/µL)	1.795E-02	1:25 of stock A	200µL in 5mL
Working CaffB: (µg/µL)	1.795E-04	1:100 of Working A	100µL in 10mL
Estradiol			
Stock A: (µg/µL)	0.6516		
Working E2A (µg/µL)	1.303E-02	1:50 of stock A	100µL in 5mL
Working E2B (ng/µL)	1.303E-03	1:10000 of working A	10µ in 100mL

Table 38: Phase 1 working solutions in acetonitrile (used to spike onto homogenates)

Compound	Concentration (µg/L)	Dilution Strategy	Dilution Execution
Triclosan	17.952	1:25 Stock A Triclosan	200µL into 5mL
Caffeine	20.336	1:25 Stock A Caffeine	200µL into 5mL

Table 39: Phase 1 GC-MS neat solution "Mix A" in acetonitrile

Sample Name	Compound	Volume Stock A (µL) dried	Volume Hexane Final (µL)	Concentration Der. Stock (mg/L)	Dilution*	Final Concentration (mg/L)
DN_E2	E2	20	300	43.44	10	4.344
DN_Caff	Caffeine	30	300	44.88	10	4.488
DN_EE2	EE2	40	300	48.16	10	4.816
DN_E3	E3	30	300	49.36	10	4.936
DN_Non	Nonlyphenol	30	300	48.88	10	4.888
					*20µL in 200µL hexane	

Table 40: Derivatized neat samples (using derivatization method A) in silinized glassware

A.3: Working solutions for Phase 2 investigation

Working Solution Designation	Concentration Stock (µg/µL)	Dilution to make working	Concentration Working (µg/µL)	Dilution Execution
Caffeine B1.1	0.5326	50	1.065E-02	200µL in 10mL
Triclosan B1.1	0.5374	100	5.374E-03	100µL in 10mL
Estradiol B1.1	0.5770	10000	5.770E-05	10µL in 100mL

Table 41: Phase 2 working solutions for spiking onto homogenates of sweet potato leaf

Working Solution Designation	Concentration Stock (µg/µL)	Dilution to make working	Concentration Working (µg/µL)	Dilution Execution
Caffeine B1.2	0.5326	2500	2.130E-04	20µL in 50mL
Triclosan B1.2	0.5374	2500	2.150E-04	20µL in 50mL
Estradiol B1.2	0.5770	500000	1.154E-06	Serial 25µL in 25mL; 50µL in 25mL

Table 42: Phase 2 working solutions for spiking into QuEChERS extracts of sweet potato leaf

A.4: Working solution #5 for Phase 3 analysis

Stock B Compound	Volume Stock B (µL)	Volume Acetonitrile (mL)	Concentration (µg/mL)	Concentration [µg/µL)
E2	0.5	10	2.885E-02	2.885E-05
Caffeine	110	10	5.859E+00	5.859E-03
Triclosan	55	10	2.956E+00	2.956E-03
Nonlyphenol	800	10	3.910E+01	3.910E-02

Table 43: Working solution (#5) for delivering homogenate-spikes and spikes into QuEChERS extracts of Phase 3 Investigation (made on 2/6/2012)

A.5: Working solutions in acetonitrile for homogenate-spikes and spikes into QuEChERS extracts of greenhouse samples

Stock B Compound	Volume Stock B (µL)	Volume Acetonitrile (mL)	Concentration (µg/mL)	Concentration [µg/µL)
E2	50	5	5.770E+00	5.770E-03
Caffeine	50	5	5.326E+00	5.326E-03
EE2	50	5	5.526E+00	5.526E-03
E3	100	5	6.242E+00	6.242E-03
Triclosan	50	5	5.374E+00	5.374E-03
Nonlyphenol	50	5	4.888E+00	4.888E-03

Table 44: Greenhouse samples extraction working solution (#1) for delivering homogenate-spikes and spikes into QuEChERS extracts (made on 10/6/2011)

Stock B Compound	Volume Stock B (µL)	Volume Acetonitrile (mL)	Concentration (µg/mL)	Concentration [µg/µL)
E2	335	5	3.866E+01	3.866E-02
Caffeine	335	5	3.568E+01	3.568E-02
EE2	335	5	3.702E+01	3.702E-02
E3	670	5	4.182E+01	4.182E-02
Triclosan	335	5	3.601E+01	3.601E-02
Nonlyphenol	335	5	3.275E+01	3.275E-02

Table 45: Greenhouse samples extraction working solution (#2) for delivering homogenate-spikes and spikes into QuEChERS extracts (made on 10/9/2011)

Stock B Compound	Volume Stock B (µL)	Volume Acetonitrile (mL)	Concentration (µg/mL)	Concentration [µg/µL)
E2	665	10	3.837E+01	3.837E-02
Caffeine	665	10	3.542E+01	3.542E-02
EE2	665	10	3.675E+01	3.675E-02
E3	1330	10	4.151E+01	4.151E-02
Triclosan	665	10	3.574E+01	3.574E-02
Nonlyphenol	665	10	3.251E+01	3.251E-02

Table 46: Greenhouse samples extraction working solution (#3) for delivering homogenate-spikes and spikes into QuEChERS extracts (made on 10/26/2011)

Stock B Compound	Volume Stock B (µL)	Volume Acetonitrile (mL)	Concentration (µg/mL)	Concentration [µg/µL)
E2	665	10	3.837E+01	3.837E-02
Caffeine	665	10	3.542E+01	3.542E-02
EE2	665	10	3.675E+01	3.675E-02
E3	1330	10	4.151E+01	4.151E-02
Triclosan	665	10	3.574E+01	3.574E-02
Nonlyphenol	665	10	3.251E+01	3.251E-02

Table 47: Greenhouse samples extraction working solution (#4) for delivering homogenate-spikes and spikes into QuEChERS extracts (Made on 12/1/2011)

APPENDIX B:

PHASE 1 INVESTIGATION

B.1: Phase 1 investigation; execution of spikes onto sweet potato homogenates

	Spiking Solution Used (See Appendix G2)			Volume spike delivered (µL)			Concentration from spike (µg/g) per sample		
Sample Letter	Triclosan	Caffeine	Estradiol	Triclosan	Caffeine	Estradiol	Triclosan	Caffeine	Estradiol
A	Stock A	Stock A	Stock A	196.8	223	153.4	20.01	20.02	19.99
B	Stock A	Stock A	Stock A	98.4	111.4	76.8	10.01	10.00	10.01
C	Stock A	Stock A	Stock A	39.4	44.6	30.8	4.01	4.00	4.01
D	Stock A	Stock A	Stock A	19.8	22.4	15.4	2.01	2.01	2.01
E	Stock A	Stock A	Stock A	9.8	11.2	7.6	1.00	1.01	0.99
F	Working TricA	Working CaffA	Working E2A	123	139.2	191.8	0.50	0.50	0.50
G	Working TricA	Working CaffA	Working E2A	24.6	27.8	38.4	0.10	0.10	0.10
H	Working TricB	Working CaffB	Working E2B	61.5	139	95.9	2.50E-02	4.99E-03	2.50E-02
K	None	None	None	0	0	0	0	0	0

Table 48: Execution of homogenate spikes onto sweet potato homogenate and concentration of samples created

Sample	Spike (µg/g) onto homogneate	Dilution	Caffeine in Dilute Extract (µg/L) from ELISA Calibration Curve			Caffeine recovered from homogenate (ng/g for K) (µg/g for F and D)			Spike Recovery (%)		
			Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
K	0	10	0.63	0.72	0.55	6.3	7.2	5.5			
K*	0	50				0.0	0.0	0.0			
F	0.500	1000	1.06	1.17	0.95	1.06	1.17	0.95	210%	233%	189%
F	0.500	250	1.68	1.89	1.51	0.42	0.47	0.38	83%	93%	74%
F*	0.500	250	1.87	2.31	1.49	0.47	0.58	0.37	92%	114%	73%
F	0.500	100	3.02	3.40	2.65	0.30	0.34	0.26	59%	67%	52%
D	2.011	1000	4.45		3.87	4.45	0.00	3.87	221%		192%
*Samples were run separately with two calibration points that showed a best match the Aug 8th Calibration Curve											

Table 49: Caffeine ELISA responses and recovery of spikes onto grocery store sweet potato homogenates

Sample	Spike (µg/g) onto homogneate	Dilution	Triclosan in Dilute Extract (µg/L) from ELISA Calibration Curve			Triclosan recovered from homogenate (µg/g)			Spike Recovery (%)		
			Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
K	0	10	0.00	0.00	0.00	0	0	0			
B	10.01	25000	0.35	0.37	0.34	8.86	9.31	8.42	89%	93%	84%
B	10.01	10000	0.62	0.66	0.58	6.15	6.57	5.78	62%	66%	58%
A	20.01	8000	1.94	2.43	1.67	15.48	19.46	13.40	77%	97%	67%

Table 50: Triclosan ELISA responses and recovery of spikes onto grocery store sweet potato homogenates

Sample	Spike (µg/g) onto homogneate	Dilution	Estradiol in Dilute Extract (ng/L)			Estradiol recovered from homogenate (ng/g for K) (µg/g for G)			Spike Recovery (%)		
			Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
K	0	10	15.82	21.74	11.84	0.16	0.22	0.12			
K*	0	10	12.64	16.92	9.79	0.13	0.17	0.10			
K*	0	30	5.12	8.86		0.15	0.27				
G	0.100	10000	9.59	13.84		0.10	0.14		96%	138%	
G	0.100	4000	13.37	18.98	9.24	0.05	0.08	0.04	53%	76%	37%

*Samples in grey were run separately with two calibration points that showed a best match the Sept 8th Calibration Curve

Table 51: Estrdiol ELISA responses and recovery of spikes onto grocery store sweet potato homogenates

B.3: Phase 1 investigation; GC-ECD retention time and peak area response to QuEChERS extracts of sweet potato, homogenates spiked with known masses of triclosan

Sample Letter Assigned	Triclosan spike (µg/g)
A	20.01
B	10.01
C	4.006
D	2.013
E	0.996
F	0.500
G	0.100
H	2.50E-02
K	0

Table 52: Triclosan spike delivered (µg/g) onto homogenates of sweet potato samples (taken from Table 48)

Sample Letter Assigned	Retention Time	Peak Area
A	43.879	2093812
B	43.880	1224810.5
C	43.881	657438
D	43.882	316458.438
E	43.879	7892.18555
F	43.882	113413.219
G	43.880	31679.6738
H	43.881	8832.63965
K	43.872	12047.7275

Table 53: GC-ECD retention time and peak area responses for QuEChERS extracts of sweet potato with known triclosan spikes delivered onto homogenate prior to extraction

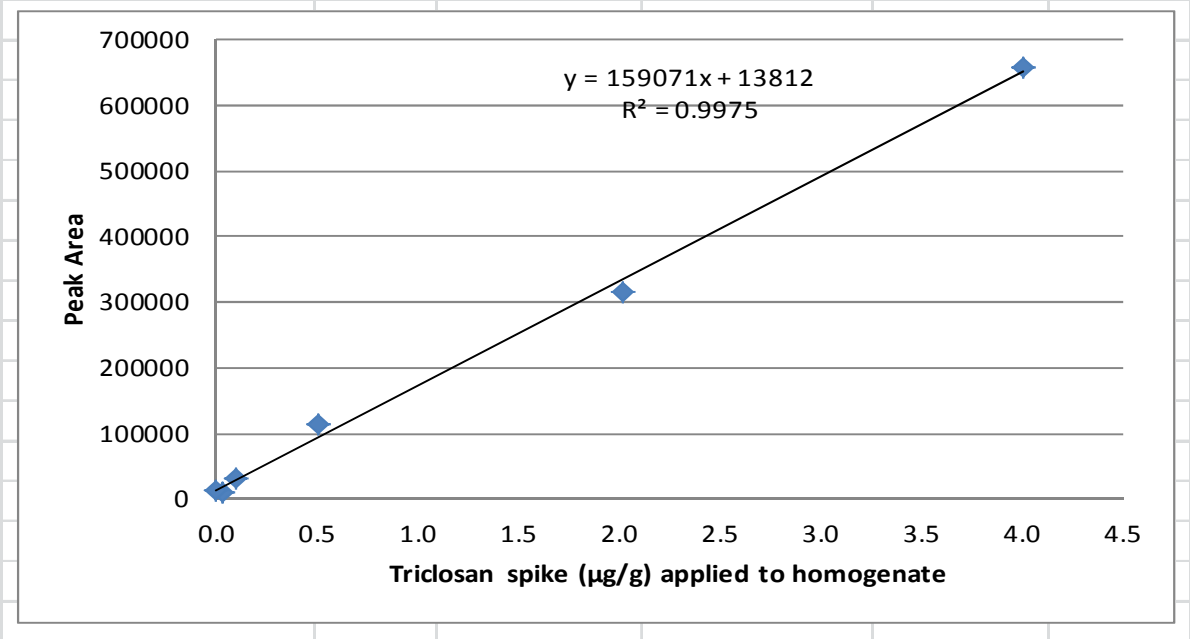

Figure 18: Triclosan spike applied to homogenate vs. GC-ECD peak area response

B.4: Phase 1 investigation; GC-ECD peak area responses to neat solutions of triclosan in acetonitrile

Dilution of Working TricA*	Concentration (mg/L)	Retention Time	Peak Area
None	2.034E+01	43.881	1582548.75
1 in 5	4.067E+00	43.875	118923.5
1 in 10	2.034E+00	43.876	57040.10547
1 in 20	1.017E+00	43.876	33685.57813
1 in 40	5.084E-01	43.877	20538.51758
1 in 200	1.017E-01	43.878	5022.739258
*Refer to Table 38			

Table 54: Retention time and peak area of neat solutions of triclosan in acetonitrile

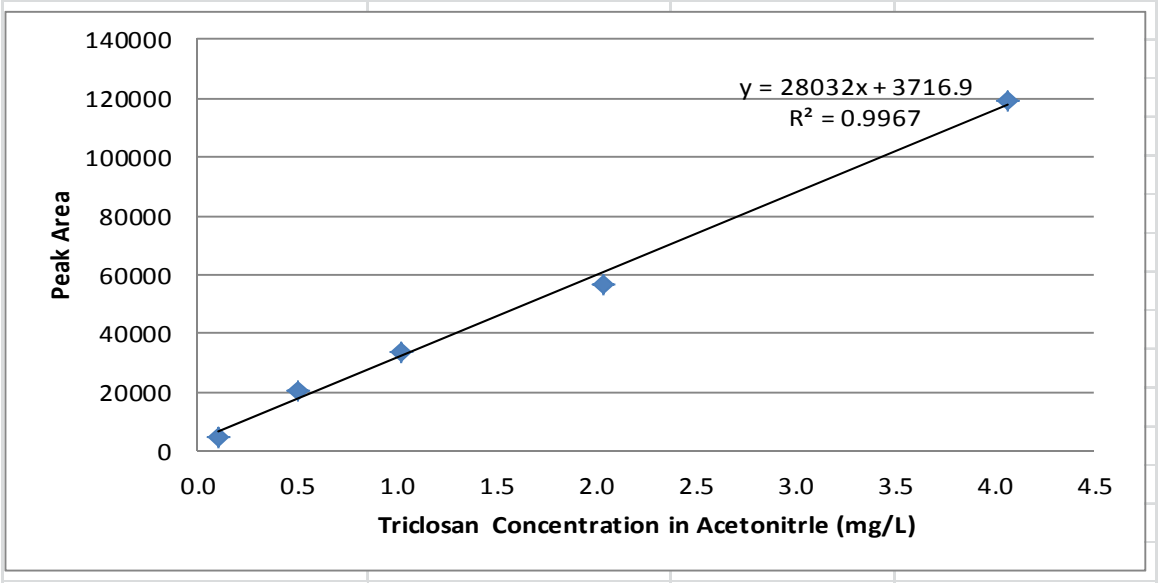

Figure 19: Concentration of neat solutions of triclosan in acetonitrile vs. GC-ECD peak area response

B.5: Phase 1 investigation; GC-Ion-Trap-MS signal to noise responses to neat standards of target compounds and determination of instrument detection limits (IDL)

Sample*	Compound	Derivatized (Y/N)	Concentration (mg/L)	S/N	Instrument Dection Limit IDL (mg/L)
Mix A	Caffeine	N	1.795E+01	1249	0.144
Mix A	Triclosan	N	2.034E+01	5381	0.038
DN_E2	Estradiol	Y	4.344E+00	6808	0.006
*See Table 39					

Table 55: GC-Ion-Trap-MS signal to noise responses for target compounds within neat standards in acetonitrile (Mix A) and hexane (DN_2)

B.6: Phase 1 investigation; GC-Ion-Trap-MS signal to noise responses to homogenate spiked sweet potato samples and determination of practical detection limits (PDL)

Sweet Potato Extract	Compound	Homogenate Spike Delivered (µg/g)	Derivatized (Y/N)	Concentration After Derivatization (µg/g)	S/N	Practical Detection Limit (µg/g)
A	Caffeine	20.02	N	N/A	195	1.026
A	Triclosan	20.01	N	N/A	3167	0.063
A	Triclosan	20.01	Y	33.351	2507	0.133
A	Estradiol	19.99	Y	33.318	2250	0.148
C	Triclosan	4.01	Y	4.674	701	0.067
C	Estradiol	4.01	Y	4.683	396	0.118

Table 56: GC-Ion-Trap-MS signal to noise responses for target compounds within extracts of homogenate spiked sweet potato. Derivatized samples were prepared using derivatization Method A (500uL sample A and 350uL sample C blown to dryness and made up in a final volume of 300uL hexane)

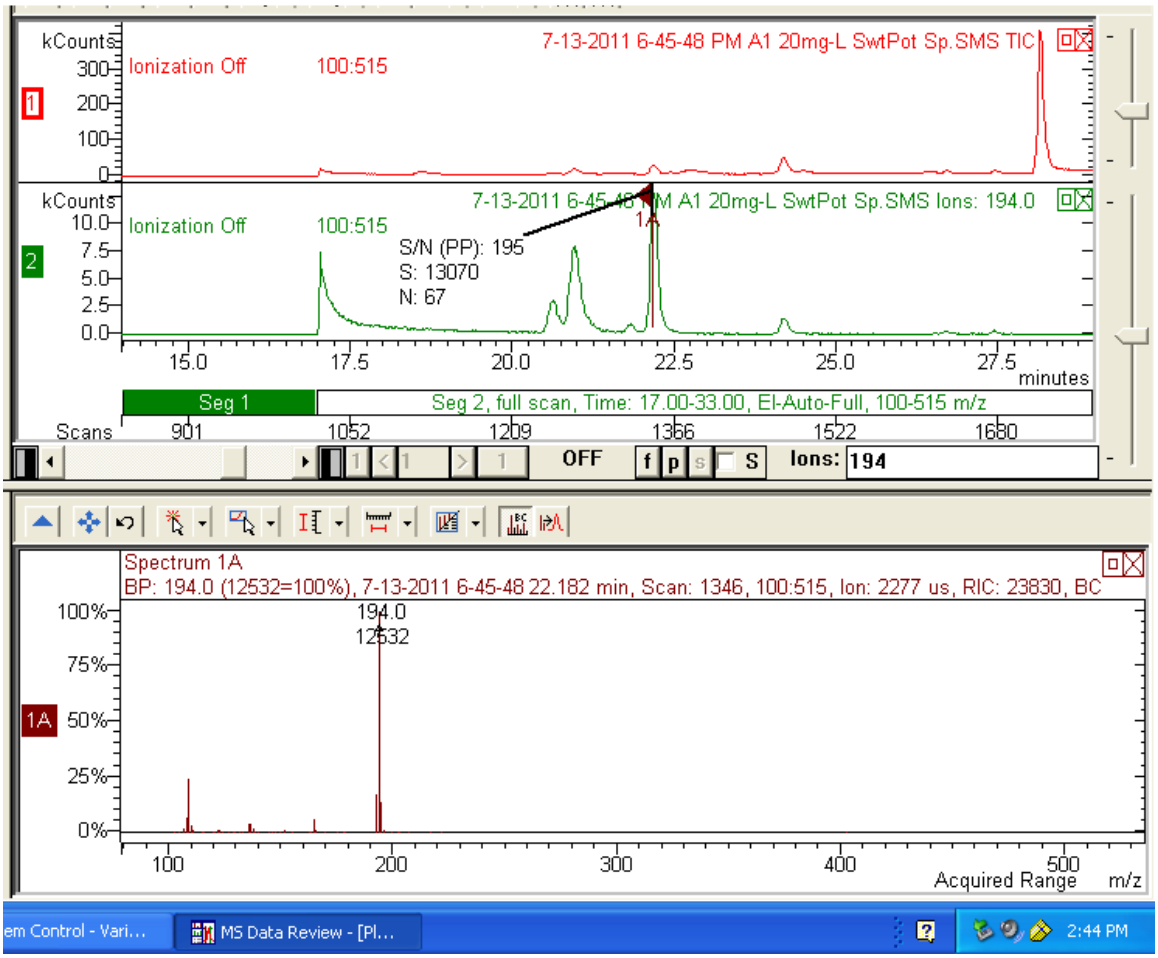

Figure 20: GC-Ion-Trap-MS chromatogram and response to caffeine within extract of homogenate-spiked sweet potato sample A (20.02µg/g): Not Derivatized.

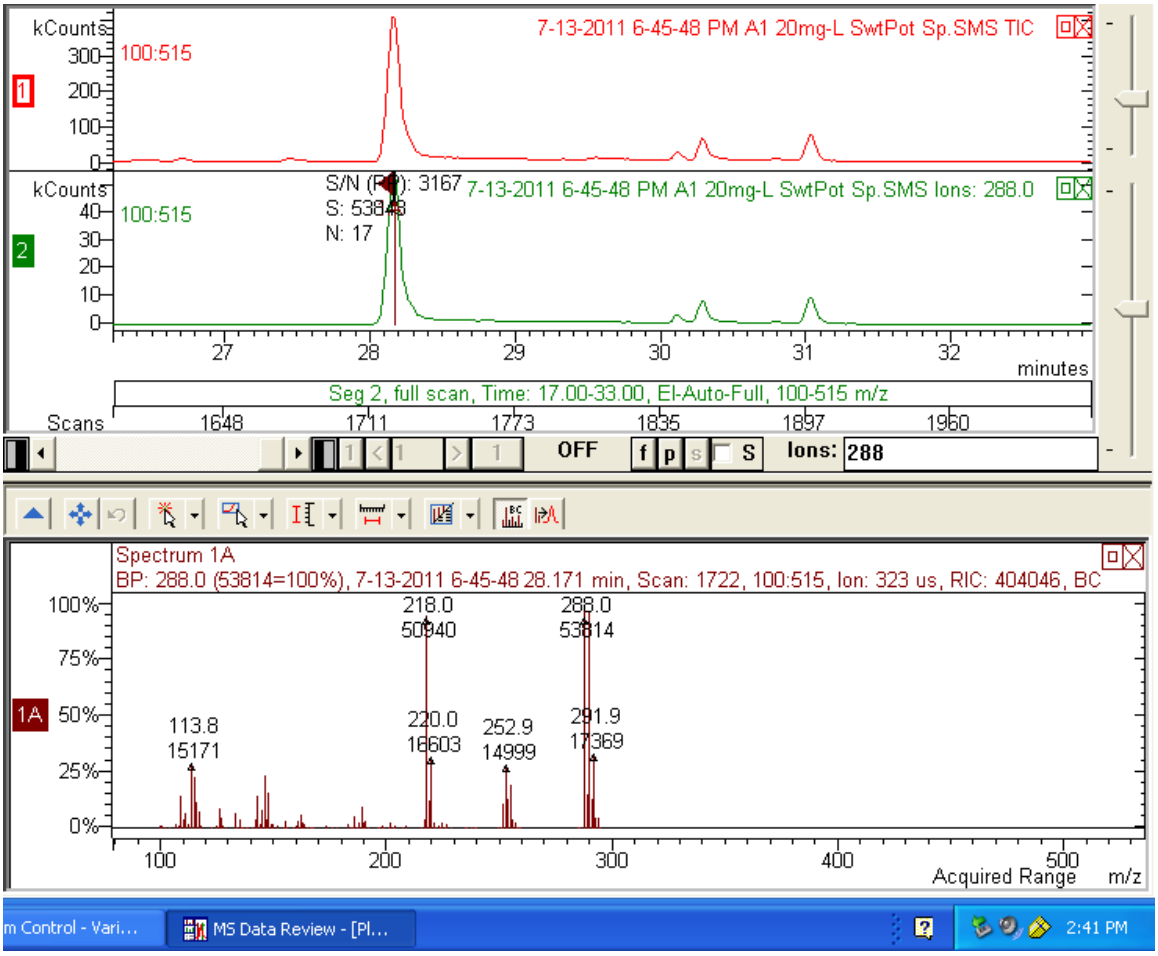

Figure 21: GC-Ion-Trap-MS chromatogram and response to triclosan within extract of homogenate-spiked sweet potato sample A (20.01µg/g): Not Derivatized.

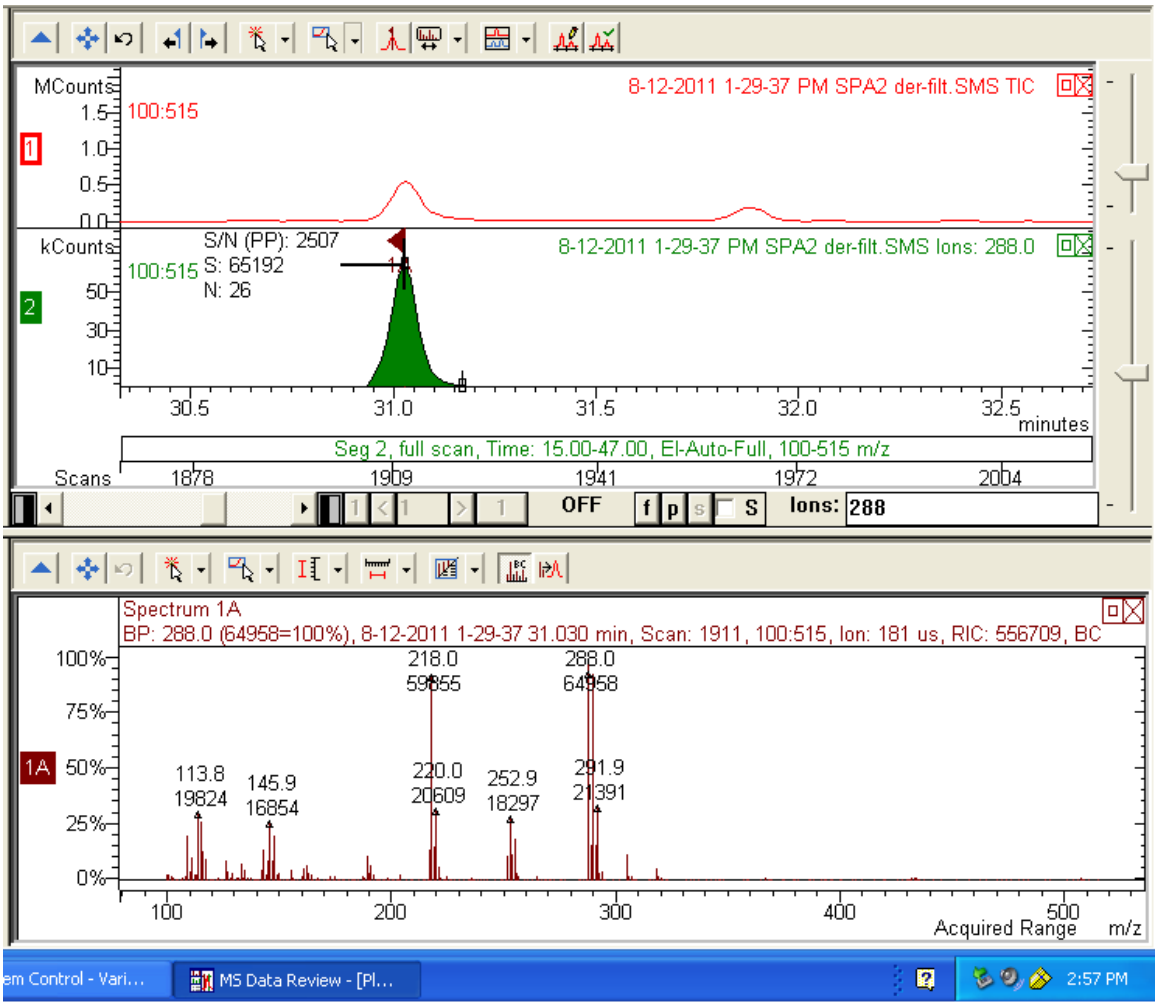

Figure 22: GC-Ion-Trap-MS chromatogram and response to triclosan within extract of homogenate-spiked sweet potato sample A (20.01µg/g): Derivatized.

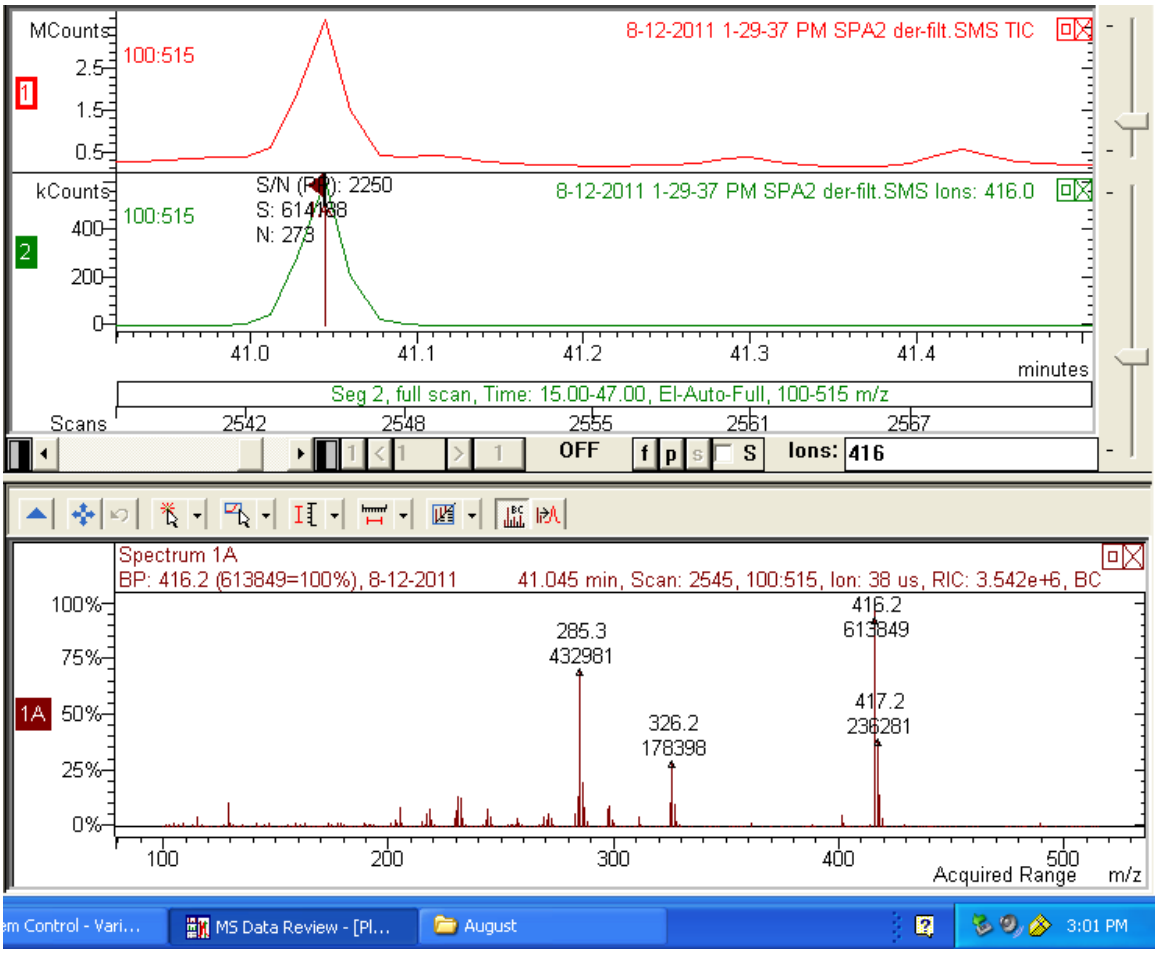

Figure 23: GC-Ion-Trap-MS chromatogram and response to estradiol within extract of homogenate-spiked sweet potato sample A (19.99µg/g): Derivatized.

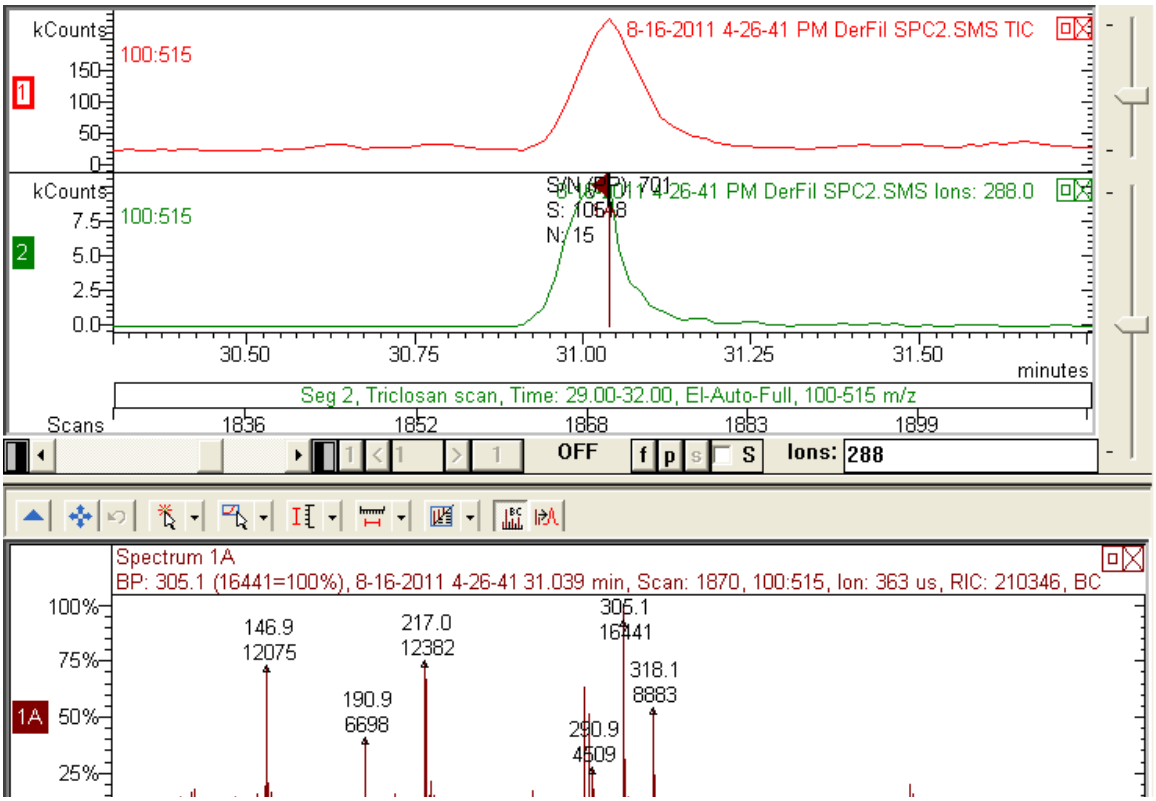

Figure 24: GC-Ion-Trap-MS chromatogram and response to triclosan within extract of homogenate-spiked sweet potato sample C (4.01µg/g): Derivatized.

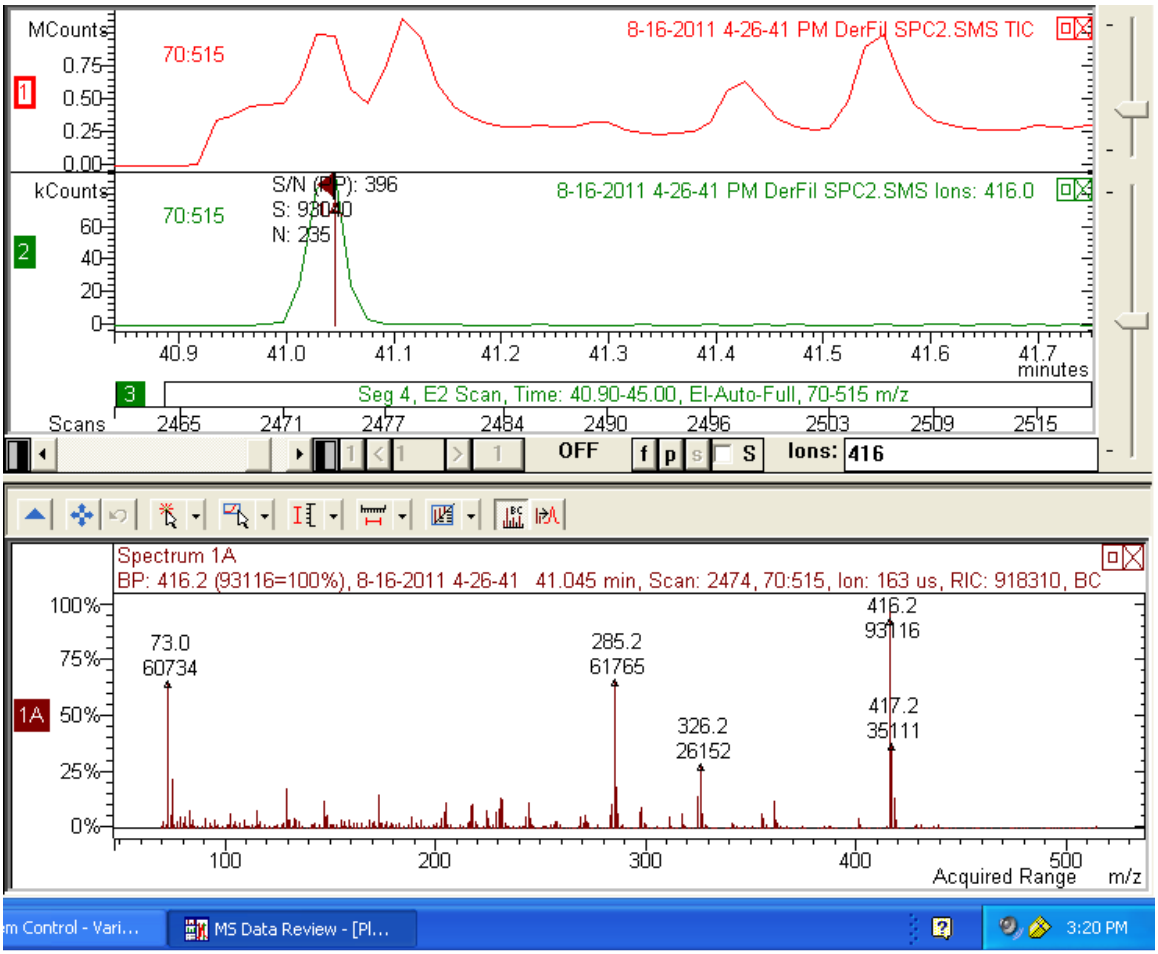

Figure 25: GC-Ion-Trap-MS chromatogram and response to estradiol within extract of homogenate-spiked sweet potato sample C (4.01µg/g): Derivatized.

APPENDIX C:

PHASE 2 INVESTIGATION

C.1: Phase 2 investigation; homogenate and extract preparation and execution

	Volume(µL) Working Solutions (B1.2) spiked into 2 mL dilute extract*			Concentration Contribution Delivered from Spike		
Sample 1	Caffeine	Triclosan	Estradiol	Caffeine (µg/L)	Triclosan (µg/L)	Estradiol (ng/L)
+A	10	15	30	5.33E+01	1.61E+00	1.73E+01
+B	40	10	10	2.13E+02	1.07E+00	5.77E+00
+C	20	20	20	1.07E+02	2.15E+00	1.15E+01
	*Refer to Appendix A.3					

Table 57: Execution of standard addition spikes into unspiked-homogenate extract without dSPE clean up (Sample 1)

Sample Designation	Volume(µL) Working Solutions (B1.1)* spiked onto 5 gram homogenates			Mass Spiked (µg) onto 5 gram homogenates			Concentration (ng/g) Spiked onto Homogenate		
	Caffeine	Triclosan	Estradiol	Caffeine	Triclosan	Estradiol	Caffeine	Triclosan	Estradiol
Sample 2	0	0	0				N/A	N/A	N/A
Sample 4	23.5	11.7	21.7	2.50E-01	6.29E-02	1.25E-03	5.01E+01	1.26E+01	2.50E+02
Sample 6	11.8	11.7	21.7	1.26E-01	6.29E-02	1.25E-03	2.51E+01	1.26E+01	2.50E+02
Sample 8	23.5	11.7	10.9	2.50E-01	6.29E-02	6.29E-04	5.01E+01	1.26E+01	1.26E+02
Sample 10	11.8	11.7	10.9	1.26E-01	6.29E-02	6.29E-04	2.51E+01	1.26E+01	1.26E+02
	*Refer to Appendix A.3								

Table 58: Execution of spikes onto sweet potato leaf homogenates for samples that DID undergo dSPE

Sample Designation	Volume(µL) Working Solutions (B1.1)* spiked onto 5 gram homogenates			Mass Spiked (µg) onto 5 gram homogenates			Concentration (ng/g) Spiked onto Homogenate		
	Caffeine	Triclosan	Estradiol	Caffeine	Triclosan	Estradiol	Caffeine	Triclosan	Estradiol
Sample 1	0	0	0				N/A	N/A	N/A
Sample 3	23.5	23.3	21.7	2.50E-01	1.25E-01	1.25E-03	5.01E+01	2.50E+01	2.50E+02
Sample 5	11.8	23.3	21.7	1.26E-01	1.25E-01	1.25E-03	2.51E+01	2.50E+01	2.50E+02
Sample 7	23.5	23.3	10.9	2.50E-01	1.25E-01	6.29E-04	5.01E+01	2.50E+01	1.26E+02
Sample 9	11.8	23.3	10.9	1.26E-01	1.25E-01	6.29E-04	2.51E+01	2.50E+01	1.26E+02
	*Refer to Appendix A.3								

Table 59: Execution of spikes onto sweet potato leaf homogenates for samples that DID NOT undergo dSPE

Spike Delivered into Extract (µg/L)	Caffeine Concentration (µg/L) from ELISA Calibration Curve			Concentration (µg/L) above Background			% Response Expected Above Background		
	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	3.25	3.69	2.82						
1.07	4.17	4.95	3.65	0.91	1.25	0.83	86%	117%	78%
1.07	4.04	4.73	3.54	0.79	1.03	0.72	74%	97%	67%
2.13	4.81		4.19	1.56		1.37	73%		64%
4.26	High								
4.26	High								

Table 60: Caffeine ELISA Responses to Standard Addition Spikes into QuEChERS Extracts of Sweet Potato Leaves

Spike Delivered into Extract (µg/L)	Triclosan Concentration (µg/L) from ELISA Calibration Curve			Concentration (µg/L) above Background			% Response Expected Above Background		
	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	0.41	0.56	0.31						
1.07	2.02		1.59	1.60		1.28	149%		119%
1.61	2.33		1.39	1.91		1.08	119%		67%
2.15	2.94		1.93	2.53		1.62	118%		76%

Table 61: Triclosan ELISA Responses to Standard Addition Spikes into QuEChERS Extracts of Sweet Potato Leaves

Spike Delivered into Extract (µg/L)	Estradiol Concentration (ng/L) from ELISA Calibration Curve			Concentration (µg/L) above Background			% Response Expected Above Background		
	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	30.17		16.07						
5.77	High								
11.54	High								
17.31	High								

Table 62: Estradiol ELISA Responses to Standard Addition Spikes into QuEChERS Extracts of Sweet Potato Leaves

Caffeine concentration (ng/g) spiked onto homogenate	Caffeine Concentration (µg/L) in Diluted Extract from Calibration Curve			Dilution Factor	Caffeine recovered from homogenate (ng/g)			Caffeine recovered from homogenate (ng/g) above unspiked sample			% Recovery		
	Fit	Upper	Lower		Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	0.66	0.77	0.56	13.33	8.8E+00	1.0E+01	7.4E+00						
5.01E+01	2.26	2.60	1.96	13.33	3.0E+01	3.5E+01	2.6E+01	2.1E+01	2.4E+01	1.9E+01	42%	49%	37%
2.51E+01	1.61	1.85	1.41	13.33	2.1E+01	2.5E+01	1.9E+01	1.3E+01	1.4E+01	1.1E+01	50%	57%	46%
5.01E+01	2.19	2.53	1.90	13.33	2.9E+01	3.4E+01	2.5E+01	2.0E+01	2.3E+01	1.8E+01	41%	47%	36%
2.51E+01	1.48	1.70	1.31	13.33	2.0E+01	2.3E+01	1.7E+01	1.1E+01	1.2E+01	1.0E+01	44%	49%	40%

Table 63: Caffeine ELISA Responses and Recovery of Homogenate Spikes onto Sweet Potato Leaves with dSPE cleanup

Triclosan concentration (ng/g) spiked onto homogenate	Triclosan Concentration (µg/L) in Diluted Extract from Calibration Curve			Dilution Factor	Triclosan recovered from homogenate (ng/g)			Triclosan recovered from homogenate (ng/g) above unspiked sample			% Recovery		
	Fit	Upper	Lower		Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0				13.33									
1.26E+01	0.19	0.26	0.13	13.33	2.5E+00	3.5E+00	1.7E+00	2.5E+00	3.5E+00	1.7E+00	20%	28%	14%
1.26E+01	0.18	0.25	0.12	13.33	2.3E+00	3.3E+00	1.6E+00	2.3E+00	3.3E+00	1.6E+00	19%	26%	13%
1.26E+01	0.09	0.14	0.06	13.33	1.2E+00	1.9E+00	8.1E-01	1.2E+00	1.9E+00	8.1E-01	10%	15%	6%
1.26E+01	0.19	0.26	0.13	13.33	2.5E+00	3.5E+00	1.7E+00	2.5E+00	3.5E+00	1.7E+00	20%	28%	14%

Table 64: Triclosan ELISA Responses and Recovery of Homogenate Spikes onto Sweet Potato Leaves with dSPE cleanup

Estradiol concentration (pg/g) spiked onto homogenate	Estradiol Concentration (ng/L) in Diluted Extract from Calibration Curve			Dilution Factor	Estradiol recovered from homogenate (pg/g)			Estradiol recovered from homogenate (pg/g) above unspiked sample			% Recovery		
	Fit	Upper	Lower		Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0				13.33									
2.50E+02	11.02	14.68	8.30	13.33	1.5E+02	2.0E+02	1.1E+02	1.5E+02	2.0E+02	1.1E+02	59%	78%	44%
2.50E+02	9.83	13.01	7.17	13.33	1.3E+02	1.7E+02	9.6E+01	1.3E+02	1.7E+02	9.6E+01	52%	69%	38%
1.26E+02	7.57	10.45		13.33	1.0E+02	1.4E+02		1.0E+02	1.4E+02	0.0E+00	80%	111%	
1.26E+02	8.48	11.33	5.69	13.33	1.1E+02	1.5E+02	7.6E+01	1.1E+02	1.5E+02	7.6E+01	90%	120%	60%

Table 65: Estradiol ELISA Responses and Recovery of Homogenate Spikes onto Sweet Potato Leaves with dSPE cleanup

Caffeine concentration (ng/g) spiked onto homogenate	Caffeine Concentration (µg/L) in Diluted Extract from Calibration Curve			Dilution Factor	Caffeine recovered from homogenate (ng/g)			Caffeine recovered from homogenate (ng/g) above unspiked sample			% Recovery		
	Fit	Upper	Lower		Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	3.25	3.69	2.82	13.33	4.3E+01	4.9E+01	3.8E+01						
5.01E+01	4.68		4.09	13.33	6.2E+01		5.5E+01	1.9E+01		1.7E+01	38%		34%
2.51E+01	3.99	4.65	3.49	13.33	5.3E+01	6.2E+01	4.7E+01	9.8E+00	1.3E+01	9.0E+00	39%	50%	36%
5.01E+01	4.08	4.80	3.58	13.33	5.4E+01	6.4E+01	4.8E+01	1.1E+01	1.5E+01	1.0E+01	22%	29%	20%
2.51E+01	4.17	4.96	3.66	13.33	5.6E+01	6.6E+01	4.9E+01	1.2E+01	1.7E+01	1.1E+01	49%	67%	45%

Table 66: Caffeine ELISA responses and recovery of homogenate spikes onto sweet potato leaves without dSPE cleanup

Triclosan concentration (ng/g) spiked onto homogenate	Triclosan Concentration (µg/L) in Diluted Extract from Calibration Curve			Dilution Factor	Triclosan recovered from homogenate (ng/g)			Triclosan recovered from homogenate (ng/g) above unspiked sample			% Recovery		
	Fit	Upper	Lower		Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	0.41	0.56	0.31	13.33	5.5E+00	7.5E+00	4.1E+00						
2.50E+01	1.00	1.43	0.72	13.33	1.3E+01	1.9E+01	9.6E+00	7.9E+00	1.2E+01	5.4E+00	31%		22%
2.50E+01	1.11	1.57	0.78	13.33	1.5E+01	2.1E+01	1.0E+01	9.2E+00	1.4E+01	6.3E+00	37%	54%	25%
2.50E+01	0.64	0.90	0.48	13.33	8.5E+00	1.2E+01	6.4E+00	2.9E+00	4.5E+00	2.2E+00	12%	18%	9%
2.50E+01	1.06	1.50	0.75	13.33	1.4E+01	2.0E+01	1.0E+01	8.6E+00	1.3E+01	5.9E+00	34%	50%	23%

Table 67: Triclosan ELISA responses and recovery of homogenate spikes onto sweet potato leaves without dSPE cleanup

Estradiol concentration (pg/g) spiked onto homogenate	Estradiol Concentration (ng/L) in Diluted Extract from Calibration Curve			Dilution Factor	Estradiol recovered from homogenate (pg/g)			Estradiol recovered from homogenate (pg/g) above unspiked sample			% Recovery		
	Fit	Upper	Lower		Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	30.17		16.07	13.33	4.0E+02	0.0E+00	2.1E+02						
2.50E+02	18.27		13.10	13.33	2.4E+02	0.0E+00	1.7E+02	-1.6E+02	0.0E+00	-4.0E+01	-63%		-16%
2.50E+02	17.19		12.63	13.33	2.3E+02	0.0E+00	1.7E+02	-1.7E+02	0.0E+00	-4.6E+01	-69%		-18%
1.26E+02	11.61	15.50	8.86	13.33	1.5E+02	2.1E+02	1.2E+02	-2.5E+02	2.1E+02	-9.6E+01	-197%	164%	-76%
1.26E+02	15.49		11.78	13.33	2.1E+02	0.0E+00	1.6E+02	-2.0E+02	0.0E+00	-5.7E+01	-156%		-45%

Table 68: Estradiol ELISA responses and recovery of homogenate spikes onto sweet potato leaves without dSPE cleanup (Background left AS READ)

Estradiol concentration (pg/g) spiked onto homogenate	Estradiol Concentration (ng/L) in Diluted Extract from Calibration Curve			Dilution Factor	Estradiol recovered from homogenate (pg/g)*			Estradiol recovered from homogenate (pg/g) above unspiked sample			% Recovery		
	Fit	Upper	Lower		Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	Removed			13.33	8.5E+01	1.6E+02	1.5E+01						
2.50E+02	18.27		13.10	13.33	2.4E+02		1.7E+02	1.6E+02	-1.6E+02	1.6E+02	63%		64%
2.50E+02	17.19		12.63	13.33	2.3E+02		1.7E+02	1.4E+02	-1.6E+02	1.5E+02	58%		61%
1.26E+02	11.61	15.50	8.86	13.33	1.5E+02	2.1E+02	1.2E+02	7.0E+01	5.2E+01	1.0E+02	56%	41%	82%
1.26E+02	15.49		11.78	13.33	2.1E+02		1.6E+02	1.2E+02	-1.6E+02	1.4E+02	97%		113%

Table 69: Estradiol ELISA responses and recovery of homogenate spikes onto sweet potato leaves without dSPE cleanup (Background estimate from Figure 28 in Appendix C.5)

C.5: Phase 2 investigation; determining the impact of dSPE on ELISA response

no-dSPE Sample	dSPE Sample	Caffeine concentration (ng/g) spiked onto homogenate	Caffeine "Recovered" from homogenate (ng/g) no-dSPE	Caffeine Recovered from homogenate (ng/g) with dSPE	Response Difference (ng/g) (no-dSPE less dSPE)
			Fit	Fit	
Sample 1	Sample 2	0	4.3E+01	8.8E+00	3.5E+01
Sample 3	Sample 4	5.01E+01	6.2E+01	3.0E+01	3.2E+01
Sample 5	Sample 6	2.51E+01	5.3E+01	2.1E+01	3.2E+01
Sample 7	Sample 8	5.01E+01	5.4E+01	2.9E+01	2.5E+01
Sample 9	Sample 10	2.51E+01	5.6E+01	2.0E+01	3.6E+01
			Average Response Difference		3.1E+01
			Difference Unspiked Samples		3.5E+01

Table 70: Determining the difference in caffeine ELISA response for extracts without dSPE cleanup vs. those with dSPE

```
> t.test(caff.no.dspe[-1],caff.dspe[-1],paired=TRUE)

        Paired t-test

data:  caff.no.dspe[-1] and caff.dspe[-1]
t = 14.1397, df = 3, p-value = 0.0007663
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 24.2359 38.3141
sample estimates:
mean of the differences
                 31.275
```

Figure 26: Paired t-test run in R, testing null hypothesis that the caffeine ELISA response with dSPE (light grey column Table 70) is equal to the response without dSPE (dark grey column in Table 70); also given 95% confidence intervals for the true difference

Triclosan concentration (ng/g) spiked onto homogenate (no-dSPE)	Triclosan concentration (ng/g) spiked onto homogenate with dSPE	Triclosan "recovered" from homogenate (ng/g) no-dSPE	Normalized Triclosan recovered from homogenate (ng/g) no-dSPE	Triclosan recovered from homogenate (ng/g) with dSPE	Difference (ng/g) (normalized no-dSPE less dSPE)
		Fit	Fit	Fit	
0	0	5.5E+00	5.5E+00	0.00E+00	5.5E+00
2.50E+01	1.26E+01	1.3E+01	6.7E+00	2.5E+00	4.2E+00
2.50E+01	1.26E+01	1.5E+01	7.4E+00	2.3E+00	5.0E+00
2.50E+01	1.26E+01	8.5E+00	4.2E+00	1.2E+00	3.0E+00
2.50E+01	1.26E+01	1.4E+01	7.1E+00	2.5E+00	4.5E+00
			Average Response Difference		4.2E+00
			Difference Unspiked Samples		5.5E+00

Table 71: Determining the difference in triclosan ELISA response for extracts without dSPE cleanup vs. those with dSPE

```
> t.test(tric.no.dspe[-1],tric.dspe[-1])

        Welch Two Sample t-test

data:  tric.no.dspe[-1] and tric.dspe[-1]
t = 5.3168, df = 4.058, p-value = 0.00578
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 2.031041 6.418959
sample estimates:
mean of x mean of y
    6.350     2.125
```

Figure 27: Two sample t-test run in R, testing null hypothesis that the triclosan ELISA response with dSPE (light grey column Table 71) is equal to the response without dSPE (dark grey column in Table 71); also given 95% confidence intervals for the true difference

no-dSPE Sample	dSPE Sample	Estradiol concentration (pg/g) spiked onto homogenate	Estradiol "recovered" from homogenate (pg/g) no-dSPE	Estradiol recovered from homogenate (pg/g) with dSPE	Concentration Difference (pg/g) (no dSPE less dSPE)
			Fit	Fit	
Sample 1	Sample 2	0.00E+00	4.02E+02	0.00E+00	
Sample 3	Sample 4	2.50E+02	2.44E+02	1.5E+02	9.67E+01
Sample 5	Sample 6	2.50E+02	2.29E+02	1.3E+02	9.81E+01
Sample 7	Sample 8	1.26E+02	1.55E+02	1.0E+02	5.39E+01
Sample 9	Sample 10	1.26E+02	2.07E+02	1.1E+02	9.34E+01
			Average Response Difference		8.6E+01
			Difference Unspiked Samples		4.0E+02

Table 72: Determining the difference in estradiol ELISA response for extracts without dSPE cleanup vs. those with dSPE

```
> t.test(e.no.dspe[-1],e.dspe[-1],conf=0.99,paired=TRUE)

        Paired t-test

data:  e.no.dspe[-1] and e.dspe[-1]
t = 7.1414, df = 3, p-value = 0.005653
alternative hypothesis: true difference in means is not equal to 0
99 percent confidence interval:
  15.47927 154.52073
sample estimates:
mean of the differences
                     85
```

Figure 28: Paired t-test run in R, testing null hypothesis that the estradiol ELISA response with dSPE (light grey column Table 72) is equal to the response without dSPE (dark grey column in Table 72); also given 99% confidence intervals for the true difference*

*Mean and 99%CI from t-test put into estradiol Table 69 in Appendix C.4

```
> t.test(e.no.dspe[-1],e.dspe[-1],paired=TRUE)

        Paired t-test

data:  e.no.dspe[-1] and e.dspe[-1]
t = 7.1414, df = 3, p-value = 0.005653
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  47.12131 122.87869
sample estimates:
mean of the differences
                     85
```

Figure 29: Paired t-test run in R, testing null hypothesis that the estradiol ELISA response with dSPE (light grey column Table 72) is equal to the response without dSPE (dark grey column in Table 72); also given 95% confidence intervals for the true difference**

**95% confidence intervals for mean difference reported in main text section 3.2.2.5

From Appendix C.4	From Appendix C.3
no.dsp.recovery	dsp.recovery
Caffeine	
38%	42%
39%	50%
22%	41%
49%	44%
Triclosan	
31%	20%
37%	19%
12%	10%
34%	20%
Estradiol	
63%	59%
58%	52%
56%	80%
97%	90%

Table 73: Collated recovery rates from sweet potato leaf homogenate (with and without dSPE) for all target analytes

```
> t.test(caff.no.dsp.recovery[1:4],caff.dsp.recovery[1:4])

        Welch Two Sample t-test

data:  caff.no.dsp.recovery[1:4] and caff.dsp.recovery[1:4]
t = -1.2155, df = 3.818, p-value = 0.2939
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -0.23876164  0.09526164
sample estimates:
mean of x mean of y
  0.37025   0.44200
```

Figure 30: T-test comparing the percent recovery caffeine dSPE vs. no dSPE (see Table 73)

```
> t.test(tric.no.dsp.recovery[1:4],tric.dsp.recovery[1:4])

        Welch Two Sample t-test

data:  tric.no.dsp.recovery[1:4] and tric.dsp.recovery[1:4]
t = 1.8269, df = 4.064, p-value = 0.1406
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -0.05805736  0.28555736
sample estimates:
mean of x mean of y
  0.28550   0.17175
```

Figure 31: T-test comparing the percent recovery triclosan dSPE vs. no dSPE (see Table 73)

```
> t.test(e.no.dsp.recovery,e.dsp.recovery)

        Welch Two Sample t-test

data:  e.no.dsp.recovery and e.dsp.recovery
t = -0.157, df = 5.963, p-value = 0.8805
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -0.3405625  0.2995625
sample estimates:
mean of x mean of y
   0.6825    0.7030
```

Figure 32: T-test comparing the percent recovery estradiol dSPE vs. no dSPE (see Table 73)

APPENDIX D:

PHASE 3 INVESTIGATION

D.1: Phase 3 investigation; sample designation, preparation, and execution of extractions from matrices

	Homogenate mass (g) of each duplicate sample	Volume (µL) working #5 applied to homogenate-spiked	Volume (mL) of acetonitrile used for extraction
VSO	10.00	75	15
VSA	10.00	75	15
L:G:SO:L Extra	0.82	50	10
L:S:SO:L Extra	0.99	50	10
P:G:SO:P Extra	10.00	50	10
P:S:SO:P Extra	10.00	50	10
	ELISA label given (See Appendix D2)	Extract Preparation	Preparation Done
Virgin Sand Samples (VSO)			
	VSO D	Extract Spiked	(10µL working #5 into 2mL)
	VSO C	Unspiked	
	VSO H	Homogenate Spiked	(75µL working #5 onto 10.00g)
Virgin Sand Samples (VSA)			
	VSA D	Extract Spiked	(10µL working #5 into 2mL)
	VSA C	Unspiked	
	VSA H	Homogenate Spiked	(75µL working #5 onto 10.00g)
L:G:SO:L Extra (Consumed)			
	LGL D	Extract Spiked	(10µL working #5into 2mL)
	LGL C	Unspiked	
	LGL H	Homogenate Spiked	(50µL working #5 onto .82g or "9.65g")
L:S:SO:L Extra (Consumed)			
	LSL D	Extract Spiked	(10µL working #5 into 2mL)
	LSL C	Unspiked	
	LSL H	Homogenate Spiked	(50µL working #5 onto 0.99g or "24.8g")
P:S:SO:P Extra (Consumed)			
	SP D	Extract Spiked	(10µL working #5 into 2mL)
	SP C	Unspiked	
	SP H	Homogenate Spiked	(50µL working #5 onto 10g)
P:G:SO:P Extra (Consumed)			
	GP D	Extract Spiked	(10µL working #5 into 2mL)
	GP C	Unspiked	
	GP H	Homogenate Spiked	(50µL working #5 onto 10g)

Table 74: Phase 3 investigation; sample designation, preparation, and execution of extractions from matrices

D.2: Phase 3 investigation; extract dilutions and designations for ELISA analysis

Input Sample Key	
C =	Unspiked
H =	Homogenate spiked
D =	Extract spiked

ELISA Sample Designation	Input Sample (Refer to Appendix D1 and Key above)	Glassware volume (mL)	Input volume (mL)	Dilution of Original Extract
A1	VSO D	2	0.2	10
A2	A1	2	1	20
A3	A2	2	1	40
A4	A3	2	1	80
B1	LGL D	2	0.2	10
B2	B1	2	1	20
B3	B2	2	1	40
B4	B3	2	1	80
C1	GP D	2	0.2	10
C2	C1	2	1	20
C3	C2	2	1	40
C4	C3	2	1	80
D1	VSO C	2	0.2	10
D2	D1	2	1	20
F1	VSO H	2	0.2	10
F2	F1	2	1	20
H1	VSA C	2	0.2	10
H2	H1	2	1	20
J1	VSA H	2	0.2	10
J2	J1	2	1	20
L1	LGL C	2	0.2	10
L2	L1	2	1	20
N1	LGL H	2	0.2	10
N2	N1	2	1	20
P1	LSL C	2	0.2	10
P2	P1	2	1	20
R1	LSL H	2	0.2	10
R2	R1	2	1	20
T1	GP C	2	0.2	10
T2	T1	2	1	20

Table 75: Phase 3 investigation; extract dilutions and designations for ELISA analysis

ELISA Sample Designation	Input Sample (Refer to Appendix D1 and Key above)	Glassware volume (mL)	Input volume (mL)	Dilution of Original Extract
V1	GP H	2	0.2	10
V2	V1	2	1	20
X1	SP C	2	0.2	10
X2	X1	2	1	20
Z1	SP H	2	0.2	10
Z2	Z1	2	1	20
Previously stored extracts also analyzed (refer to labbook)				
BB1	L:G:SO:S	2	0.2	10
BB2	BB1	2	1	20
BB3	BB2+1µL working #5	2	1	40
CC1	P:G:SO:S	2	0.2	10
CC2	CC1	2	1	20
CC3	CC2+1µL working #5	2	1	40
DD1	P:F:SO:S	2	0.2	10
DD2	DD1	2	1	20
DD3	DD2+1µL working #5	2	1	40
EE1	L:F:SO:L	2	0.2	10
EE2	EE1	2	1	20
EE3	EE2+1µL working #5	2	1	40
FF1	L:F:SO:S	2	0.2	10
FF2	FF1	2	1	20
FF3	FF2+1µL working #5	2	1	40
GG1	L:S:SO:L	2	0.2	10
GG2	GG1	2	1	20
GG3	GG2+1µL working #5	2	1	40
HH1	P:S:SO:S	2	0.2	10
HH2	HH1	2	1	20
HH3	HH2+1µL working #5	2	1	40
LGW1	Working #5	2	1µL	2000
LGW2	LGW1	2	1	4000
LGW3	LGW2	2	1	8000

Table 75 (Continued): Phase 3 investigation; extract dilutions and designations for ELISA analysis

D.3: Phase 3 investigation; ELISA analysis of working solution #5 used to spike onto homogenates and into finished extracts

Sample	Dilution	Expected Caffeine Concenration in Working Solution (Appendix A2) (µg/L)	Caffeine Concentration (µg/L) and 99% CI for Original Working Solution #5			Concentration (µg/L) expected in 2 mL extract with 10µL #5 spiked in (GraphPad)		
			Fit	Upper	Lower	Fit	Upper	Lower
Working Solution # 5 in LGW	2000	5858	4660	6418	3344	23.3	32.1	16.7
Working Solution # 5 in LGW	4000	5858	4655	6375	3542	23.3	31.9	17.7
Working Solution # 5 in LGW	8000	5858	5638	7514	3905	28.2	37.6	19.5
		Average	4985	6769	3597	24.9	33.8	18.0
Average (fit) Caffeine Concentration (µg/L) of working solution used for spiking					4985			
Concentration (µg/L) expected from 10µL spiked into 2mL extracts					24.9		+8.9	-6.9

Table 76: ELISA analysis of caffeine within dilutions of working solution #5; and determining the concentration bump that should be observed upon adding a 10µL spike of #5 into a 2mL sample

```
> t.test(neat.caff)

        One Sample t-test

data:  neat.caff
t = 15.2653, df = 2, p-value = 0.004264
alternative hypothesis: true mean is not equal to 0
95 percent confidence interval:
 17.90567 31.96100
sample estimates:
mean of x
 24.93333
```

Figure 33: Using a one sample t-test in R to determine if the differences observed in the normalized dilutions of working solution #5 (10µL spike in 2mL sample) are statistically significant, and determining the 95% confidence interval of the concentration bump that should be observed upon adding a 10µL spike of #5 into a 2mL sample: Caffeine.

Base Sample	Dilution	Expected Triclosan Concenration in Working Solution (Appendix A2) (µg/L)	Triclsoan Concentration (µg/L) and 99% CI for Original Working Solution #5			Concentration (µg/L) expected in 2 mL extract with 10µL #5 spiked in (GraphPad)		
			Fit	Upper	Lower	Fit	Upper	Lower
Working Solution # 5 in LGW	2000	2956	2656	3439	2038	13.3	17.2	10.2
Working Solution # 5 in LGW	4000	2956	3576	4627	2810	17.9	23.1	14.0
Working Solution # 5 in LGW	8000	2956	3202	3947	2606	16.0	19.7	13.0
		Average	3145	4004	2484	15.7	20.0	12.4
Average (fit) triclosan concentration (µg/L) of working solution used for spiking					3145			
Concentration (µg/L) expected from 10µL spiked into 2mL extracts					15.7		+4.3	-3.3

Table 77: ELISA analysis of triclosan within dilutions of working solution #5; and determining the concentration bump that should be observed upon adding a 10µL spike of #5 into a 2mL sample

```
> t.test(neat.tric)

        One Sample t-test

data:  neat.tric
t = 11.789, df = 2, p-value = 0.007119
alternative hypothesis: true mean is not equal to 0
95 percent confidence interval:
  9.991087 21.475579
sample estimates:
mean of x
 15.73333
```

Figure 34: Using a one sample t-test in R to determine if the differences observed in the normalized dilutions of working solution #5 (10µL spike in 2mL sample) are statistically significant, and determining the 95% confidence interval of the concentration bump that should be observed upon adding a 10µL spike of #5 into a 2mL sample: Triclosan.

Base Sample	Dilution	Expected Estradiol Concenration in Working Solution (Appendix A2) (ng/L)	Estradiol Concentration (µg/L) and 99% CI for Original Working Solution #5			Concentration (µg/L) expected in 2 mL extract with 10µL #5 spiked in (GraphPad)		
			Fit	Upper	Lower	Fit	Upper	Lower
Working Solution # 5 in LGW	2000	28850	High					
Working Solution # 5 in LGW	4000	28850	84116		72146	421		361
Working Solution # 5 in LGW	8000	28850	84407	103211	68967	422	516	345
		Average	84262	103211	70557	421	516	353
Average (fit) estradiol concentration (ng/L) of working solution used for spiking					84262			
Concentration (ng/L) expected from 10µL spiked into 2mL extracts					421		+95	-69

Table 78: ELISA analysis of estradiol within dilutions of working solution #5; and determining the concentration bump that should be observed upon adding a 10µL spike of #5 into a 2mL sample

D.4: Phase 3 investigation; caffeine ELISA analysis of extract-spiked samples and unspiked homogenate extracts

Matrix and sample prepartion	Dilution	Expected caffeine concentration (μg/L) from 10μL spike into 2mL original extract*	Concentration (μg/L) and 99% CI range of original extract		
			Fit	Upper	Lower
Virgin Soil (VSO)					
Extract Spiked	10	24.9 +/-7.0	23.2	32.0	16.7
Extract Spiked	20	24.9 +/-7.0	21.6	29.3	16.3
Extract Spiked	40	24.9 +/-7.0	26.3	35.3	18.0
Extract Spiked	80	24.9 +/-7.0	37.3	53.5	24.1
Unspiked Homogeante Extract	10	0.0	No Detect	No Detect	No Detect
Unspiked Homogeante Extract	20	0.0	No Detect	No Detect	No Detect
		*From Appendix D.3			

Table 79: Caffeine ELISA analysis of extracts of Virgin Soil samples (VSO)

```
> t.test(neat.caff,vso.caff)

        Welch Two Sample t-test

data:  neat.caff and vso.caff
t = -0.5561, df = 4.134, p-value = 0.6069
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -12.847207   8.513874
sample estimates:
mean of x mean of y
 24.93333  27.10000
```

Figure 35: Two sample t-test run in R, testing the null hypothesis that there is no significant difference between the caffeine concentration observed in the VSO extract spikes samples (Table 79) and the expected values based on the analysis of working solution #5 (Table 76)

Matrix and sample prepartion	Dilution	Expected caffeine concentration (μg/L) from 10μL spike into 2mL original extract*	Concentration (μg/L) and 99% CI range of original extract		
			Fit	Upper	Lower
L:G:SO:L (Fresh Extraction)					
Extract Spiked	10	24.9 +/-7.0	27.1	37.5	19.0
Extract Spiked	20	24.9 +/-7.0	25.8	35.8	19.7
Extract Spiked	40	24.9 +/-7.0	35.1	46.6	25.5
Extract Spiked	80	24.9 +/-7.0	35.0	50.9	22.1
Unspiked Homogeante Extract	10	0.0	No Detect	No Detect	No Detect
Unspiked Homogeante Extract	20	0.0	No Detect	No Detect	No Detect
		*From Appendix D.3			

Table 80: Caffeine ELISA analysis of extracts of lettuce eaf samples (grown in soil, irrigate with tap water)

```
> t.test(neat.caff,lgl.caff)

        Welch Two Sample t-test

data:  neat.caff and lgl.caff
t = -1.9495, df = 4.799, p-value = 0.1111
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -13.584029   1.950696
sample estimates:
mean of x mean of y
 24.93333  30.75000
```

Figure 36: Two sample t-test run in R, testing the null hypothesis that there is no significant difference between the caffeine concentration observed in the L:G:SO:L extract spikes samples (Table 80) and the expected values based on the analysis of working solution #5 (Table 76).

Matrix and sample prepartion	Dilution	Expected caffeine concentration (μg/L) from 10μL spike into 2mL original extract	Concentration (μg/L) and 99% CI range of original extract			Concentration (μg/L) and 99% CI range of original extract LESS average unspiked resposne		
			Fit	Upper	Lower	Fit	Upper	Lower
P:G:SO:P (Fresh Extraction)								
Extract Spiked	10	24.9 +/-7.0	28.8	40.6	20.0	24.3	33.5	16.7
Extract Spiked	20	24.9 +/-7.0	31.3	43.9	23.7	26.8	36.8	20.4
Extract Spiked	40	24.9 +/-7.0	44.5	60.5	33.7	39.9	53.4	30.3
Extract Spiked	80	24.9 +/-7.0	34.4	50.3	21.6	29.9	43.2	18.2
Unspiked Homogeante Extract	10	0.0	5.1	7.2	3.4			
Unspiked Homogeante Extract	20	0.0	3.9	7.0	No Detect			
		*From Appendix D.3						

Table 81: Caffeine ELISA analysis of extracts of sweet potato tissue (grown in soil, irrigate with tap water)


```
> neat.caff
[1] 23.3 23.3 28.2   NA
> pgp.caff
[1] 24.3 26.8 39.9 29.9
> t.test(neat.caff,pgp.caff)

        Welch Two Sample t-test

data:  neat.caff and pgp.caff
t = -1.3954, df = 4.196, p-value = 0.2322
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -15.629299   5.045966
sample estimates:
mean of x mean of y
 24.93333  30.22500
```

Figure 37: Two sample t-test run in R, testing the null hypothesis that there is no significant difference between the caffeine concentration observed in the P:G:SO:P extract spikes samples (Table 81) and the expected values based on the analysis of working solution #5 (Table 76).

D.5: Phase 3 investigation; Triclosan ELISA analysis of extract-spiked samples and unspiked homogenate extracts

Matrix and sample prepartion	Dilution	Expected triclosan concentration (μg/L) from 10μL spike into 2mL original extract*	Concentration (μg/L) and 99% CI range of original extract		
			Fit	Upper	Lower
Virgin Soil (VSO)					
Extract Spiked	10	15.7 +/- 5.7	11.9	15.3	9.2
Extract Spiked	20	15.7 +/- 5.7	11.6	14.8	9.5
Extract Spiked	40	15.7 +/- 5.7	14.4	17.6	11.6
Extract Spiked	80	15.7 +/- 5.7	12.7	16.3	9.6
Homogenate Only	10	0.0	No Detect	No Detect	No Detect
Homogenate Only	20	0.0	No Detect	No Detect	No Detect
		*From Appendix D.3			

Table 82: Triclosan ELISA analysis of extracts of Virgin Soil samples (VSO)

```
> t.test(neat.tric,vso.tric)

        Welch Two Sample t-test

data:  neat.tric and vso.tric
t = 2.0906, df = 2.889, p-value = 0.1312
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -1.714244  7.880911
sample estimates:
mean of x mean of y
 15.73333  12.65000
```

Figure 38: Two sample t-test run in R, testing the null hypothesis that there is no significant difference between the triclosan concentration observed in the VSO extract spikes samples (Table 82) and the expected values based on the analysis of working solution #5 (Table 77).

Matrix and sample prepartion	Dilution	Expected triclosan concentration (μg/L) from 10μL spike into 2mL original extract*	Concentration (μg/L) and 99% CI range of original extract		
			Fit	Upper	Lower
L:G:SO:L (Fresh Extraction)					
Extract Spiked	10	15.7 +/- 5.7	12.9	16.7	9.9
Extract Spiked	20	15.7 +/- 5.7	21.7	28.0	16.8
Extract Spiked	40	15.7 +/- 5.7	24.3	31.1	19.7
Extract Spiked	80	15.7 +/- 5.7	17.0	21.3	13.1
Homogenate Only	10	0.0	No Detect	No Detect	No Detect
Homogenate Only	20	0.0	No Detect	No Detect	No Detect
		*From Appendix D.3			

Table 83: Triclosan ELISA analysis of extracts of lettuce leaf samples (grown in soil, irrigate with tap water)

```
> t.test(neat.tric,lgl.tric)

        Welch Two Sample t-test

data:  neat.tric and lgl.tric
t = -1.1346, df = 4.395, p-value = 0.3146
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -10.901214   4.417881
sample estimates:
mean of x mean of y
 15.73333  18.97500
```

Figure 39: Two sample t-test run in R, testing the null hypothesis that there is no significant difference between the triclosan concentration observed in the L:G:SO:L extract spikes samples (Table 83) and the expected values based on the analysis of working solution #5 (Table 77).

Matrix and sample prepartion	Dilution	Expected triclosan concentration (μg/L) from 10μL spike into 2mL original extract*	Concentration (μg/L) and 99% CI range of original extract		
			Fit	Upper	Lower
P:G:SO:P (Fresh Extraction)					
Extract Spiked	10	15.7 +/- 5.7	21.7		16.6
Extract Spiked	20	15.7 +/- 5.7	20.1	26.0	15.7
Extract Spiked	40	15.7 +/- 5.7	22.2	28.1	18.1
Extract Spiked	80	15.7 +/- 5.7	17.3	21.6	13.2
Homogenate Only	10	0.0	No Detect	No Detect	No Detect
Homogenate Only	20	0.0	No Detect	No Detect	No Detect
		*From Appendix D.3			

Table 84: Triclosan ELISA analysis of extracts of sweet potato samples (grown in soil, irrigate with tap water)

```
> t.test(neat.tric,pgp.tric)

        Welch Two Sample t-test

data:  neat.tric and pgp.tric
t = -2.6517, df = 4.322, p-value = 0.05252
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -9.26127859  0.07794526
sample estimates:
mean of x mean of y
 15.73333  20.32500
```

Figure 40: Two sample t-test run in R, testing the null hypothesis that there is no significant difference between the triclosan concentration observed in the P:G:SO:P extract spikes samples (Table 84) and the expected values based on the analysis of working solution #5 (Table 77).

D.6: Phase 3 investigation; estradiol ELISA analysis of extract-spiked samples and unspiked homogenate extracts

Matrix and sample prepartion	Dilution	Expected estradiol concentration (ng/L) from 10μL spike into 2mL original extract*	Concentration (ng/L) and 99% CI range of original extract		
			Fit	Upper	Lower
Virgin Soil (VSO)					
Extract Spiked	10	421 +/- 95	134	159	110
Extract Spiked	20	421 +/- 95	No Detect	No Detect	No Detect
Extract Spiked	40	421 +/- 95	429	524	351
Extract Spiked	80	421 +/- 95	506	638	385
Homogenate Only	10	0.0	No Detect	No Detect	No Detect
Homogenate Only	20	0.0	No Detect	No Detect	No Detect
		*From Appendix D.3			

Table 85: Estradiol ELISA analysis of extracts of Virgin Soil samples (VSO)

```
> t.test(neat.e,vso.e)

        Welch Two Sample t-test

data:  neat.e and vso.e
t = 0.5731, df = 2, p-value = 0.6244
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -422.7865  552.7198
sample estimates:
mean of x mean of y
 421.3000  356.3333
```

Figure 41: Two sample t-test run in R, testing the null hypothesis that there is no significant difference between the estradiol concentration observed in the VSO extract spikes samples (Table 85) and the expected values based on the analysis of working solution #5 (Table 78).

Matrix and sample prepartion	Dilution	Expected estradiol concentration (ng/L) from 10μL spike into 2mL original extract*	Concentration (ng/L) and 99% CI range of original extract		
			Fit	Upper	Lower
L:G:SO:L (Fresh Extraction)					
Extract Spiked	10	421 +/- 95	336		
Extract Spiked	20	421 +/- 95	303	356	251
Extract Spiked	40	421 +/- 95	425	520	348
Extract Spiked	80	421 +/- 95	314	423	
Homogenate Only	10	0.0	300		245
Homogenate Only	20	0.0	297	349	246
		*From Appendix D.3			

Table 86: Estradiol ELISA analysis of extracts of lettuce leaf samples (grown in soil, irrigate with tap water)

```
> t.test(lgl.e.extract.read,lgl.e.homog.read)

        Welch Two Sample t-test

data:  lgl.e.extract.read and lgl.e.homog.read
t = 1.6584, df = 3.018, p-value = 0.1953
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -41.98139 133.98139
sample estimates:
mean of x mean of y
    344.5     298.5
```

Figure 42: Two sample t-test run in R, testing the null hypothesis that there is no significant difference between the estradiol concentration observed in the L:G:SO:L extract spikes samples (Table 86) and the spiked lettuce extracts (Table 86).

Matrix and sample prepartion	Dilution	Expected estradiol concentration (ng/L) from 10μL spike into 2mL original extract*	Concentration (ng/L) and 99% CI range of original extract		
			Fit	Upper	Lower
P:G:SO:P (Fresh Extraction)					
Extract Spiked	10	421 +/- 95	153		127
Extract Spiked	20	421 +/- 95	156	195	125
Extract Spiked	40	421 +/- 95	196	252	
Extract Spiked	80	421 +/- 95	No Detect	No Detect	No Detect
Homogenate Only	10	0.0	84	105	68
Homogenate Only	20	0.0	107	137	70
		*From Appendix D.3			

Table 87: Estradiol ELISA analysis of extracts of sweet potato samples (grown in soil, irrigate with tap water)

```
> t.test(pgp.e.extract.read,pgp.e.homog.read)

        Welch Two Sample t-test

data:  pgp.e.extract.read and pgp.e.homog.read
t = 4.044, df = 2.927, p-value = 0.0285
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  14.70138 130.96529
sample estimates:
mean of x mean of y
 168.3333   95.5000
```

Figure 43: Two sample t-test run in R, testing the null hypothesis that there is no significant difference between the estradiol concentration observed in the P:G:SO:P extract spikes samples (Table 87) and the unspiked sweet potato extracts (Table 87).

D.7: Phase 3 investigation; caffeine ELISA homogenate spike-recovery analysis

Virgin Soil (VSO) matrix		Caffeine Concentration and 99%CI (µg/L) within Original Extract			Mass Recovered (ng) within extract			% Recovery		
mass (ng) spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	10									
0	20									
374	10	7.9	10.5	5.6	119	158	84	32%	42%	23%
374	20	7.8	11.6	4.5	116	175	67	31%	47%	18%

Table 88: Homogenate spike recovery analysis from virgin soil matrix (note blank cells indicate an outside detection response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

Virgin Sand (VSA) matrix		Caffeine Concentration and 99%CI (µg/L) within Original Extract			Mass Recovered (ng) within extract			% Recovery		
ng spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	10									
0	20									
374	10	15.8	22.1	12.0	236	332	179	63%	89%	48%
374	20	18.6	24.7	13.6	279	371	204	75%	99%	55%

Table 89: Homogenate spike recovery analysis from virgin sand matrix (note blank cells indicate an outside detection response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

L:G:SO:L (Lettuce Leaf) matrix		Caffeine Concentration and 99%CI (µg/L) within Original Extract			Mass Recovered (ng) within extract			% Recovery		
mass (ng) spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	10									
0	20									
249	10	4.8	6.9	3.2	48.4	68.8	31.5	19%	28%	13%
249	20	7.2	11.0		71.6	109.6		29%	44%	

Table 90: Homogenate spike recovery analysis from lettuce leaf matrix (note blank cells indicate an outside detection response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

L:S:SO:L (Lettuce Leaf) matrix		Caffeine Concentration and 99%CI (µg/L) within Original Extract			Mass Recovered (ng) within extract			% Recovery		
mass (ng) spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	10									
0	20									
249	10	9.3	12.4	6.8	92.9	123.7	68.1	37%	50%	27%
249	20	10.5	14.7	7.0	105.4	147.2	69.9	42%	59%	28%

Table 91: Homogenate spike recovery analysis from lettuce leaf matrix (note blank cells indicate an outside detecion response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

P:G:SO:P (Sweet Potato) matrix		Caffeine Concentration and 99%CI (µg/L) within Original Extract			Mass Recovered (ng) within extract			% Recovery *			Concentration (ng/g) within potato matrix (% Recovery Adjusted)			Mass (ng) per plant within treatment group		
mass (ng) spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	10	5.1	7.2	3.4	51	72	34				8.8	8.6	8.0	807	795	736
0	20	3.9	7.0		39	70					6.7	8.4		619	776	
249	10	30.5	45.8	21.1	305	458	211	104%	155%	71%						
249	20	27.0	37.6	20.7	270	376	207	90%	122%	69%						
								*(Mass recovered spike-mass recovered unspiked)/mass spiked								

Table 92: Homogenate spike recovery analysis from sweet potato matrix, grown in soil, irrigate with tap water (note blank cells indicate an outside detection response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

P:S:SO:P (Sweet Potato) matrix		Caffeine Concentration and 99%CI (µg/L) within Original Extract			Mass Recovered (ng) within extract			% Recovery *			Concentration (ng/g) within potato matrix (% Recovery Adjusted)			Mass (ng) per plant within treatment group		
mass (ng) spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	10	2.8	4.5		28	45					5.2	6.3		1049	1279	
0	20	4.4	7.6		44	76					8.2	10.6		1662	2150	
249	10	22.2	30.7	16.1	222	307	161	75%	99%	64%						
249	20	29.0	40.6	22.1	290	406	221	102%	139%	89%						
								*(Mass recovered spike-mass recovered unspiked)/mass spiked								

Table 93: Homogenate spike recovery analysis from sweet potato matrix, grown in soil, irrigate with spiked-reclaimed water (note blank cells indicate an outside detecion response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

D.8: Phase 3 investigation; triclosan ELISA homogenate spike-recovery analysis

Virgin Soil (VSO) matrix		Triclosan Concentration and 99%CI (µg/L) within Original Extract			Mass Recovered (ng) within extract			% Recovery		
mass (ng) spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	10									
0	20									
236	10	4.1	5.1	3.4	62	77	51	26%	32%	21%
236	20	4.2	5.2	3.2	63	79	48	27%	33%	20%

Table 94: Homogenate spike recovery analysis from virgin soil (VSO) (note blank cells indicate an outside detection response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

Virgin Sand (VSA) matrix		Triclosan Concentration and 99%CI (µg/L) within Original Extract			Mass Recovered (ng) within extract			% Recovery		
ng spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
	10									
	20									
236	10	5.5	7.0	4.5	83	105	68	35%	45%	29%
236	20	5.4	6.6	4.2	81	99	63	34%	42%	27%

Table 95: Homogenate spike recovery analysis from virgin sand (VSA) (note blank cells indicate an outside detection response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

L:G:SO:L (Lettuce Leaf) matrix		Triclosan Concentration and 99%CI (µg/L) within Original Extract			Mass Recovered (ng) within extract			% Recovery		
mass (ng) spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	10									
0	20									
157	10	6.0	7.7	4.9	60.3	77.1	48.9	38%	49%	31%
157	20	7.3	9.0	5.9	73.2	89.8	59.2	47%	57%	38%

Table 96: Homogenate spike recovery analysis from lettuce leaf matrix, grown in soil, irrigate with tap water (note blank cells indicate an outside detection response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

L:S:SO:L (Lettuce Leaf) matrix		Triclosan Concentration and 99%CI (µg/L) within Original Extract			Mass Recovered (ng) within extract			% Recovery		
mass (ng) spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	10									
0	20									
157	10	5.0	6.3	4.1	50.2	63.1	41.1	32%	40%	26%
157	20	7.1	8.7	5.7	70.9	86.8	57.1	45%	55%	36%

Table 97: Homogenate spike recovery analysis from lettuce leaf matrix, grown in soil, irrigate with spiked reclaimed water (note blank cells indicate an outside detection response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

P:G:SO:P (Sweet Potato) matrix		Triclosan Concentration and 99%CI (µg/L) within Original Extract			Mass Recovered (ng) within extract			% Recovery			Concentration (ng/g) within potato matrix (% Recovery Adjusted)			Mass (ng) per plant within treatment group		
mass (ng) spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	10															
0	20															
157	10	16.2	22.0	12.4	162	220	124	103%	140%	79%						
157	20	13.1	16.8	10.6	131	168	106	83%	107%	67%						
								*(Mass recovered spike-mass recovered unspiked)/mass spiked								

Table 98: Homogenate spike recovery analysis from sweet potato tissue, grown in soil, irrigate with tap water (note blank cells indicate an outside detection response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

P:S:SO:P (Sweet Potato) matrix		Triclosan Concentration and 99%CI (µg/L) within Original Extract			Mass Recovered (ng) within extract			% Recovery *			Concentration (ng/g) within matrix (% Recovery Adjusted)			Mass (ng) per plant within treatment group		
mass (ng) spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	10	2.2	2.7	1.7	22	27	17				3.9	3.8	3.3	783	772	674
0	20	2.1	2.8	1.7	21	28	17				3.8	3.9	3.3	760	797	659
157	10	17.4		13.3	174		133	97%		85%						
157	20	16.7	21.6	13.2	167	216	132	93%	120%	84%						
								*(Mass recovered spike-mass recovered unspiked)/mass spiked								

Table 99: Homogenate spike recovery analysis from sweet potato tissue, grown in soil, irrigate with spiked reclaimed water (note blank cells indicate an outside detection response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

D.9: Phase 3 investigation; estradiol ELISA homogenate spike-recovery analysis

Virgin Soil (VSO) matrix		Estradiol Concentration (ng/L) and 99%CI Original			Mass Recovered (pg) within extract			% Recovery		
pg spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	10									
0	20									
6320	10	234.2		200.5	3512		3008	56%		48%
6320	20									

Table 100: Homogenate spike recovery analysis from Virgin Soil (VSO) (note blank cells indicate an outside detection response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

Virgin Sand (VSA) matrix		Estradiol Concentration (ng/L) and 99%CI Original Extract			Mass Recovered (pg) within extract			% Recovery		
pg spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
	10									
	20									
6320	10	64	81	49	959	1209	733	15%	19%	12%
6320	20	130	164	100	1953	2461	1506	31%	39%	24%

Table 101: Homogenate spike recovery analysis from Virgin Sand (VSA) (note blank cells indicate an outside detection response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

L:G:SO:L (Lettuce Leaf) matrix		Estradiol Concentration (ng/L) and 99%CI Original Extract			Mass Recovered (pg) within extract			% Recovery		
pg spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
	10	300		245	3005		2449			
	20	297	349	246	2967	3492	2457			
4213	10	268		225	2676		2247	-7%	-83%	-5%
4213	20	280	331	231	2797	3310	2306	-4%	-4%	-3%

Table 102: Homogenate spike recovery analysis from lettuce leaf, grown in soil, irrigate with tap water (note blank cells indicate an outside detection response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

L:S:SO:L (Lettuce Leaf) matrix		Estradiol Concentration (ng/L) and 99%CI Original Extract			Mass Recovered (pg) within extract			% Recovery		
pg spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
	10	56	71	39	563	714	391			
	20	93	120		928	1204				
4213	10	92	114	75	916	1135	747	4%	4%	8%
4213	20	118	149	85	1176	1488	852	10%	13%	11%

Table 103: Homogenate spike recovery analysis from lettuce leaf, grown in soil, irrigate with spiked-reclaimed water (note blank cells indicate an outside detection response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

P:G:SO:P (Sweet Potato) matrix		Estradiol Concentration (ng/L) and 99%CI Original Extract			Mass Recovered (pg) within original extract			% Recovery		
pg spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	10	84	105	68	840	1047	682			
0	20	107	137	70	1072	1365	697			
4213	10	168	197	141	1681	1973	1410	17%	18%	17%
4213	20	190	235	155	1903	2351	1553	22%	27%	20%

Table 104: Homogenate spike recovery analysis from sweet potato matrix, grown in soil, irrigate with tap water (note blank cells indicate an outside detection response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

P:S:SO:P (Sweet Potato) matrix		Estradiol Concentration (ng/L) and 99%CI Original Extract			Mass Recovered (pg) within original extract			% Recovery		
pg spiked onto homogenate	Dilution	Fit	Upper	Lower	Fit	Upper	Lower	Fit	Upper	Lower
0	10	108	131	88	1075	1312	879			
0	20	99	127		988	1270				
4213	10	145	171	119	1446	1706	1195	10%	10%	7%
4213	20	162	203	131	1622	2027	1314	14%	17%	10%

Table 105: Homogenate spike recovery analysis from sweet potato matrix, grown in soil, irrigate with spiked-reclaimed water (note blank cells indicate an outside detection response from the ELISA or that the GraphPad algorithm could not create an upper/lower 99% confidence interval range value)

D.10: Phase 3 investigation; caffeine ELISA analysis of stored extracts

ELISA Sample Designation	Fall Extract Designation*	Dilution	Expected Concentration (µg/L) from spike delivered	Original Extract Concentration (µg/L) and 99%CI Range			% expected response of extract-spiked (fit) above unspiked
				Fit	Upper	Lower	
BB1	L:G:SO:S	10					
BB2		20					
BB3	+ 1µL #5	10	24.9 +/-7.0	17.9	25.0	13.4	72%
CC1	P:G:SO:S	10					
CC2		20					
CC3	+ 1µL #5	10	24.9 +/-7.0	19.3	26.9	14.3	78%
DD1	P:F:SO:S	10					
DD2		20					
DD3	+ 1µL #5	10	24.9 +/-7.0	21.1	29.2	15.4	85%
EE1	L:F:SO:L	10		2.0	3.5		
EE2		20					
EE3	+ 1µL #5	10	24.9 +/-7.0	17.9	25.1	13.4	72%
FF1	L:F:SO:S	10					
FF2		20					
FF3	+ 1µL #5	10	24.9 +/-7.0	20.7	28.8	15.2	83%
GG1	L:S:SO:L	10					
GG2		20					
GG3	+ 1µL #5	10	24.9 +/-7.0	18.5	25.9	13.8	74%
HH1	P:S:SO:S	10					
HH2		20					
HH3	+ 1µL #5	10	24.9 +/-7.0	14.6	20.4	11.1	58%
	*Note: All "+ 1µL #5" are extracts dilute 10 fold (200µL in 2mL) into which 1µL working #5 was spiked in after the dilution						

Table 106: Phase 3 investigation; caffeine ELISA analysis of stored extracts

D.11: Phase 3 investigation; triclosan ELISA analysis of stored extracts

ELISA Sample Designation	Fall Extract Designation*	Dilution	Expected Concentration (µg/L) from spike delivered	Original Extract Concentration (µg/L) and 99%CI Range			% expected response of extract-spiked (fit) above unspiked
				Fit	Upper	Lower	
BB1	L:G:SO:S	10					
BB2		20					
BB3	+ 1µL #5	10	15.7 +/- 5.7	9.2	11.9	7.2	58%
CC1	P:G:SO:S	10		0.7	0.9		
CC2		20					
CC3	+ 1µL #5	10	15.7 +/- 5.7	11.2	14.4	8.7	71%
DD1	P:F:SO:S	10					
DD2		20					
DD3	+ 1µL #5	10	15.7 +/- 5.7	8.2	10.6	6.5	52%
EE1	L:F:SO:L	10					
EE2		20					
EE3	+ 1µL #5	10	15.7 +/- 5.7	8.6	11.1	6.8	55%
FF1	L:F:SO:S	10					
FF2		20					
FF3	+ 1µL #5	10	15.7 +/- 5.7	10.0	12.9	7.8	64%
GG1	L:S:SO:L	10					
GG2		20					
GG3	+ 1µL #5	10	15.7 +/- 5.7	9.0	11.7	7.1	57%
HH1	P:S:SO:S	10		4.4	5.4	3.6	
HH2		20		4.6	5.7	3.6	
HH3	+ 1µL #5	10	15.7 +/- 5.7	15.2	20.2	11.7	68%
	*Note: All "+ 1µL #5" are extracts dilute 10 fold (200µL in 2mL) into which 1µL working #5 was spiked in after the dilution						

Table 107: Phase 3 investigation; triclosan ELISA analysis of stored extracts

D.12: Phase 3 investigation; estradiol ELISA analysis of stored extracts

ELISA Sample Designation	Fall Extract Designation*	Dilution	Expected Concentration (ng/L) from spike delivered	Original Extract Concentration (ng/L) and 99%CI Range			% expected response of extract-spiked (fit) above unspiked
				Fit	Upper	Lower	
BB1	L:G:SO:S	10					
BB2		20					
BB3	+ 1μL #5	10	421	129	154	106	31%
CC1	P:G:SO:S	10					
CC2		20					
CC3	+ 1μL #5	10	421	189	227	160	45%
DD1	P:F:SO:S	10					
DD2		20		57	86		
DD3	+ 1μL #5	10	421	150	177	124	36%
EE1	L:F:SO:L	10		90	112	73	
EE2		20		133	168	104	
EE3	+ 1μL #5	10	421	135	160	111	11%
FF1	L:F:SO:S	10		42	55		
FF2		20					
FF3	+ 1μL #5	10	421	125	150	102	20%
GG1	L:S:SO:L	10		58	73	41	
GG2		20		90	117		
GG3	+ 1μL #5	10	421	123	148	101	15%
HH1	P:S:SO:S	10					Above Detection
HH2		20		454		390	
HH3	+ 1μL #5	10	421				Above Detection
	*Note: All "+ 1μL #5" are extracts dilute 10 fold (200μL in 2mL) into which 1μL working #5 was spiked in after the dilution						

Table 108: Phase 3 investigation; estradiol ELISA analysis of stored extracts

APPENDIX E:

ANALYSIS OF RESERVOIRS

Date Tested	Date Collected	Caffeine (µg/L)	Triclosan (µg/L)	Estradiol (ng/L)
26-Jan	4-Nov	-	-	NT
26-Jan	1-Dec	-	0.162	NT
8-Aug	21-Jul	0.315	0.108	-
8-Aug	29-Jul	0.302	0.091	-
8-Aug	5-Aug	-	0.078	-
13-Jul	11-Jul	-	0.095	-
6-Jun	5-Jun	NT	NT	NT
Estimate Average Concentration		1.0E-01	8.9E-02	0.0E+00
Estimate Mass Delivered to Sweet Potatoes (35.1L)		3.6E+00	3.1E+00	0.0E+00
Estimate Mass Delivered to Lettuce (8.3L)		8.5E-01	7.4E-01	0.0E+00

Key	
#	Sample tested exceeded the upper limit of quantitation
-	Tested but below below the LOD
NT	Not tested

Table 109: Concentration of target analytes observed in tap water reservoir

Date Tested	Date Collected	Caffeine (µg/L)	Triclosan (µg/L)	Estradiol (ng/L)
26-Jan	4-Nov	-	0.73	NT
26-Jan	1-Dec	-	0.8	NT
8-Aug	21-Jul	0.375	0.064	2.7
8-Aug	29-Jul	0.748	0.107	-
8-Aug	5-Aug	1.28	2.5	25
13-Jul	11-Jul	-	0.56	-
6-Jun	5-Jun	1	0.85	NT
Estimate Average Concentration		4.9E-01	8.0E-01	6.9E+00
Estimate Mass Delivered to Sweet Potatoes (35.1L)		1.7E+01	2.8E+01	2.4E+02
Estimate Mass Delivered to Lettuce (8.3L)		4.0E+00	6.7E+00	5.7E+01

Table 110: Concentration of target analytes observed in reclaimed water reservoir

Date Tested	Date Collected	Caffeine (µg/L)	Triclosan (µg/L)	Estradiol (ng/L)
26-Jan	4-Nov	-	9.24	NT
26-Jan	1-Dec	-	3.36	NT
8-Aug	21-Jul	7.07	0.646	-
8-Aug	29-Jul	6.568	0.417	-
8-Aug	5-Aug	-	5.66	3100
13-Jul	11-Jul	5	1.06	25
6-Jun	5-Jun	11	3	NT
Estimate Average Concentration		4.2E+00	3.3E+00	7.8E+02
Estimate Mass Delivered to Sweet Potatoes (35.1L)		1.5E+02	1.2E+02	2.7E+04
Estimate Mass Delivered to Lettuce (8.3L)		3.5E+01	2.8E+01	6.5E+03

Table 111: Concentration of target analytes observed in spiked-reclaimed water reservoir

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