UPTAKE, ACCUMULATION AND METABOLISM OF CHEMICALS OF EMERGING CONCERN IN VEGETABLES

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ABSTRACT

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Pharmaceuticals have been most commonly used as medicine to treat human and animal diseases, and as animal feed supplements to promote growth. These applications have rendered the ubiquitous presence of pharmaceuticals in animal excretions and their discharges from wastewater treatment plants (WWTPs). Land application of animal manures and biosolids from WWTPs and crop irrigation with reclaimed water result in the dissemination of these pharmaceuticals in agricultural soils and waters. Crops and vegetables could take up pharmaceuticals from soil and water, leading to the accumulation of trace-level pharmaceuticals in fresh produce. The pharmaceutical concentrations in crops and vegetables are much lower than the dosage for effective therapy. However, the impacts of long-term consumption of pharmaceutical-tainted crops/vegetables to human and animal health remain nearly unknown. Currently, the mechanism of plant uptake of pharmaceuticals from soil and water is not clear, which impedes the development of effective measures to mitigate contamination of food crops by pharmaceuticals. We hypothesize that water flow is the primary carrier for pharmaceuticals to enter plants, and plant physiological characteristics, pharmaceutical physicochemical properties as well as plant-pharmaceutical interactions (e.g., sorption affinity) collectively influence pharmaceutical accumulation and transport in plants. In this work, a sensitive and effective extraction method was first developed to quantify the uptake of thirteen pharmaceuticals by lettuce (*Lactuca sativa*) from water. The results indicated that small-sized pharmaceuticals with molecular weight (MW) < 300 g mol^{-1} could enter lettuce, and those pharmaceuticals with low affinity to lettuce roots such as caffeine and

carbamazepine could substantially transport to shoots. A strong positive linear relation was observed between their mass accumulation in shoots and the amount of transpiration water. Lamotrigine and trimethoprim are also small-sized pharmaceuticals; however, their relatively strong affinity to lettuce roots mitigated the amount of transfer to shoots. Large-sized pharmaceuticals (MW > 400 g mol^{-1}) such as lincomycin, monensin sodium, oxytetracycline and tylosin were primarily accumulated in lettuce roots with limited amount to transfer to shoots. The results of mass balance showed that acetaminophen, β-estradiol, carbadox, estrone, and triclosan were readily metabolized in lettuce with ≥ 90% loss during 144-h exposure period. A workflow for identification of non-targeted metabolites was developed using liquid chromatography coupled to hybrid triple quadrupole-linear ion trap mass spectrometer, and this method was applied to investigate the metabolism of caffeine in lettuce. The results revealed that caffeine underwent metabolism in lettuce with > 50% loss during 144-h exposure period. The major metabolism reaction was demethylation forming the products of losing one, two or three methyl functional groups, and these products accounted for 20% of the initial dosage of caffeine. Caffeine also underwent oxidation and hydroxylation reactions in lettuce. Overall, this study sheds new light to uptake pathways and transport characteristics of pharmaceuticals in vegetables (e.g. lettuce). The mechanistic insights obtained could help form a framework for exposure modelling of diverse pharmaceutical compounds, and facilitate the development of scientifically informed management strategies to mitigate pharmaceutical accumulation in agricultural food produce.

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LITERATURE REVIEW

Pharmaceutical Production

The increasing global human population has resulted in increased production of pharmaceuticals for human and veterinary disease prevention/treatment, and as livestock feed supplements to ensure continuously sufficient food supply in the world.[1] In the Early 1990s, in Germany, around 2900 human drugs were approved for use.[2] According to the annual report questionnaire, the estimated number of drug users (aged 15-64) increased from 208 to 255 millions during the period of 2006-2015. In the United States, each year approximately two-thirds of 36 millions of beef cattle received growth promoters in order to meet the needs of meat production.[3] Moreover, in the summary report from U.S. Food and Drug Administration, the amount of actively marketed antimicrobial drugs, approved for use in food-producing animals, increased from 12.8 to 15.6 million kilograms between 2009-2015.[4, 5] The annual consumption of tetracyclines, a class of frequently used antibiotics, could be as much as 658 tons in European countries.[6] Overall, substantial amounts of pharmaceuticals in production, trade, and consumption have been executed during the past century and expected to continue to increase in the coming years.

Pharmaceutical Release to the Environment

The long-time consumption of vast amount of pharmaceuticals by humans and animals serves as the continuous source for dissemination of these bioactive contaminants in territorial and water systems, which leads to adverse impacts to ecosystem service and human health.[2, 7-10] Pharmaceutical residues have been frequently found in farmland soils, meats, non-target aquatic organisms and aquatic environments.[2, 7-10] The ubiquity of pharmaceuticals (now considered as chemicals of emerging concern) in the environment originated primarily from the direct excretion of livestock, land application of biosolids (sewage sludge) and discharge of wastewater effluents

from municipal wastewater treatment plants (WWTPs).[9, 11-17] In one study, tetracyclines and sulfadiazine were detected in swine manures in eastern parts of China with the concentrations up to 139.4 and 7.1 mg kg^{-1},[18] and tetracyclines were up to 46 mg kg^{-1} in Austria.[19] In the influents of municipal WWTPs, pharmaceuticals were frequently detected, which could originate from improper disposals of unused or expired medications by flushing in toilet or sink,[15, 16] and from excreta from humans through taking or injecting medicines.[17] Most currently operating procedures in WWTPs are not specifically designed to remove the pharmaceuticals from the influents, therefore, certain portions of pharmaceuticals could remain in sewage sludge and in the treated effluents.[2, 20-22] Carbamazepine, an anticonvulsant, was most commonly found in the treated wastewater with concentrations up to 6.3 μg L^{-1}.[2, 23, 24] Antidiabetic II medicine metformin was also commonly detected in treated wastewater with concentration range of 2.2 to 21 μg L^{-1}.[25, 26] Nonsteroidal anti-inflammatory drugs such as diclofenac and ibuprofen were detected in the land-applied biosolids with the concentrations of 22 and 217 μg kg^{-1}.[27] Each year, approximately 8 to 11 million dry tons of biosolids are generated in the European Union and the United States, and over 2 million tons of sewage sludge are produced in Japan.[28-30] Land application of biosolids to agricultural lands is the major practice for their fertility values to agricultural crops, and as a convenient approach to disposal as well. This has been approved as the primary pathway to disseminate pharmaceuticals in the environment.

Climate change, urbanization and exponential growth of human populations cause water scarcity at the global scale, which increases drought events and fresh water demands in many arid and semi-arid regions.[22, 31, 32] Approximately 20% of the populations live with limited water resources, and over 50% of the populations are predicted to confront with water shortage by 2025.[20, 33] An average of 70% freshwater are used for agricultural production in the world; in some

arid regions such as Egypt, up to 98% of the available freshwater is required for irrigation. The rest of fresh water is used for industrial manufacturing and domestic purposes.[34, 35] In many regions, crop irrigation with reclaimed water to croplands become common practice in order to increase water use efficiency and alleviate the pressure of water shortage.[21, 28-30] However, irrigation with reclaimed water for crop production could result in the uptake of pharmaceuticals by crops and vegetables.[21, 36, 37] Plant uptake of pharmaceuticals originating from irrigation with reclaimed water has been documented in many previous studies. For example, the accumulation of carbamazepine in cucumber was measured at around 20.4 μg kg^{-1} in leaves and 1.0 μg kg^{-1} in fruits after irrigation with the reclaimed water containing 2.99 μg L^{-1} of carbamazepine. Apple trees and alfalfa were also found to have caffeine residues (15.5 and 13.9 μg kg^{-1}) resulting from the irrigation with reclaimed water (0.24 μg L^{-1} of caffeine).[24, 36]

In general, the detected pharmaceutical concentrations in edible vegetables are at the levels of parts per trillion to parts per billion. The consumption of these vegetables poses very low or no risks associated with these pharmaceuticals on the basis of the suggested dosage for medical treatment purpose.[38, 31, 39, 40] However, the potentials of synergistic effects from a mixture of pharmaceuticals under long-term exposure and the specific toxic effects are unclear, thereby not included in the risk assessment.[22, 24, 41]

Analytical Methods for Pharmaceutical Residues in Vegetables

A better understanding of plant uptake of pharmaceuticals could provide information necessary for estimating the magnitude and distribution of pharmaceuticals in crops and vegetables for risk assessment. It is a prerequisite to develop an accurate and efficient analytical method to qualify and quantify the pharmaceutical residues in plants. To analyze pharmaceuticals in the complex plant matrices, pharmaceutical extraction, cleanup procedure, and instrumental analysis

are the essential three steps during the overall process. Generally, extraction methods include solid-liquid extraction (SLE), ultrasonic liquid extraction (ULE), Soxhlet extraction (SE), and accelerated solvent extraction (ASE) (or pressurized liquid extraction).[31, 42, 43] The common cleanup procedures are liquid-liquid extraction (LLE), solid-phase extraction (SPE), and gel permeation chromatography (GPC), which intends to minimize the impacts of plant matrices prior to instrumental analysis.[42] Gas or liquid chromatography coupled to tandem mass spectrometer (GC-MS/MS or LC-MS/MS) is the most common instrument used to analyze pharmaceutical residues in plants.[44, 45] These analytical procedures manifested varying extraction efficiencies of pharmaceuticals from vegetables. For example, SE coupled to GPC or hydrophilic–lipophilic balanced (HLB) SPE cleanup step revealed good extraction recoveries for most of the 10 studied pharmaceuticals from carrot and lettuce, but low and reproducible extraction recoveries for some studied pharmaceuticals.[46] The ASE combined with the cleanup using Biotage Evolute ABN cartridges could achieve the extraction efficiencies of > 87% for 5 pharmaceuticals from soybean.[21] The SLE using different organic solvents combined with the cleanup step of LLE and HLB SPE achieved the extraction efficiencies from 42.8% to 96.9% for 13 pharmaceuticals from pea and cucumber.[30] The ULE followed with SPE cleanup achieved the extraction efficiencies ranging from 87.1% to 123.5% for 16 pharmaceuticals and 3 personal care products from lettuce.[47] Although these procedures had received reasonable success in extracting pharmaceuticals from plants, it still remains a challenge to develop extraction method to achieve high extraction efficiency, low matrix effect, versatility to pharmaceuticals with different chemical properties, and reasonable analytical time.

Recently, a quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction technique has become popular for extracting organic analytes from various matrices.[48-52] The QuEChERS

method was originally designed to extract pesticide residues from fruits and vegetables using small amount of organic solvents in the presence of salts that enhance the extraction efficiency because of the salting-out effects and to remove residual water. To reduce the plant matrix effect, the cleanup step is performed using dispersive solid phase extraction (d-SPE) sorbents consisting of $MgSO_4$, primary-secondary amine (PSA), C18 and graphitized carbon black (GCB). These are the most common ingredients and targeted the removal of slightly polar and nonpolar components in the matrices, e.g., PSA and C18 used for the removal of lipids, chlorophyll, pigments, sterols, and GCB for the effective removal of planar-structured compounds.[50, 53] This QuEChERS extract method could recover 85% to 101% of > 20 pesticides from lettuce and strawberry with the standard deviations of < 5%.[48] It has been accepted as a standard extraction method by Association of Official Analytical Chemists (AOAC) in 2007, and approved for determination of pesticide residues in food by European Committee for Standardization in 2008.[54, 55] Currently, the QuEChERS method has been extended to determine pharmaceuticals in various matrices such as in blood (with limit of detection < 20 ng mL^{-1}),[49] as well as in sewage sludge, eggs, meats, fish.[51, 52, 56, 57] Although the QuEChERS extraction method has been applied to pharmaceutical analysis in many dimensions; however, only few studies focused on extraction from vegetables and crops [28]. Based on the success in applying this method in other studies, it is reasonable to assume that the QuEChERS method could be adapted and optimized for analysis of pharmaceuticals in plants, which is essential for the next-step study of elucidating the mechanism of uptake and accumulation of pharmaceuticals in plants.

Mechanism of Plant Uptake of Pharmaceuticals

The accumulation of organic compounds and pesticides in plants is commonly described by bioconcentration factor (BCF) defined as the ratio of concentration in plant to that in the

surrounding media. Briggs et al. (1982) studied uptake of non-ionized pesticides by barley, and found that root concentration factor was linearly related to octanol-water partitioning coefficient (log K_{ow}) of these pesticides. The extend of transport from roots to shoots described as transpiration stream concentration factor demonstrated as a Gaussian-curve relationship as a function of log K_{ow}.[58] Burken and Schnoor (1998) also found the similar relationship for the uptake and translocation of twelve organic contaminants in hybrid poplar trees.[59] Most pharmaceuticals are characterized as hydrophilic nature containing multiple polar and/or ionic functional groups. The root uptake and subsequent transport within plants was reported to relate with their lipophilicity (D_{ow}, K_{ow} adjusted to neutral species at a given pH),[30, 31, 60-62] which generally agree with the previously proposed partition model of describing uptake of lipophilic compounds.[21, 30, 31, 63, 64] In these previous studies a positive relationship was found between the logarithm of root concentration factor (RCF, the ratio of pharmaceutical concentration in roots to growing media) and log D_{ow}, and a negative relationship observed between the logarithm of translocation factor (TF) that is defined as the ratio of pharmaceutical concentration in aerial parts to that in roots, and log D_{ow}.[21, 28, 30, 31] However, these results from different experimental settings (including plant species, hydroponic or pot studies, applied pharmaceutical concentration, and exposure periods) revealed that for a given pharmaceutical the RCF or TF values could vary up to two orders of magnitude, and no apparent relationship between RCF (or TF) and log D_{ow} was reported in several literature reviews.[44, 45] Pharmaceutical speciation (e.g. neutral vs. ionic fractions) is also taken into consideration for the accumulation in plants.[31, 32, 37, 40, 65] Ionized pharmaceutical species manifest less uptake than the neutral pharmaceutical species, probably their difficulty to penetrate the hydrophobic plasma membranes.[30, 65] Anionic pharmaceuticals are repelled from cell membranes because the negative electrical potentials on cell membranes repel the approximation of negatively

charged pharmaceutical species.[31, 32] Cationic pharmaceuticals could be adsorbed on the negatively charged sites derived from the head groups (phosphate) of phospholipids on cell membrane.[66] As a result, most of these pharmaceuticals could be retained on roots limiting their transport to stems and leaves.[31, 44] In addition, the fluids in plant tissues vary in pH, which could cause the shifts of pharmaceutical species, hence impacting their accumulation and transport. In tomato, the pH value is 5.5 between cellular space and cell wall, 7.2 in cytosol, and 5.5 in vacuole.[67] Lamotrigine (an organic base with p*K*a 5.7) is predominant as neutral form in cytosol, and presents as cationic and neutral forms in vacuoles. The formed cationic lamotrigine in vacuole could be trapped in tonoplast, and cannot penetrate back to cytosol from tonoplast.[31, 40, 44, 65]

Water movement in plants (e.g., transpiration stream) could influence the accumulation and transport of pharmaceuticals in plants.[31, 40, 65, 68] Water enter plant roots via apoplast, symplast and transcellular/transmembrane.[44, 67, 69] Apoplast pathway refers to water movement through cell wall and intercellular space. Symplast pathway refers to water movement between cells through narrow thread of plasmodesmata. The transcellular or transmembrane pathway refers to water cross cell membrane by osmosis or through aquaporin. In general, water in plant roots could penetrate the hydrophobic Casparian strip at endodermis via symplast (or also transmembrane) pathway, and enter xylem where water moves upward to shoots. It is still unclear how transpiration stream influences pharmaceutical transport in plants.[14, 24, 68] The movement of water in the plant-atmosphere continuum is driven by water potential. The difference of vapor pressure between the atmosphere (low water vapor pressure) and air space in leaves (high water pressure) leads the release water vapor from leaves into the atmosphere through the stomata.[66, 69] Increase in transpiration rate could lead to more accumulation of organic contaminants in plants. For example, higher accumulation of 2,2-bis(chlorophenyl)-1,1,1-trichloroethane (DDT) was found in the

shoots of pumpkin and zucchini than that in alfalfa, rye grass, and tall fescue, which was assumed to be caused by the larger-sized pumpkin and zucchini leaves (i.e., greater transpiration).[70] The transport of bromacil to soybean shoots increased with transpiration rate.[71] Dodgen *et al.* revealed that more pharmaceuticals were transported to carrot, lettuce, and tomato growing in warm and dry environments than that in cool and humid conditions, which could be attributed to the lower transpiration rates in the latter experimental settings.[68] Because plant transpiration rates are distinct in different regions (arid vs. humid regions), the amount of pharmaceuticals accumulated in plants could be also different at different weather conditions. Hence, understanding the effect of transpiration stream on plant uptake of pharmaceuticals could be beneficial for predicting models of accumulation and transport of pharmaceuticals in plants. Besides, the uptake of pharmaceuticals from roots to shoots moves with water flow via the routes of the three water-moving pathways in which pharmaceuticals could interact directly with root constituents (e.g., cellulose, hemicellulose, pectin, proteins, and lignin). This interaction could reduce the movement of pharmaceuticals in plants, but no sufficient data are currently available.

Pharmaceutical Metabolism in Plants

Pharmaceuticals enter plants result primarily from the practices of land application of biosolids and crop irrigation with reclaimed water in agricultural fields. After being taken up, pharmaceuticals could be metabolized in plants,[72, 73] which commonly could be deduced from the pharmaceutical mass discrepancy between input and output, or more directly by identification and measurement of their metabolites.[30, 65, 74, 75] It was documented that in pea, around 10%−90% mass was lost for more than a dozen of pharmaceuticals within 24 hours.[30 74] Two major carbamazepine metabolites 10,11-epoxide-carbamazepine and 10,11-dihydro-10,11-dihydroxy-carbamazepine were commonly determined in tomato and cucumber, which accounted for > 40% of the initially

added carbamazepine in the vegetables.[65] Acetaminophen could conjugate with glutathione and glycoside in Indian mustard with an increase of enzyme activity (glutathione S transferases) in leaf at higher acetaminophen concentration.[74] The metabolites of pharmaceuticals could have equivalent or even higher potency than parent compounds.[76, 77] For instance, 10,11-epoxide-carbamazepine demonstrated equivalent anticonvulsant and neurotoxic effects to carbamazepine.[78] Therefore, identifying and quantifying metabolites of pharmaceuticals in plants is necessary for the assessment of risks to human health. To identify pharmaceutical metabolites, liquid chromatography coupled with hybrid mass spectrometry (e.g., triple quadrupole-QQQ, Orbitrap, linear ion trap or time-of-flight) are currently essential tools for analyzing plant and environmental samples.[79-83] However, the knowledge on pharmaceutical metabolites in plants is very limited, which could be due to the complex plant matrices impeding instrumental analysis for metabolites, and lack of an effective and efficient approaches, sufficient instrumental analysis, and data processing for metabolites.[84, 85]

RESEARCH OBJECTIVES AND HYPOTHESES

The research objectives of this study are to elucidate the mechanism of plant uptake of pharmaceuticals, to examine the relationship between transpiration rate and pharmaceutical accumulation and upward transport in lettuce, and to identify pharmaceutical metabolites in plants. We hypothesize that (1) water flow is the primary carrier for pharmaceuticals to enter plants, and facilitates their distributions to different plant parts, (2) plant physiological characteristics, pharmaceutical physicochemical properties, and the interaction of pharmaceuticals with plant roots could collectively influence pharmaceutical accumulation and translocation in plants, (3) increases in transpiration rates could lead to more uptake and transport of pharmaceuticals in plants, and (4) pharmaceuticals in plants could be metabolized. To test these hypotheses, the uptake of a wide

array of pharmaceuticals in lettuce was measured using hydroponic experimental settings. Pharmaceutical sorption by lettuce roots was also determined to evaluate its influence on pharmaceutical transport from roots to shoots. Lettuce transpiration rate was alleviated via supplementing plant hormone abscisic acid (ABA) in hydroponic solution to reduce the stomatal conductance in lettuce leaves. Metabolites of caffeine in lettuce were identified using LC-QTrap-MS/MS under the mode of enhanced mass scan with information dependent acquisition criteria followed by enhanced product ion (EMS-IDA-EPI) scan in the analysis.

The results from this study include the relation of pharmaceutical accumulation in lettuce with plant physiological characteristics, pharmaceutical physicochemical properties, and pharmaceutical affinity to plant roots provide innovative mechanistic insights into pharmaceutical uptake and movement in plants. The relationship between transpiration rates and pharmaceutical accumulation in plant tissues could be important to predictive model for pharmaceutical uptake and transport in plants. The established framework for identification of caffeine metabolites in plants including kinetic study and LC-QTrap-MS/MS analysis could be extended to other types of pharmaceuticals and agrochemicals for metabolism studies in agricultural crops and vegetables. The results obtained from this study could provide information necessary for the risk assessment of food safety and human health related to dietary consumption of pharmaceutical-tainted agricultural fresh products.

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CHAPTER II

COMPARISON OF ACCELERATED SOLVENT EXTRACTION AND QUICK, EASY, CHEAP, EFFECTIVE, RUGGED AND SAFE METHOD FOR EXTRACTION AND DETERMINATION OF PHARMACEUTICALS IN VEGETABLES

ABSTRACT

Land application of biosolids and irrigation with reclaimed water in agricultural production could result in accumulation of pharmaceuticals in vegetable produce. To better assess the potential human health impact from long-term consumption of pharmaceutical-contaminated vegetables, it is important to accurately quantify the amount of pharmaceuticals accumulated in vegetables. In this study, a quick, easy, cheap, effective, rugged and safe (QuEChERS) method was developed and optimized to extract multiple classes of pharmaceuticals from vegetables, which were subsequently quantified by liquid chromatography coupled to tandem mass spectrometry. For the eleven target pharmaceuticals in celery and lettuce, the extraction recovery of the QuEChERS method ranged from 70.1 to 118.6% with relative standard deviation < 20%, and the method detection limit was achieved at the levels of ng g^{-1}. The results revealed that the performance of the QuEChERS method was comparable to, or better than that of accelerated solvent extraction (ASE) method for extraction of pharmaceuticals from plants. The two optimized extraction methods were applied to quantify the uptake of pharmaceuticals by celery and lettuce growing hydroponically. The results showed that all the eleven target pharmaceuticals could be absorbed by the vegetables from water. Compared to the ASE method, the QuEChERS method offers the advantages of short time and reduced costs of sample preparation, and less amount of organic solvents used. The established QuEChERS method could be used to determine the accumulation of multiple classes of pharmaceutical residues in vegetables and other plants, which is needed to evaluate the quality and safety of agricultural produce consumed by humans.

INTRODUCTION

Pharmaceuticals could be taken up by crop/vegetable produce from agricultural lands, and enter humans and animals through food chains.[1-5] The human health impacts of chronic exposure to trace levels of bioactive pharmaceutical chemicals are largely unknown. These pharmaceuticals widely found in agricultural production fields originate primarily from land application of animal manures and sewage biosolids, and crop irrigation with reclaimed water (effluents) from wastewater treatment plants (WWTPs). Many pharmaceuticals are commonly used in animal feeding operations for disease treatment and prevention, as well as growth promotion. The Food and Drug Administration estimated that in 2015 more than 15.5 million kilograms of antimicrobial drugs were approved for use in livestock production in the U.S.[6] Large fractions of pharmaceuticals used in animal production are excreted into manures either as parent compounds or bioactive metabolites.[2, 7-11] In addition, pharmaceuticals administered to humans are typically transported into wastewater streams that flow into municipal WWTPs. Due to the insufficient treatments for pharmaceuticals at WWTPs, certain fractions of pharmaceuticals are discharged in effluents and sorbed by sewage sludge. In many arid and semi-arid regions, irrigation with reclaimed water has been increasingly adopted so as to relieve fresh water shortage and to improve the sustainability and profitability of crop production. Undoubtedly, pharmaceuticals are introduced into agroecosystems through land application of manure and biosolids, as well as crop irrigation with reclaimed water.

Agricultural production has been benefited greatly from these practices. However, several recent studies demonstrated that human and veterinary pharmaceuticals (e.g. amoxicillin, caffeine, carbamazepine, ciprofloxacin, diphenhydramine, florfenicol, ibuprofen, levamisole, trimethoprim, and sulfamethazine) can enter vegetables and crops (e.g. carrot, lettuce, soybeans and alfalfa) from

soil and water.[1, 5, 12-14] Sabourin et al.[15] reported that pharmaceuticals in the soils originating from fertilization with municipal biosolids at agronomic rates could be accumulated in tomato, carrot, potato or sweet corn with the concentrations ranging from 0.33 to 6.25 ng g^{-1} dry weight. Wu et al.[5] demonstrated that vegetables irrigated with reclaimed water could accumulate 0.8 ng g^{-1} of caffeine, 1.4 ng g^{-1} of carbamazepine, 0.26 ng g^{-1} of naproxen, 1.3 ng g^{-1} of triclosan in mature edible parts of vegetables. These results together indicate that crops and vegetables can take up pharmaceuticals under common farming practice, though the amounts consumed by humans are estimated to be much lower than the acceptable daily intake.

In order to assess the food quality and safety in terms of accumulation of bioactive pharmaceutical chemicals, a quick, selective and sensitive analytical protocol is needed to quantify the pharmaceuticals in vegetable produce. Determination of trace levels of pharmaceuticals in vegetables is of great challenge owing to the complex composition of plant tissues including pigment, fat, cellulose and wax constituents, which may interfere with sample extraction and instrumental analysis.[16, 17] Several methods have been developed for extraction of pharmaceuticals from plants, including solid-liquid extraction (SLE), ultrasonic liquid extraction (ULE), Soxhlet extraction (SE), pressurized liquid extraction (PLE)/accelerated solvent extraction (ASE).[18, 19] The subsequent cleanup step is critically needed due to the complexity of plant tissue matrices; the cleanup methods include liquid-liquid extraction (LLE), solid-phase extraction (SPE), and gel permeation chromatography (GPC).[18] The SLE with HCl and KCl solution coupled to the SPE cleanup achieved the extraction efficiencies of 56-61% for carbamazepine and 67-98% for ibuprofen from ryegrass roots.[13, 20] The PLE method using acetonitrile/water mixtures (55/45 and 85/15, v/v) manifested good extraction efficiencies for pharmaceuticals from carrot and cabbage ranging from 46 to 176% after corrected with internal standard (carbamazepine-^{13}C, ^{15}N).[10] The

combination of ULE and SPE cleanup was recently established with extraction efficiencies of 87.1 to 123.5% for 19 pharmaceuticals from lettuce, and the detection limits ranged from 0.04 to 3 ng g^{-1}.[17]

Recently, a quick, easy, cheap, effective, rugged and safe (QuEChERS) method received more attention on the efficient extraction of organic analytes from sludge, animal and plant tissues.[21-29] The QuEChERS method was designed to extract pesticide residues from fruits and vegetables using relatively polar organic solvents (e.g. acetonitrile) in the presence of a large amount of salts. The salting-out effect could enhance the transfer of analytes to organic extractant phase. The extracts were then cleaned up by mixing with dispersive solid phase extraction (d-SPE) sorbents consisting of $MgSO_4$, primary-secondary amine (PSA), C18 and graphitized carbon black (GCB).[21] This simple extraction procedure could achieve excellent accuracy (recovery between 85 to 101%) and high precision (variation < 5%) when quantifying 250 ng g^{-1} of over twenty polar and basic pesticides in lettuce and strawberry.[21] Therefore, it has been adopted as an official extraction method by Association of Official Analytical Chemists (AOAC) in 2007, and approved for determination of pesticide residues in food by European Committee for Standardization in 2008.[30, 31]

Currently, the QuEChERS method has been extended to determine pharmaceuticals in eggs, meats, fish and sewage sludge;[25, 32, 33] however, few studies were conducted to quantify pharmaceuticals in vegetables and crop produce using the QuEChERS method.[34] Salvia at el.[35] showed that both ASE and a modified QuEChERS method could effectively extract multiple classes of pharmaceuticals from soils. In this study, we developed a quick and sensitive method to quantify the accumulation of multiple classes of pharmaceuticals in vegetables by coupling QuEChERS extraction to analysis using liquid chromatography-tandem mass spectrometry (LC-

MS/MS). The performance of QuEChERS extraction method was compared with ASE and ULE methods by evaluating their extraction recovery of pharmaceuticals from vegetables, relative standard deviation (RSD), and method detection limit (MDL). The method was also validated by measuring the uptake of pharmaceuticals from water by lettuce and celery that grew hydroponically in laboratory.

MATERIALS AND METHODS

Chemicals and Materials

Eleven pharmaceuticals acetaminophen, caffeine, carbadox, carbamazepine, lincomycin, monensin sodium, oxytetracycline, sulfadiazine, sulfamethoxazole, trimethoprim and tylosin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The chemical structures and selected physicochemical properties of the pharmaceuticals are listed in Table 2.1. These pharmaceuticals were dissolved in methanol (HPLC grade) to prepare stock solutions with concentrations ranging from 10 to 100 µg mL^{-1}. The internal standard of simeton was purchased from Absolute Standards, Inc. (Hamden, CT, USA). Acetonitrile and anhydrous sodium sulfate (Na_2SO_4) were purchased from EMD Chemicals (Gibbstown, NJ, USA). Ceramic homogenizers, C18, PSA and GCB were purchased from Agilent Technologies (Santa Clara, California, USA). Disodium ethylenediaminetetraacetate (Na_2EDTA), formic acid, and sodium chloride (NaCl) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Waters Oasis hydrophilic–lipophilic balance (HLB) cartridge was purchased from Waters Corporation (Milford, MA, USA). Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Billerica, MA, USA).

Table 2.1. Physicochemical properties of pharmaceuticals used in this study.

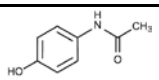
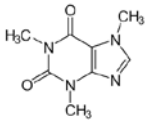
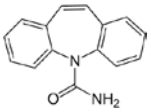
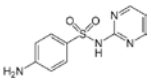
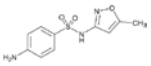
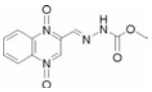
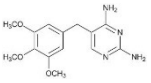
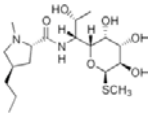
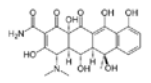
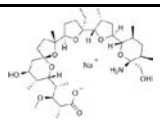
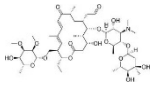
Pharmaceuticals	Molecular Weight ($g\ mol^{-1}$)	Chemical Structure	Water Solubility[a] ($mg\ L^{-1}$)	pK_a[a]	logK_{ow}[a]
Acetaminophen	151.16		14000	9.38	0.46
Caffeine	194.19		21600	10.4	-0.07
Carbamazepine	236.27		18	2.3, 13.9[b]	2.45
Sulfadiazine	250.28[c]		77[b]	2.01, 6.99[c]	-0.09[c]
Sulfamethoxazole	253.28		610	1.6, 5.7	0.89
Carbadox	262.22[d]		1755[d]	1.8, 10.5[e]	-1.22[e]
Trimethoprim	290.32		400	7.12	0.91
Lincomycin	406.54		927	7.6	0.2
Oxytetracycline	460.43		313	3.57, 7.49, 9.44[f]	-0.9

Table 2.1. (cont'd)

Pharmaceuticals	Molecular Weight ($g\ mol^{-1}$)	Chemical Structure	Water Solubility[a] ($mg\ L^{-1}$)	pK_a[a]	logK_{ow}[a]
Monensin sodium	692.87[g]		Slightly soluble[e]	4.3[g]	5.43[g]
Tylosin	916.10		5	7.73	3.27[d]

[a] From TOXNET database: http://toxnet.nlm.nih.gov/index.html.
[b] Calisto and Esteves (2012).[36]
[c] From Drugbank database: http://www.drugbank.ca/drugs/DB00564.
[d] From ChemSpider database: http://www.chemspider.com/Chemical-Structure.10606106.html.
[e] Song et al. (2010).[37]
[f] Rakshit et al. (2013).[38]
[g] From Guidechem database: http://www.guidechem.com/reference/dic-20635.html.

Preparation of Vegetable Samples

Vegetables celery and lettuce were purchased from a local supermarket, and the samples were frozen at -85 °C in a freezer for 6 hours, and freeze-dried for 3 days using a VirTis freeze mobile lyophilizer (SP Scientific, *Warminster, PA,* USA). The dried vegetable samples were then ground using a Smartgrind coffee grinder (Black & Decker, Middleton, WI, USA) to obtain powders, and stored in a desiccator. For each vegetable sample (500 mg), 200 µL of methanol solution containing all tested pharmaceuticals (500 ng mL^{-1} for each) was spiked to the sample, and vortexed for 1 min. The samples were then placed in a fume hood to allow methanol evaporated, and the concentration was 200 ng g^{-1} for each pharmaceutical in the vegetable samples.

Extraction and Cleanup

The prepared vegetable samples were subject to the extraction by ASE, ULE, and QuEChERS methods for comparison. For the ASE procedure, the prepared vegetable samples (500.0 mg) were added in 22-mL stainless steel cells with glassfiber filter placed at the bottom end of the cell, and extracted using a Dionex ASE 200 accelerated solvent extractor (Sunnyvale, CA, USA). The samples were preheated for 5 min, extracted at 80^{o}C under the pressure of 1500 psi. The static extraction period was set at 5 min with a flush volume of 60% of cell volume, purged with N_2 for 120 s. This extraction step was repeated with two cycles. To select the extractant for ASE, the mixture of water, acetonitrile and methanol at different ratios was tested to identify the solvent mixture that achieved the highest extraction recovery. The collected extracts were dried under a gentle nitrogen flow and then reconstituted with 150 mg L^{-1} Na_2EDTA aqueous solution to 20.0 mL, followed by a cleanup procedure using SPE method. The reconstituted solution passed through a HLB cartridge which was preconditioned with 3.0 mL methanol and 5.0 mL water. The

cartridge was then washed with 5.0 mL of water, and the target pharmaceuticals were eluted from the cartridge using 5.0 mL of methanol.

For the ULE procedure, the prepared vegetable samples (500.0 mg) were placed in 30-mL glass centrifuge tubes and mixed with 20 mL of acetonitrile. The tubes were sonicated in a Fisher Scientific FS110H ultrasonic water bath (Pittsburgh, PA, USA) for 20 min, and centrifuged at 1080 g for 20 min. The supernatants were collected, and the residual vegetable samples were extracted one more time. The extracts were combined, dried under a gentle nitrogen stream, and reconstituted to 20.0 mL with 150 mg L^{-1} of Na_2EDTA solution followed by a cleanup using SPE method as described above.

The prepared vegetable samples were also extracted using QuEChERS procedure. The prepared vegetable samples (500.0 mg) and 2.0 mL of 150 mg L^{-1} of Na_2EDTA solution were placed in 50-mL polypropylene centrifuge tubes with two ceramic homogenizers, and mixed by vortex for 1 min. Five milliliters of acetonitrile and methanol mixtures with volume ratios of 100/0, 85/15, 75/25, 65/35, and 50/50 were added to the centrifuge tubes in order to test extraction recovery. The volume ratios of the extractant mixtures were 71.4/0/28.6, 60.7/10.7/28.6, 53.6/17.9/28.6, 46.4/25.0/28.6 and 35.7/35.7/28.6 for acetonitrile/methanol/150 mg L^{-1} Na_2EDTA solution. After shaking for 1 min, anhydrous Na_2SO_4 (2.0 g) and NaCl (0.5 g) were added into the centrifuge tubes, and vortexed with the vegetable samples for 1.5 min in order to reduce water content in the sample and enhance extract efficiency. All the samples were centrifuged at 2990 *g* for 10 min, and 1.3 mL of the supernatants were collected in 1.5-mL polypropylene centrifuge tubes to which the d-SPE sorbents consisting of C18 (12.5 mg), PSA (12.5 mg) and Na_2SO_4 (225 mg) were added. The samples were mixed vigorously for 1 min, centrifuged at 9240 *g* for 10 min, and 1.0 mL of the supernatants was transferred to clean glass vials for LC-MS/MS analysis.

LC-MS/MS Analysis

The vegetable extraction samples were analyzed for pharmaceuticals using a Shimadzu Prominence high-performance liquid chromatography (Columbia, MD, USA) coupled with an Applied Biosystems Sciex 3200 triple quadrupole mass spectrometer (Foster City, CA, USA). The Phenomenex Luna C18 column (50 mm×4.6 mm, particle size 3 μm, Torrance, CA, USA) was used to separate target pharmaceuticals. Binary mobile phase consisted of phase A water containing 0.3% formic acid and phase B acetonitrile/methanol (1/1, v/v) containing 0.3% formic acid. The flow system was pre-equilibrated with 100% phase A for 5 min, and the gradient program (with respect to phase B) was set as 0.1–2.0 min phase B increased to 40%; 2.0–2.5 min phase B increased to 60%; 2.5–3.0 min phase B increased to 80%; 3.0–4.0 min phase B increased to 84%; 4.0-7.0 min phase B increased to 100% and stop at 7.0 min. The flow rate was 0.35 mL per min, and the sample injection volume was 10 μL. The target pharmaceuticals were quantified using multiple reaction monitoring (MRM) mode. The Turbo IonSpray source of the mass spectrometer was operated in positive mode with ionspray voltage of 5000 V and temperature at 700 ˚C. Curtain gas pressure was 20 psi, collision gas pressure was 6 psi, and ion source gas pressure was 60 psi.

Method Validation

To validate the analytical methods, the eleven target pharmaceuticals were spiked to the celery sample with concentration of 200 ng g^{-1} for each pharmaceutical, extracted by ULE, optimized ASE and QuEChERS methods, and quantified by the LC-MS/MS, as described above. The optimized method was evaluated by considering instrumental detection limit (IDL), linearity of standard curves, MDL, extraction recovery and RSD. The IDL was estimated via diluting the eleven pharmaceuticals with methanol to the range of 0.01 to 10 ng mL^{-1}, and obtaining the signal to noise ratios (S/N) at 3. The standard solutions were prepared with the range of 0.01 to 250 ng

mL^{-1} in the matching extraction matrices using the extracts from the pharmaceutical-free vegetables by ULE, ASE and QuEChERS methods. The MDL value was determined by spiking a small amount of pharmaceuticals to celery, then extracted and analyzed by the optimized methods. The pharmaceutical concentration with the response of S/N = 3 was referred to as the MDL. The extraction recovery was determined using the celery samples with the pharmaceutical concentration of 200 ng g^{-1}, and the RSD was calculated using the results from triplicate samples.

Method Application to Analyze Pharmaceuticals in Vegetables

The optimized ASE and QuEChERS methods were applied to analyze uptake of the target pharmaceuticals by celery and lettuce growing hydroponically. The celery seeds (Generic Seeds, Springville, UT, USA) and lettuce seeds (Burpee, Warminster, PA, USA) were wetted on paper tissue for 3 to 5 days until they began to sprout, and then transferred to moist sands. After they reached the growth stage of 3 to 4 leaves (~10 days), the celery and lettuce seedlings were transferred to hydroponic growth system, and allowed to grow in nutrient solution (Hydrodynamics International, Lansing, MI, USA) for 14 days at 18^{o}C with the cycle of 16 h light and 8 h in dark per day. Air was continuously purged to the nutrient solution using fusion air pumps. At day 28, the solution was replaced with the nutrient solution containing the eleven target pharmaceuticals with 100 ng mL^{-1} for each pharmaceutical. The glass containers were wrapped with aluminum foil to prevent the potential photodegradation of the pharmaceuticals. After 24 h uptake, the vegetables were collected, rinsed with water, and immediately frozen at -85^{o}C in a freezer for 3 h to obtain the dried samples. These vegetable samples were ground, extracted by the optimized ASE and QuEChERS methods, and analyzed by LC-MS/MS using the protocols described above. The experiments were conducted in triplicate including the controls without the

added pharmaceuticals. The statistical analysis were conducted using Microsoft Office Excel (version 2013).

RESULTS AND DISCUSSION

LC-MS/MS Analysis

The retention time for the target pharmaceuticals is listed in Table 2.2. For each pharmaceutical the precursor ion and two product ions were simultaneously monitored with the greater abundance transition (in bold in Table 2.2) used for quantification. This operation could achieve the identification points (IPs) of four which meets the criteria of IPs ≥ 3 for unambiguous identification of veterinary pharmaceuticals required by European Commission Decision.[39] To improve instrumental sensitivity and selectivity, in the tandem mass spectrometer declustering potential (DP), entrance potential (EP), cell entrance potential (CEP), collision energy CE) and collision cell exit potential (CXP) were optimized in the MRM transitions for each pharmaceutical (Table 2.2). As a result, the instrumental detection limits of the pharmaceuticals ranged from 0.1 to 3.5 pg (Table 2.2), which are comparable to the ranges reported in several previous studies.[17, 40]

Table 2.2. Precursor, product ions and mass spectrometer parameters used in identification and quantification of the pharmaceuticals, retention time (RT) and instrumental detection limit (IDL).

Chemicals	Precursor ion (m/z)	Product ion[a] (m/z)	DP[b] (V)	EP[c] (V)	CEP[d] (V)	CE[e] (V)	CXP[f] (V)	RT (min)	IDL(pg)
Acetaminophen	152.0	**110.0**	34.0	4.5	11.0	20.5	9.0	2.8	1.8
		93.0	34.0	4.5	11.0	30.0	7.0		
Caffeine	195.0	**138.0**	39.0	4.9	12.0	25.0	12.0	3.2	0.5
		110.0	39.0	4.9	12.0	31.5	9.0		
Carbamazepine	237.2	**194.0**	41.0	6.5	19.0	26.0	15.0	4.4	0.1
		192.0	41.0	6.5	19.0	31.0	14.4		
Sulfadiazine	251.1	**92.2**	38.0	4.0	15.0	36.0	8.0	2.9	0.6
		108.1	38.0	4.0	15.0	31.5	9.0		
Sulfamethoxazole	254.0	**108.1**	38.0	4.4	14.2	32.0	9.0	3.7	0.6
		156.2	38.0	4.4	14.2	12.0	12.0		
Carbadox	263.0	**231.0**	45.0	4.6	15.0	16.5	19.0	3.5	2.8
		159.1	45.0	4.6	15.0	26.0	13.0		
Trimethoprim	291.4	**230.4**	68.0	4.0	10.0	31.0	20.0	2.8	0.4
		261.3	68.0	4.0	10.0	33.0	22.0		
Lincomycin	407.2	**126.2**	32.0	3.5	13.5	41.0	11.0	2.6	0.4
		359.3	32.0	3.5	13.5	26.0	30.0		
Oxytetracycline	461.3	**426.3**	30.0	5.0	20.0	29.3	36.0	2.8	1.3
		283.1	30.0	5.0	20.0	50.0	22.0		

Table 2.2. (cont'd)

Chemicals	Precursor ion (m/z)	**Product ion[a] (m/z)**	DP[b] (V)	EP[c] (V)	CEP[d] (V)	CE[e] (V)	CXP[f] (V)	RT (min)	IDL(pg)
Monensin sodium	693.5	**461.4**	80.0	10.0	24.0	70.0	37.5	6.3	0.2
		479.6	80.0	10.0	24.0	70.0	37.5		
Tylosin	916.6	**174.3**	68.0	11.0	33.0	41.0	15.0	3.8	3.5
		101.3	68.0	11.0	33.0	69.0	7.5		

[a] The product ion coupled with precursor used to qualify and quantify pharmaceuticals (product ions in bold used for quantification).
[b] DP: Declustering potential.
[c] EP: Entrance potential.
[d] CEP: Cell entrance potential.
[e] CE: Collision energy.
[f] CXP: Collision cell exit potential.

Comparison of Extraction Methods

In this study, ASE, ULE and QuEChERS methods have been tested and compared for their extraction recovery of pharmaceuticals from vegetables. The former two methods have been shown to effectively extract pharmaceuticals from plant tissues.[1, 10, 17, 41-43] In this study we began with ASE and ULE methods to extract pharmaceuticals from celery tissues using acetonitrile. The ASE and ULE methods generally demonstrated similar extraction efficiencies for most of the target pharmaceuticals (Figure 2.1). The ASE method achieved greater extraction recovery for acetaminophen, sulfadiazine and sulfamethoxazole than the ULE method (Figure 2.1). The RSD values for the majority of target pharmaceuticals were less than 20% indicating acceptable precision for both methods. For acetaminophen the RSDs by ULE and ASE were 7.1% and 2.2%, indicating that the ASE method might provide a better precision than the ULE method. Moreover, the ASE method manifested greater extraction recovery for sulfadiazine (38.0 ± 5.7%) and sulfamethoxazole (35.5 ± 6.8%) than the ULE method (23.9 ± 1.9% and 21.0 ± 2.7%, respectively). Therefore, ASE method was selected for further optimization in order to achieve acceptable extraction recovery and precision.

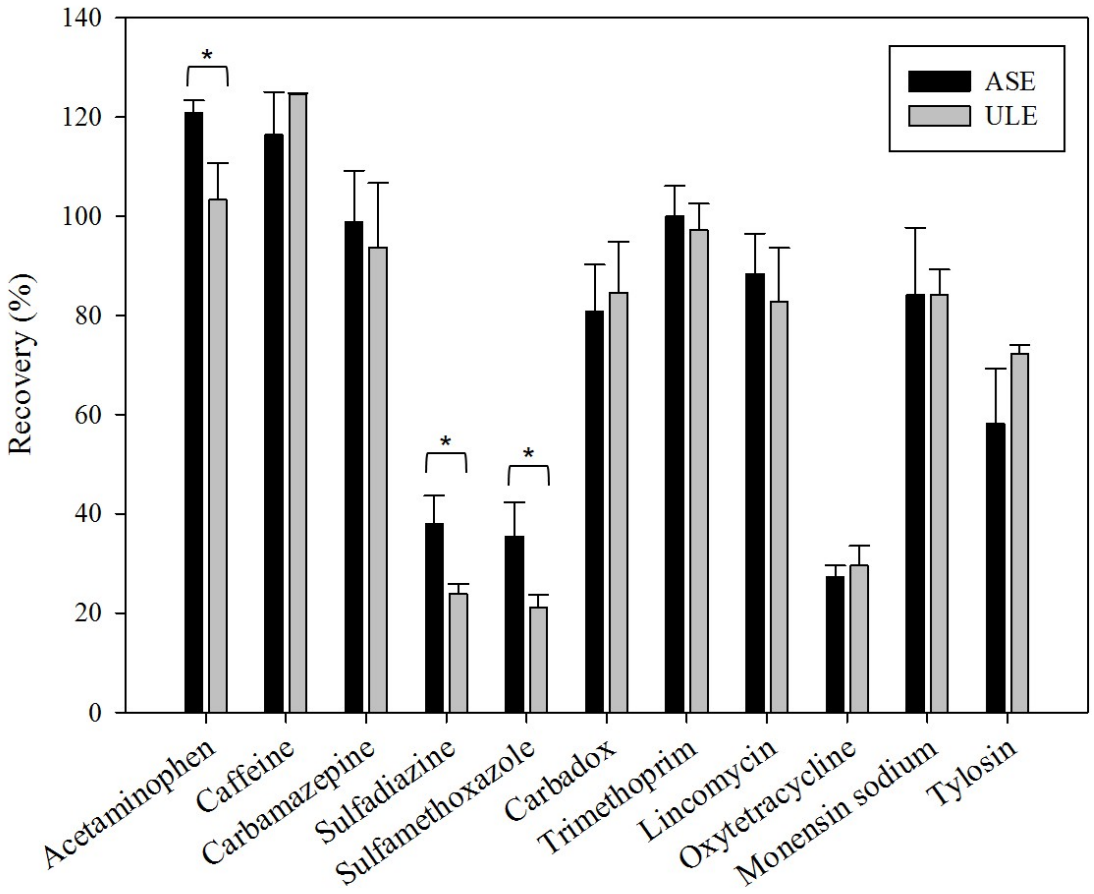


Figure 2.1. Comparison of extraction recovery of pharmaceuticals from celery with acetonitrile using accelerated solvent extraction (ASE) and ultrasonic liquid extraction (ULE). Error bars represent standard deviations (n = 3). Asterisk (*) indicates significant difference between ASE and ULE extractions ($p < 0.05$).

According to the recommendation guideline by European Union, the extraction recovery is suggested to reach 70-120% with RSD < 20% for analysis of pesticide residues in food and feed.[44] In this study the extraction recoveries of the ASE method were relatively low for sulfadiazine (38.0% with RSD of 14.9%), sulfamethoxazole (35.5% with RSD of 19.3%), oxytetracycline (27.2% with RSD of 8.5%) and tylosin (58.1% with RSD of 19.3%). Therefore, mixture of extraction solvents was utilized to enhance the extraction recovery for target pharmaceuticals from vegetables. Wu et al.[17] used two-step extraction with methyl tert-butyl ether (MTBE) and acetonitrile sequentially, and improved extraction recovery of triclosan from 70 to 110% and acetaminophen from 20% to 60%, compared to with acetonitrile only. Ding et al.[2, 45] mixed acetonitrile and water (v/v = 7/3), and achieved reasonable extraction recovery for sulfadiazine, sulfamethoxazole, lincomycin and oxytetracycline from biosolids (>52%). In addition, methanol was shown to effectively extract tylosin from soils and plants.[13] In this study all the mixed extractant containing acetonitrile, methanol and water could achieve the acceptable extraction efficiencies > 70% for carbamazepine, trimethoprim and monensin sodium (Table 2.3). Among the tested solvent combinations, the mixture of acetonitrile/methanol/water of 72:8:20 (v/v/v) could achieve the extraction recovery > 70%, and the corresponding RSDs were < 20% for all pharmaceuticals (Table 2.3). Therefore, the ASE with the mixture of acetonitrile/methanol/water = 72/8/20 was identified as the optimized extractant with the extraction procedure described in the experimental section.

Table 2.3. Extraction recovery of accelerated solvent extraction with different solvents for pharmaceuticals from celery.

Pharmaceuticals	Acetonitrile		A[a]:M[b]:W[c] (81:9:10)[d]		A:M:W (72:8:20)		A:M:W (72:18:10)		A:M:W (65:16:20)	
	Recovery (%)	RSD (%)[e]	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Acetaminophen	120.7	2.2	73.4	4.9	133.5	8.7	63.7	19.6	103.9	8.0
Caffeine	116.4	7.5	83.8	1.6	130.3	17.3	55.0	7.5	99.3	8.5
Carbamazepine	98.7	10.6	89.5	5.1	116.9	10.7	88.6	6.5	95.5	4.6
Sulfadiazine	38.0	14.9	64.1	11.3	71.4	6.4	86.7	11.2	52.8	13.8
Sulfamethoxazole	35.5	19.3	79.7	17.0	71.3	14.3	78.5	12.6	46.4	9.7
Carbadox	80.7	11.7	66.7	14.6	103.3	4.1	69.2	19.0	82.0	4.7
Trimethoprim	100.0	6.1	101.0	19.5	111.7	13.1	95.6	14.0	95.3	2.2
Lincomycin	88.4	9.2	36.5	35.2	91.0	18.7	19.1	8.0	97.0	2.6
Oxytetracycline	27.2	8.5	56.1	3.3	79.5	13.7	47.2	8.5	48.5	13.0
Monensin sodium	84.1	16.1	84.5	13.6	101.1	12.3	94.3	10.4	94.7	3.2
Tylosin	58.1	19.3	86.4	10.3	92.9	4.6	105.6	14.3	92.9	9.6

[a] A: acetonitrile, [b] M: methanol, [c] W: water, [d] volume ratio, and [e] RSD: relative standard deviation.

The prepared vegetable samples were also extracted with QuEChERS method. Vegetable samples were shaken with 150 mg L^{-1} of Na_2EDTA and ceramic homogenizers in polypropylene centrifuge tubes. The eleven target pharmaceuticals were found to have negligible adsorption by the polypropylene centrifuge tube in the extraction solvent. Na_2EDTA solution could enhance the extraction of polar chemicals such as pharmaceuticals from plants,[24] and reduce the complexation of tetracyclines with metal cations hence enhance the extraction recovery of oxytetracycline. [2, 25, 45, 46] Ceramic chips helped grind vegetables and pulverize salt agglomerates, which better homogenized the samples.[46] The addition of NaCl enhances the salting-out effect, and anhydrous Na_2SO_4 reduces water content in the samples, both of which facilitate the transfer of pharmaceuticals from plant tissues and water to acetonitrile/methanol phase. In the classical QuEChERS method $MgSO_4$ is commonly used to remove the residual water. In this study anhydrous Na_2SO_4 was used instead of $MgSO_4$ because $MgSO_4$ could potential diminish the extraction efficiency of tetracyclines and macrolides (e.g. tylosin).[47, 48] After the extraction step in the QuEChERS method, the supernatant is cleaned up using d-SPE sorbents including PSA, C18 and GCB. PSA can remove organic acids, sugar and polar pigments, C18 can adsorb proteins and lipids, and GCB can reduce chlorophyll and sorb planar-structured chemicals from the extracts of plant and animal tissues.[21, 23, 49] To select the appropriate cleanup sorbents, we tested sorption of the target pharmaceuticals by these d-SPE sorbents in acetonitrile/methanol mixture (65/35, v/v). The results revealed that PSA and C18 demonstrated negligible sorption for the target pharmaceuticals. However, GCB manifested relatively strong affinity to several pharmaceuticals; approximately 39% of caffeine, 98% of carbadox, 25% of trimethoprim, 71% of oxytetracycline, 73% of monensin sodium and 59% of tylosin were sorbed by GCB from the acetonitrile/methanol

mixture. Therefore, PSA and C18 were selected as d-SPE sorbents, and GCB was excluded though it could help remove chlorophyll from the extracts of plant tissues.

To further optimize the QuEChERS procedure, the extractant mixtures with varying acetonitrile/methanol ratios were tested for the extraction efficiency of pharmaceuticals from vegetable tissues. Among the mixed solvents with varying volume ratios of acetonitrile/methanol/Na_2EDTA, the mixture with volume ratio of 46.4/25.0/28.6 demonstrated the highest extraction recovery, which ranged from 70.1 to 118.6% for the target pharmaceuticals, and the corresponding RSDs were less than 20% (Table 2.4). Therefore, this mixture was selected as the extraction solvent in the QuEChERS method used in the following studies.

Table 2.4 Extraction recovery of QuEChERS with different solvent ratio for pharmaceutical from celery.

Pharmaceuticals	A[a]:M[b]:W[c] (71.4/0/28.6)[d]		A:M:W (60.7/10.7/28.6)		A:M:W (53.6/17.9/28.6)		A:M:W (46.4/25.0/28.6)		A:M:W (35.7/35.7/28.6)	
	Recovery (%)	RSD (%)[e]	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Acetaminophen	84.5	10.3	83.6	7.1	95.5	13.0	91.7	5.0	121.9	8.9
Caffeine	98.9	10.1	94.3	4.1	86.1	16.5	86.6	10.7	118.0	7.9
Carbamazepine	74.4	7.2	80.0	7.2	109.2	6.2	83.8	11.5	84.6	11.0
Sulfadiazine	57.5	5.3	62.1	8.9	46.2	17.9	89.9	15.3	60.3	10.9
Sulfamethoxazole	58.8	1.5	67.5	6.4	65.9	10.6	79.0	11.7	53.0	11.1
Carbadox	63.7	7.7	51.0	0.0	53.5	16.9	74.0	9.7	69.5	18.9
Trimethoprim	89.3	4.9	86.6	9.4	50.5	17.2	80.5	19.9	62.9	4.1
Lincomycin	9.5	17.2	40.2	6.3	66.2	11.4	80.3	6.1	66.5	2.6
Oxytetracycline	0.9	6.9	2.7	16.5	49.7	10.8	79.5	14.7	68.4	12.5
Monensin sodium	91.9	6.8	116.5	3.7	87.8	11.4	70.1	5.4	29.3	10.7
Tylosin	52.6	19.7	86.2	6.6	87.4	19.3	118.6	2.7	80.1	18.7

[a] A: acetonitrile, [b] M: methanol, [c] W: water containing 150 mg L^{-1} of Na_2EDTA, [d] volume ratio, and [e] RSD: relative standard deviation.

Method Validation

The optimized ASE and QuEChERS methods were validated for extraction of pharmaceuticals from celery and lettuce samples. As for ASE method vegetable samples were extracted with the mixture of acetonitrile/methanol/water (72/8/20) using an accelerated solvent extractor. The extracts were then cleaned up using HLB SPE cartridges. For the QuEChERS procedure vegetable samples were extracted with the mixture of acetonitrile/methanol/Na_2EDTA solution (v/v/v = 46.4/25.0/28.6) in the presence of NaCl and Na_2SO_4, and cleaned up with d-SPE sorbents consisting of C18, PSA and Na_2SO_4. Table 2.5 summarizes the method detection limits (MDLs), and extraction recoveries of the ASE and QuEChERS methods for the eleven target pharmaceuticals from celery and lettuce. The standard solutions were prepared in the matrices of the extracts from the pharmaceutical-free vegetable samples. The standard curves ranging from 0.1 to 250 ng mL^{-1} demonstrated excellent linear relationship with the correlation coefficients > 0.995 for all target pharmaceuticals. The method detection limits ranged from 1.9 to 15.8 ng g^{-1} for the ASE method, and from 0.7 to 8.0 ng g^{-1} for the QuEChERS method (Table 2.5), which are comparable to the MDLs reported by Wu et al. (2012).[17] In general, the QuEChERS method demonstrated a better sensitivity for most target pharmaceuticals compared to the ASE method.

Table 2.5. Method detection limit (MDL) and extraction recovery of pharmaceuticals from celery and lettuce by the optimized accelerated solvent extraction (ASE) and QuEChERS methods.

Pharmaceuticals	MDL (ng g^{-1})		Recovery from celery (%)		Recovery from lettuce (%)	
	ASE	QuEChERS	ASE	QuEChERS	ASE	QuEChERS
Acetaminophen	4.4	8.0	133.5 ± 11.6[a]	91.7 ± 4.6	113.0 ± 1.1	73.4 ± 4.1
Caffeine	2.3	2.4	130.3 ± 22.5	86.6 ± 9.3	92.4 ± 6.1	89.8 ± 7.9
Carbamazepine	1.9	0.7	116.9 ± 12.5	83.8 ± 9.6	90.1 ± 9.5	96.5 ± 4.4
Sulfadiazine	11.9	3.7	71.4 ± 4.5	89.9 ± 13.7	78.0 ± 5.5	74.0 ± 4.0
Sulfamethoxazole	15.8	4.4	71.3 ± 10.2	79.0 ± 9.2	71.9 ± 0.2	73.6 ± 2.9
Carbadox	7.4	6.9	103.3 ± 4.2	74.0 ± 7.2	70.7 ± 0.2	91.5 ± 12.8
Trimethoprim	2.4	4.7	111.7 ± 14.6	80.5 ± 16.0	118.9 ± 2.6	81.9 ± 7.6
Lincomycin	3.1	2.2	91.0 ± 17.1	80.3 ± 4.9	100.3 ± 12.3	83.3 ± 6.6
Oxytetracycline	6.7	2.7	79.5 ± 10.9	79.5 ± 11.7	70.2 ± 4.0	72.3 ± 2.0
Monensin sodium	5.4	0.7	101.1 ± 12.4	70.1 ± 3.8	78.9 ± 14.1	83.0 ± 1.7
Tylosin	4.6	4.2	92.9 ± 4.3	118.6 ± 3.2	107.5 ± 4.5	89.0 ± 11.0

[a] Recovery: mean ± standard deviation (n=3).

For the ASE method the extraction recoveries of pharmaceuticals ranged from 71.3 to 133.5% from celery and 70.2 to 118.9% from lettuce, which were comparable to those obtained by the QuEChERS method (70.1 to 118.6% from celery and 72.3 to 96.5% from lettuce). These recoveries revealed significant difference ($p < 0.05$) between the ASE and QuEChERS methods for extracting acetaminophen, caffeine, carbamazepine, carbodox, monensin sodium, and tylosin from celery. For the extraction of pharmaceuticals from lettuce, acetaminophen, carbodox, trimethoprim and tylosin demonstrated significant difference ($p < 0.05$) in the extraction recoveries between the two extraction methods. Although there was no consistency between the ASE or QuEChERS methods in terms of extraction recovery for pharmaceuticals from celery or lettuce, all the extraction efficiencies were greater than 70% with the RSDs < 20% (n = 3). These narrow RSDs indicate that the similar precision could be achieved by either method to quantify accumulation of pharmaceuticals in vegetables. The overall extraction recoveries of pharmaceuticals ranging from 70.1 to 118.6% for the QuEChERS method meet the criteria of recovery between 70-120% as suggested in the method validation for analysis of pesticide residues in food by European Union [44]. The ASE method achieved the extraction recovery ranged from 70.2 to 133.5%, which is a little above the high end of the criteria. However, the recovery between 60-140% with RSD < 20% is still considered acceptable for routine analysis of multi-pesticide residues in plants [44].

The optimized ASE and QuEChERS methods both provide the acceptable extraction recovery and precision to quantify pharmaceuticals in vegetable tissues; however, the QuEChERS method manifests itself as a simple, quick and inexpensive extraction method for determination of pharmaceuticals in vegetables. In addition, the QuEChERS method achieved more acceptable recovery compared to the ASE method in this study. The high sample throughput of the

QuEChERS method allows to simultaneously prepare a large batch of samples. Compared to ASE, much less amount of solvent was used, and neither sophisticated extraction instrument nor cleanup device is needed to carry out the sample preparation. Overall, the QuEChERS method is a simple, effective, cheap, and high throughput procedure suitable for simultaneous extraction of a large number of vegetable samples. However, more manual operations and labor are needed in the QuEChERS method.

Method Application to Plant Uptake of Pharmaceuticals

The optimized ASE and QuEChERS methods were applied to extract the accumulation of the eleven target pharmaceuticals by living celery and lettuce cultured hydroponically. After growing for 28 days, celery and lettuce plants were transferred to the nutrient solution containing the concentration of 100 ng mL^{-1} for each pharmaceutical. After 24 h uptake, the celery and lettuce samples were extracted using the optimized ASE and QuEChERS methods, and analyzed by LC-MS/MS (Table 2.6). The reported pharmaceutical concentrations were corrected by the extraction recoveries (Table 2.5). The results showed that both celery and lettuce could take up and accumulate all target pharmaceuticals with the concentration range of 22.9 to 247.5 ng g^{-1} in celery and 52.9 to 323.7 ng g^{-1} in lettuce. In the celery, the uptake amount of pharmaceuticals extracted by the optimized ASE and QuEChERS methods decreased in the order of monensin sodium > oxytetracycline > carbamazepine > acetaminophen > caffeine > tylosin > lincomycin > trimethoprim > sulfadiazine > sulfamethoxazole > carbadox. In the lettuce, the order of pharmaceutical uptake decreased as trimethoprim > monensin sodium > oxytetracycline > acetaminophen > carbamazepine > caffeine > tylosin > lincomycin > carbadox > sulfamethoxazole > sulfadiazine, prepared by both extraction methods. Among the pharmaceuticals investigated, trimethoprim manifested the greatest uptake by the lettuce, but relatively lower uptake by celery.

Overall, these two extraction methods could effectively extract multiple classes of pharmaceuticals from vegetables, and be potentially used to determine pharmaceutical residues in vegetables and other plants for the evaluation of food quality and safety.

Overall, the established ASE and QuEChERS methods coupled to LC-MS/MS demonstrate high sensitivity and reliability for quantification of multiple classes of pharmaceutical residues in vegetables. The mixture of solvents achieved reasonable extraction efficiency (>70%) with RSD < 20% for multiple classes of pharmaceuticals. Compared with the ASE method, the QuEChERS method demonstrates the advantages as a fast, easy, cheap, environmental friendly and effective approach to extract pharmaceuticals from plant tissues. These two extraction methods have been successfully applied to analyze the uptake of pharmaceuticals by vegetables. The results revealed that pharmaceuticals could enter celery and lettuce from pharmaceutical-contaminated water, which raises the concern about the potential risk to human health by consumption of pharmaceutical-contaminated vegetable produce.

Table 2.6. Pharmaceutical uptake by celery and lettuce growing hydroponically in nutrient solution.

Pharmaceuticals	Celery (ng g^{-1})		Lettuce (ng g^{-1})	
	ASE	QuEChERS	ASE	QuEChERS
Acetaminophen	110.3 ± 14.7[a]	132.7 ± 12.1	210.3 ± 26.2	202.5 ± 19.6
Caffeine	110.1 ± 9.5	102.4 ± 1.1	188.0 ± 17.4	181.9 ± 27.7
Carbamazepine	171.5 ± 16.7	203.4 ± 13.8	190.3 ± 21.1	194.6 ± 17.4
Sulfadiazine	41.2 ± 21.9	39.4 ± 2.9	53.0 ± 7.3	57.3 ± 13.7
Sulfamethoxazole	40.3 ± 4.8	34.3 ± 2.5	56.2 ± 15.3	67.1 ± 11.6
Carbadox	22.9 ± 9.9	23.4 ± 0.5	89.3 ± 12.1	96.8 ± 20.6
Trimethoprim	47.4 ± 1.9	48.9 ± 11.7	323.7 ± 5.4	308.8 ± 3.1
Lincomycin	73.3 ± 4.2	76.9 ± 9.5	111.0 ± 20.1	111.9 ± 4.1
Oxytetracycline	212.6 ± 25.0	206.8 ± 15.2	242.0 ± 13.2	233.0 ± 26.7
Monensin sodium	239.3 ± 8.8	247.5 ± 13.0	274.9 ± 38.5	289.0 ± 64.3
Tylosin	78.7 ± 8.0	84.9 ± 5.1	156.7 ± 34.5	167.5 ± 16.5

[a] Concentration corrected by extraction recovery: mean ± standard deviation (n=3).

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CHAPTER III

MECHANISM OF UPTAKE AND ACCUMULATION OF PHARMACEUTICALS BY LETTUCE FROM WATER

ABSTRACT

Mechanism underlying plant uptake of pharmaceuticals from soil and water remains largely unknown, thus impeding the development of effective measures to mitigate contamination of food crops by pharmaceuticals. In this study, uptake of thirteen commonly used pharmaceuticals by lettuce from water was investigated. Pharmaceutical sorption by lettuce roots was also measured to evaluate its influence on the transport of pharmaceuticals to shoots. Small-sized pharmaceuticals with molecular weight (MW) < 300 g mol^{-1} entered lettuce via symplast pathway, and those pharmaceuticals with low affinity to lettuce roots manifested substantial transport to shoots which included caffeine and carbamazepine. Small-sized lamotrigine and trimethoprim had relatively high affinity to lettuce roots that reduced the amount of transport to shoots. Large-sized pharmaceuticals (MW > 400 g mol^{-1}) including lincomycin, monensin sodium, and tylosin could be excluded by cell membranes and moved primarily in apoplast pathway, resulting in predominant accumulation in lettuce roots. Large-sized oxytetracycline existed as zwitterionic species that could slowly enter lettuce via the symplast pathway; however, the relatively strong interaction with lettuce roots limited its transport to shoots. The mass balance analysis indicated that acetaminophen, β-estradiol, carbadox, estrone, and triclosan were readily metabolized in lettuce with ≥ 90% loss during the 144-h exposure period. Molecular size and ionic state of pharmaceuticals, as well as their sorption to root constituents, collectively determine their uptake, transport, and accumulation in plants.

INTRODUCTION

Pharmaceuticals are ubiquitously present in the environment at a range of concentrations, and are considered as chemicals of emerging concern. They are released into agroecosystems through livestock excretion of administered veterinary pharmaceuticals, land application of biosolids, and irrigation with reclaimed water (effluents) from municipal wastewater treatment plants (WWTPs).[1-3] To alleviate water scarcity, especially in arid and semi-arid regions, reclaimed water is increasingly used in agricultural irrigation.[4] This practice results in the dissemination of pharmaceuticals in soils that could eventually be taken up by crops/vegetables.[5-7] Biosolids and animal manures are also applied to agricultural lands as a convenient approach of waste disposal while also providing value of fertilizer. As a result, many pharmaceuticals have been found to accumulate in vegetables and agricultural products at μg kg^{-1} levels.[6, 8] These levels of pharmaceuticals are far below the suggested dosage for the therapeutic purpose, thereby posing limited risks to human health.[9, 10-12] However, pharmaceuticals can induce changes in plant hormone levels and cause detrimental impacts to plant health.[13-15] Moreover, synergistic or antagonistic effects from mixtures of pharmaceuticals under long-term dietary exposure are currently unclear, which impedes the development of appropriate risk assessment framework.[8, 16, 17]

Pharmaceutical residues in agricultural products is an essential component in the risk assessment. The bioaccumulation factor in plants is commonly described as the ratio of pharmaceutical concentration in plant tissue to that in growing media (e.g., accumulation in roots is commonly referred as root concentration factor, RCF).[18] Pharmaceutical movement within plants is represented as the translocation factor (TF) calculated as the ratio of pharmaceutical concentration in shoots to that in roots.[19, 20] In several previous studies positive relationship was

found between log RCF and log D_{ow} (octanol-water partitioning coefficient adjusted to the fraction of neutral pharmaceutical species), and negative relationship observed between log TF and log D_{ow}.[5, 11, 14, 21] However, the results from different experimental settings (including plant species, hydroponic or pot studies, applied pharmaceutical concentrations, and exposure periods) revealed that for a given pharmaceutical RCF or TF values could vary up to two orders of magnitude, and no apparent relationship between RCF (or TF) and log D_{ow} was reported in several literature reviews.[19, 20] Generally, plant uptake of neutral pharmaceuticals is greater than that of ionic species, because anionic pharmaceuticals are repelled by cell membranes with negative electrostatic potential, whereas cationic species are attracted to the cell membranes thus limiting their movement into plants.[11, 18, 22] Some pharmaceuticals could alter their charged species during their transport within plants, and are consequently trapped in plant cells because solution pH varies at various locations in plant cells, e.g., 5.5 in intercellular space, 7.2 in the cytosol, and 5.5 in vacuoles.[22, 23] For instance, neutral form of lamotrigine (pK_a 5.7) could pass through cell membranes and tonoplasts by passive diffusion. Once in vacuole, neutral lamotrigine species are converted to cationic species that has difficulty crossing the lipid bilayer tonoplast hence being trapped in the vacuole of plant cells.[19, 20, 22]

Most previous research efforts were dedicated to investigate the impact of pharmaceutical speciation and lipophilicity on their accumulation in plants, while less attention was paid to the influence of water flow on their uptake and transport.[11, 12, 22, 24] Water molecules enter plant roots via apoplast and symplast pathways.[20, 25, 26] The apoplast pathway refers to the movement of water molecules through cell walls and intercellular spaces. In the cortex, the space for apoplast water movement accounts for 8 to 25% of root volume.[18] The symplast pathway refers to the process of water molecules crossing cell membranes and moving among cells. Water flow is believed to be

the primary carrier for uptake and transport of pharmaceuticals in plants. At endodermis, the highly hydrophobic Casparian strip could block the transport of pharmaceuticals carried by water in the apoplast pathway. Only those pharmaceuticals that cross cell membranes and enter roots in the symplast pathway could move to xylem and then transport upward to plant shoots.[20, 21, 23]

In this study, we hypothesized that water flow is the primary carrier for pharmaceuticals to enter plants, and facilitates their distributions in different plant parts. Interaction of pharmaceuticals with roots could retard their upward transport to shoots. To test these hypotheses, uptake and transport of a range of pharmaceuticals in lettuce were measured using a hydroponic experimental setting. Pharmaceutical sorption by lettuce roots was also determined to evaluate its influence on pharmaceutical transport from roots to shoots. These integrated results together provide innovative mechanistic insights into pharmaceutical uptake and movement in plants.

MATERIALS AND METHODS

Chemicals and Materials

Pharmaceuticals including acetaminophen, β-estradiol, caffeine, carbadox, carbamazepine, estrone, lincomycin, monensin sodium, oxytetracycline, trimethoprim, and tylosin and HPLC-grade methanol were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Lamotrigine was obtained from Toronto Research Chemicals, Inc. (Toronto, ON, Canada), and triclosan from AK Scientific, Inc. (Union City, CA, USA). The physicochemical properties of those selected pharmaceuticals are provided in Table 3.1. The internal standard, simeton, was supplied by Absolute Standards, Inc. (Hamden, CT, USA). Acetonitrile, ammonium hydroxide (NH_4OH) and anhydrous sodium sulfate (Na_2SO_4) were obtained from EMD Chemicals (Gibbstown,NJ, USA). Disodium ethylenediaminetetraacetate (Na_2EDTA), formic acid, and sodium chloride (NaCl) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Waters Oasis hydrophilic–lipophilic balance

(HLB) cartridge was acquired from Waters Corporation (Milford, MA, USA). Ceramic homogenizers, C18, and primary secondary amine (PSA) powders were purchased from Agilent Technologies (Santa Clara, CA, USA). Ultrapure water was generated from a Milli-Q water purification system (Millipore, Billerica, MA, USA).

Table 3.1. Physicochemical properties of the pharmaceuticals studied.

Pharmaceuticals	Molecular weight ($g\ mol^{-1}$)	Molecular volume[a] (nm^3)	Water Solubility[b] ($mg\ L^{-1}$)	pK_a[b]	Predominant species (%) (pH at 5.8)	log K_{ow}[b]	log D_{ow}[c]
Acetaminophen	151.16	0.201	14000	9.38	Neutral: 100	0.46	0.46
Caffeine	194.19	0.221	21600	10.4	Cationic:100	−0.07	−4.67
Carbamazepine	236.27	0.311	18	2.3, 13.9[d]	Neutral: 100	2.45	2.45
Lamotrigine	256.1	0.271	170	5.7	Neutral: 56	0.99	0.74
Carbadox	262.22	0.301[e]	1755[e]	1.8, 10.5[f]	Neutral: 100	−1.22[f]	−1.22
Estrone	270.37	0.385	30	10.33[g]	Neutral: 100	3.13	3.13
β-Estradiol	272.38	0.387	3.9	10.33[g]	Neutral: 100	4.01	4.01
Triclosan	289.54	0.322	10	7.9	Neutral: 99	4.76	4.76
Trimethoprim	290.32	0.385	400	7.12	Cationic:95	0.91	−0.43
Lincomycin	406.54	0.520[e]	927	7.6	Cationic:98	0.2	−1.61
Oxytetracycline	460.43	0.445	313	3.57, 7.49, 9.44[h]	Zwitterionic: 97	−0.9	−0.91
Monensin sodium	692.87	0.918	Slightly soluble[h]	4.3[i]	Anionic: 97	5.43[i]	3.92
Tylosin	916.10	1.221[e]	5	7.73	Cationic:99	−1.63	0.31

[a] Calculated molar volume acquired from USEPA website: https://comptox.epa.gov/dashboard/.

[b] From TOXNET database: http://toxnet.nlm.nih.gov/index.html.

[c] Calculated at pH 5.8 (nutrient solution) by the equation from Carballa et al.[27]

[d] Calisto and Esteves.[28]

[e] From ChemSpider database: http://www.chemspider.com/.

[f] Song et al.[29]

[g] From Drugbank database: https://www.drugbank.ca/.

[h] Rakshit et al.[30]

[i] From Guidechem database: http://www.guidechem.com/.

Lettuce

Black Seeded Simpson lettuce seeds (*Lactuca sativa*) were placed on moistened paper tissues until they began to sprout (2 to 3 days). The seedlings were inserted through pre-drilled holes in thin Styrofoam plates, fixed with stonewool, and then placed in hydroponic nutrient solution. The nutrient solution contained 9.375 g of MaxiGro plant nutrient (10−5−14) (General Hydroponics, Sevastopol, CA, USA) in 15 L of deionized water. The initial pH and electrical conductivity (EC) of the nutrient solution was 5.8, and 0.4 mS/cm, respectively. During the lettuce cultivation, the pH and EC values were checked every 24 hours. Solution pH was maintained within 5.6–5.8 using diluted citric acid (Earth Juice Natural Down, Hydrofarm, Petaluma, CA, USA) and potassium bicarbonate ($KHCO_3$) (Earth Juice Natural Up, Hydrofarm, Petaluma, CA, USA), and EC gradually increased from 0.4 to 0.8 mS/cm by replenishing freshly prepared nutrient solution before exposure of lettuce to pharmaceuticals. The lighting period was set up as 16 hours per day with light intensity of 150 μmol/m^2/s using a LED Light source (Apollo Horticulture Full Spectrum 300W, Rowland Heights, CA, USA). The nutrient solution was continuously aerated with a fusion air pump. The lettuce seedlings grew for approximately 22 days at 18 ºC, and reached 35–40 cm in height and 8.0–10.5 g of biomass with well-developed roots.

Lettuce Uptake of Pharmaceuticals

The experiment of pharmaceutical uptake by lettuce was conducted using the hydroponic system described above. Two, 22-day-old, lettuce plants were transferred to each Erlenmeyer flask containing 210 mL of nutrient solution spiked with thirteen pharmaceuticals at the initial concentration of 50 ng mL^{-1} for each pharmaceutical. The flasks were wrapped with aluminum foil to minimize the potential photodegradation of the pharmaceuticals. During the exposure period, the pH and EC values of the nutrient solution were measured every 24 hours, and the water loss

from the flasks (due to transpiration by lettuce) was determined gravimetrically. Fresh nutrient solution free of pharmaceuticals was replenished to the flasks to maintain the initial volume (210 mL). The solution pH and EC were maintained at 5.8 and 0.8 mS/cm, respectively, using the methods described above. At 12, 24, 48, 72, 105 and 144 hours, three flasks were sacrificed to collect lettuce samples for extraction and analysis of pharmaceuticals. The experimental controls included lettuce plants without pharmaceutical exposure, and nutrient solution with pharmaceuticals but without lettuce. The collected lettuce was rinsed with deionized water, separated into roots and shoots, and then immediately frozen at –85 °C for 3 hours. The frozen samples were freeze-dried, ground to powders, and stored at –20 °C prior to extraction.

Lincomycin Uptake by Whole Lettuce and Lettuce Shoots

The uptake of lincomycin by whole lettuce plants from roots versus shoots only (after removal of roots) was conducted using the hydroponic system described in the manuscript. Two whole lettuce plants and lettuce shoots only were transferred to each Erlenmeyer flask containing 210 mL of nutrient solution spiked with 50 ng mL^{-1} of lincomycin. For the uptake by whole lettuce plants, the roots were immersed in the nutrient solution. For the shoot-only lettuce samples, only ~1 cm of the shoots (incised bottom) was immersed in the nutrient solution. After 48 hours of exposure, lettuce samples were collected, rinsed with deionized water, and extracted for lincomycin analysis using LC-MS/MS.

Pharmaceutical Extraction

Pharmaceuticals in solution phase were extracted using a HLB solid phase extraction (SPE) cartridge and analyzed by a Shimadzu Prominence high-performance liquid chromatography (Columbia, MD, USA) coupled with Sciex 4500 QTrap mass spectrometer (Foster City, CA, USA) (LC-MS/MS). Twenty mL of each solution sample were mixed with 1.0 mL of 3000 mg L^{-1} of

Na_2EDTA. The samples were passed through the HLB SPE cartridges that were preconditioned with 3.0 mL of methanol and 5.0 mL of water. Afterwards the cartridges were washed with 5.0 mL of water, and the retained pharmaceuticals were eluted with 5 mL of methanol. The eluted liquid samples were stored at −20 °C prior to pharmaceutical analysis using the LC-MS/MS.

Pharmaceuticals in lettuce roots and shoots were extracted using a quick, easy, cheap, effective, rugged and safe (QuEChERS) method modified from the Chapter II. Dried lettuce samples (250.0 mg of shoots or 100.0 mg of roots) were first mixed with 1.0 mL of 300 mg L^{-1} of Na_2EDTA for 1 min in 50-mL polypropylene centrifuge tubes. Two ceramic homogenizers and 1.75 mL of methanol were added to the centrifuge tubes and then vortexed for 1.5 min. Afterwards, 2.0 g of Na_2SO_4 and 0.5 g of NaCl were added to the tubes and mixed with the samples for 1.5 min to improve extraction efficiency. The extracts in methanol were collected by centrifugation at 5050 *g* for 10 min. Samples were then consecutively mixed with 3.25 mL of acetonitrile for 1.5 min and centrifuged again at 5050 *g* for 10 min. Later, 0.42 mL of the extracts in methanol and 0.78 mL of the extracts in acetonitrile were combined (total of 1.2 mL) and cleaned up using disperse SPE (d-SPE) sorbents. The d-SPE sorbents contained 12.5 mg of C18, 12.5 mg of PSA and 225 mg of Na_2SO_4. The combined 1.2 mL of extracts and d-SPE sorbents were mixed vigorously for 1 min, centrifuged at 9240 *g* for 10 min, and the supernatants were transferred to clean glass vials for pharmaceutical analysis using the LC-MS/MS. The pharmaceuticals were quantified using matrix-matched standard curves. The extraction efficiencies ranged between 85.1–116.3% for the pharmaceuticals in nutrient solution, between 87.5–106.9% for the pharmaceuticals in shoots and between 85.2−129.6% for the pharmaceuticals in roots (Table 3.2).

Table 3.2. Extraction efficiency (%) of pharmaceuticals from nutrient solution and from lettuce roots and shoots using the modified-QuEChERS method.

Pharmaceuticals	Nutrient solution	Modified-QuEChERS method	
		Shoots	Roots
Acetaminophen	100.7 ± 1.9[a]	87.5 ± 5.9	85.2 ± 10.7
Caffeine	116.3 ± 4.5	91.8 ± 4.4	106.2 ± 3.3
Carbamazepine	113.8 ± 10.4	95.8 ± 10.9	104.9 ± 1.8
Lamotrigine	109.1 ± 9.4	101.1 ± 2.7	91.7 ± 8.2
Carbadox	99.2 ± 1.9	102.1 ± 5.6	111.4 ± 6.8
Estrone	104.5 ± 7.4	102.8 ± 3.2	117.6 ± 3.8
β-Estradiol	113.3 ± 11.9	104.7 ± 5.7	115.8 ± 8.0
Triclosan	103.3 ± 17.6	92.5 ± 2.6	105.3 ± 1.3
Trimethoprim	114.0 ± 17.0	105.3 ± 6.4	102.8 ± 3.7
Lincomycin	101.4 ± 2.7	106.9 ± 5.2	129.6 ± 3.9
Oxytetracycline	97.7 ± 4.6	92.0 ± 13.1	127.2 ± 3.6
Monensin sodium	85.1 ± 7.0	86.3 ± 1.6	105.1 ± 9.2
Tylosin	112.6 ± 8.3	98.0 ± 4.4	119.2 ± 4.1

[a] Mean ± standard deviation (n = 3).

LC-MS/MS Analysis

Pharmaceuticals obtained from sample extraction were analyzed using the LC-MS/MS under either positive or negative ionization mode. An Agilent Eclipse Plus C18 column (50 mm × 2.1 mm, particle size 5μm, Santa Clara, CA, USA) was used to separate the thirteen pharmaceuticals. Under the positive ionization mode, the binary mobile phase consisted of water (Phase A) and a mixture of acetonitrile and methanol (65/35, v/v) (Phase B), and both contained 0.3% of formic acid. Following 2 min of pre-equilibration with 100% of Phase A, the gradient program was set as follows: the Phase B increased to 40% during 0.1–1.0 min, then to 70% between 1.0–2.0 min, to 80% between 2.0–3.0 min, then to 100% between 3.0–3.5 min, and finally 100% of Phase B was maintained until 7.2 min. Under the negative ionization mode, Phase A was water with 0.005% NH_4OH, and Phase B was a mixture of acetonitrile and methanol (9/1, v/v). After 2 min pre-equilibration with 100% of Phase A, Phase B increased to 5% between 0.1–2.0 min, and to 100% between 2.0–10.0 min that was held until 12.0 min. The flow rate was 0.35 mL/min, and the sample injection volume was 10 μL. In the tandem mass spectrometer, a scan type of multiple reaction monitoring (MRM) was set up for precursor and product ion transitions. The ionspray voltage, temperature, curtain gas pressure and entrance potential were 5000 V, 700 °C, 20 psi and 10 V for the positive ionization mode, and 4500 V, 700 °C, 40 psi, and −10 V for the negative ionization mode, respectively. The analytic parameters are provided in Table 3.3.

Sorption by Lettuce Roots

Pharmaceutical sorption by lettuce roots from nutrient solution was measured using a batch equilibration method. The freeze-dried lettuce root powders were passed through a 250-μm sieve, and then used in the sorption experiments. Root powder of ≤ 250 μm (25 mg) was weighed into glass centrifuge tubes and mixed with 20.0 mL of nutrient solution containing the thirteen

pharmaceuticals at concentration of 0, 10, 20, 30, 40 or 50 ng mL^{-1} for each pharmaceutical. The experimental controls consisted of the nutrient solution with pharmaceuticals devoid of lettuce root powders. The tubes were shaken on an Innova 2300 platform shaker (New Brunswick Scientific, Edison, NJ, USA) at 150 rpm for 24 hours in dark, and centrifuged at 1460 *g* for 15 min. The supernatant was collected and analyzed using the LC-MS/MS. The mass loss of each pharmaceutical between the initial and final nutrient solution was assumed to be sorbed by lettuce roots.

Table 3.3. Precursor/product ion transitions and mass spectrometer parameters used for identification and quantification of pharmaceuticals.

Chemicals	Precursor ion (m/z)	Product ion[a] (m/z)	DP[b] (V)	CE[c] (V)	CXP[d] (V)
Positive ionization mode					
Acetaminophen	151.9	**110**	**60**	**20**	**8**
		93	60	30	6
Caffeine	195.0	**138**	**60**	**30**	**10**
		110	60	30	6
Carbamazepine	237.0	**193.7**	**80**	**30**	**10**
		192	80	30	12
Lamotrigine	255.9	**210.7**	**86**	**43**	**14**
		165.7	91	37	13
Carbadox	262.9	**230.9**	**60**	**20**	**12**
		144.8	60	30	8
Trimethoprim	291.0	**261**	**80**	**30**	**12**
		230	100	30	12
Lincomycin	407.1	**126**	**60**	**30**	**8**
		359.1	80	30	6
Oxytetracycline	461.0	**426.1**	**60**	**30**	**8**
		283.1	60	50	8
Monensin sodium	694.2	**676.3**	**120**	**50**	**6**
		480	120	70	6
Tylosin	916.3	**173.8**	**100**	**40**	**10**
		83	60	100	4
Negative ionization mode					
Estrone	269.1	**145**	**−105**	**−48**	**−19**
		143	−105	−66	−15
β-Estradiol	271.0	**182.9**	**−40**	**−50**	**−12**
		144.8	−40	−50	−4
Triclosan	**286.9**	**35**	**−40**	**−30**	**−6**
	289.0	35	−40	−20	−8

[a] The product ion coupled with precursor ion used to quantify (in bold) and qualify pharmaceuticals.
[b] Declustering potential.
[c] Collision energy.
[d] Collision cell exit potential.

RESULTS

Pharmaceutical Uptake by Lettuce

In the lettuce-free controls, no apparent loss of pharmaceuticals was found during the experimental period (144 hours) (Figure 3.1). In the presence of lettuce, the pharmaceutical concentrations in nutrient solution decreased with time (Figure 3.2). Caffeine, carbadox, carbamazepine, lamotrigine, monensin sodium, and trimethoprim solution concentrations gradually decreased as low as 10% of the initial concentration during the 144 hours of exposure. Lincomycin, oxytetracycline, and tylosin concentrations were relatively stable with > 80% of the applied pharmaceuticals remaining in the nutrient solution. In contrast, acetaminophen, β-estradiol, estrone, and triclosan concentrations dramatically decreased to < 10 ng mL^{-1}, i.e., < 20% of the initially added pharmaceuticals, during the first 24 hours of exposure.

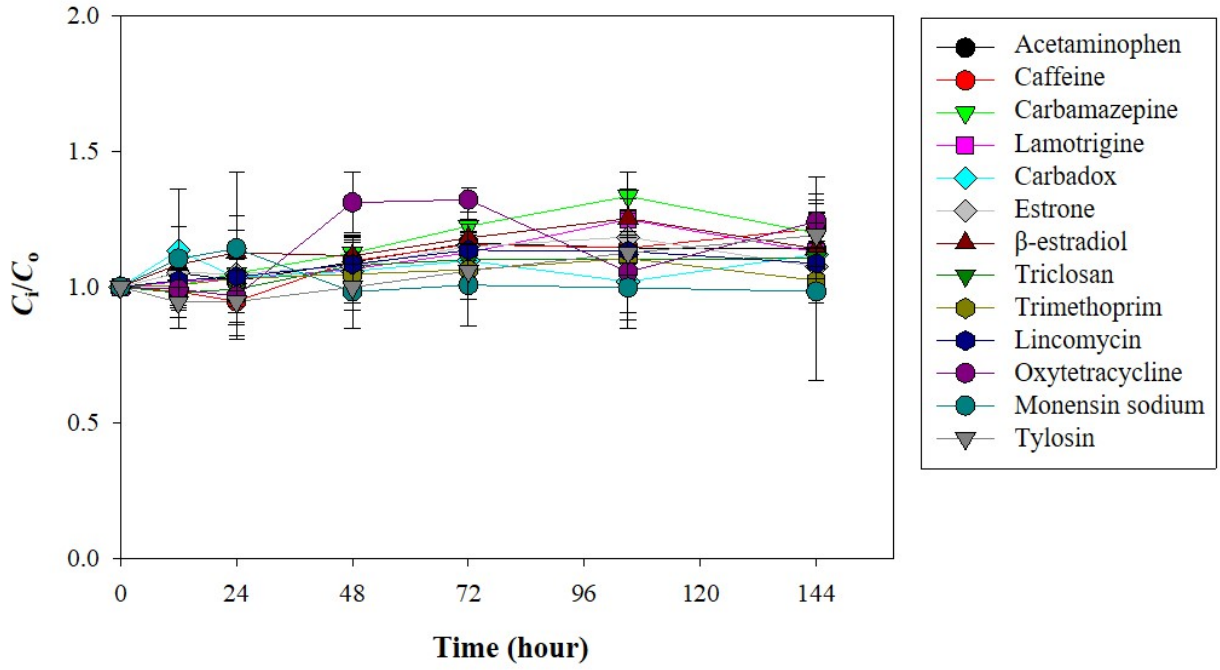

Figure 3.1. Relative concentrations (C_i/C_0) of the thirteen pharmaceuticals in lettuce-free nutrient solution through the 144 hours of experimental period. C_i is the measured concentration at a given time, and C_0 is the initial concentration.

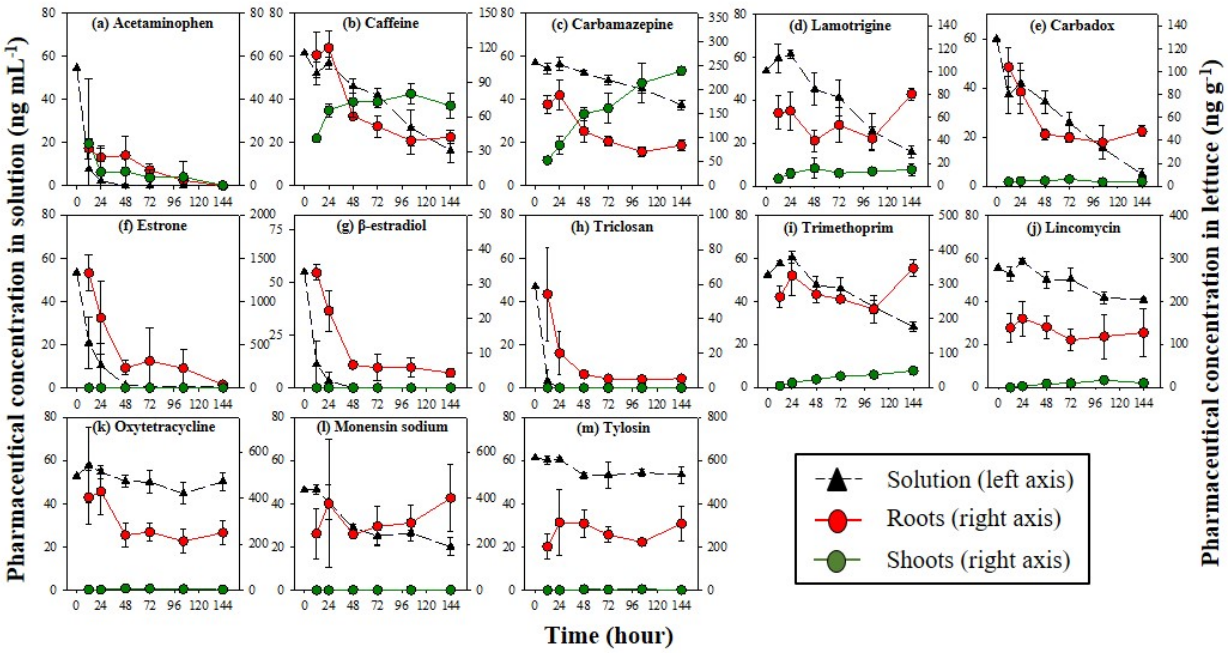

Figure 3.2. Pharmaceutical concentration in solution (left y-axis), lettuce roots and shoots (right y-axis) as a function of exposure time. The thirteen pharmaceuticals from (a) to (m) were arranged in the order of increasing molecular weight.

The exposure of lettuce plants to the pharmaceuticals in solution did not render adverse impact in appearance, or a significant difference in biomass growth compared to those in the pharmaceutical-free controls (*t*-test, $p > 0.05$) (Figure 3.3). Most pharmaceuticals accumulated in the lettuce with concentrations ranging from 23.3 to 697.9 ng g^{-1} in roots and from 3.3 to 258.2 ng g^{-1} in shoots. Relatively high concentrations in lettuce roots were found for lincomycin (80.5–189.3 ng g^{-1}), monensin sodium (209.5–565.8 ng g^{-1}), oxytetracycline (171.9–294.4 ng g^{-1}), trimethoprim (185.4–365.5 ng g^{-1}) and tylosin (207.0–362.6 ng g^{-1}). Caffeine, carbadox, carbamazepine, and lamotrigine concentrations in roots were < 136.9 ng g^{-1}. The uptake of acetaminophen, β-estradiol, estrone, and triclosan was < 10 ng g^{-1} in lettuce roots. The averaged pharmaceutical accumulation in roots (during the 48 and 144 h of uptake experiment) ranked in the order of monensin sodium > trimethoprim ≈ oxytetracycline > tylosin ≈ lincomycin > carbamazepine > lamotrigine ≈ caffeine ≈ carbadox > estrone > acetaminophen ≈ triclosan > β-estradiol. Pharmaceuticals accumulated in lettuce roots could also be transported upward to shoots. Caffeine, carbamazepine, lamotrigine, and trimethoprim concentration in shoots reached as high as 258.2 ng g^{-1} during the experimental period, whereas less amounts of acetaminophen, carbadox, lincomycin, monensin sodium, and oxytetracycline were found in shoots (e.g., < 20 ng g^{-1}). It is noted that after 48 hours of exposure the concentrations of caffeine or carbamazepine in shoots were greater than that in roots, indicating that these two pharmaceuticals could be readily transported to the upper portions of lettuce, which could be attributed to their weak affinity to lettuce roots (to be discussed below). Estrone, β-estradiol, and triclosan were found to accumulate primarily in lettuce roots. This result is consistent with two previous hydroponic studies,[11, 31] whereas several other studies reported that triclosan could enter the shoots of soybean, cabbage,

ryegrass, and radish from soils in pot experiments[5, 7, 32] due likely to higher applied concentrations and longer exposure periods.

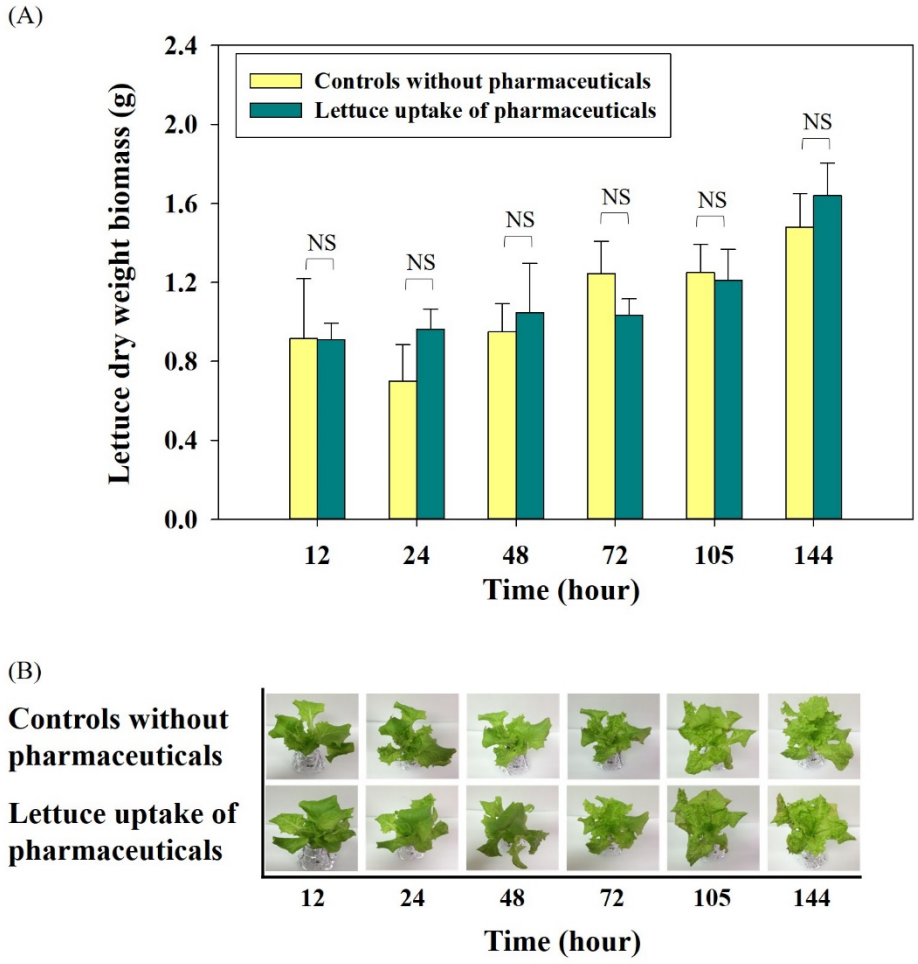

Figure 3.3. (A) Lettuce dry weight biomass and (B) appearance through the 144 hours of experimental period. NS = not significant ($p > 0.05$).

During the 144-hour exposure period, lettuce root and shoot biomass increased approximately 1.5 and 1.9 times, respectively. The increase in biomass could dilute pharmaceutical concentrations in lettuce; therefore, the accumulated pharmaceutical mass was used to calculate their distributions in nutrient solution, lettuce roots and shoots as shown in Figure 3.4. Among the tested pharmaceuticals, the sum of mass recoveries from nutrient solution, lettuce roots and shoots were > 60% for carbamazepine, lincomycin, monensin sodium, oxytetracycline, trimethoprim, and tylosin after the 144 hours of exposure. Acetaminophen, β-estradiol, estrone, and triclosan were readily metabolized with the mass recoveries of < 25% during the first 48 hours of exposure. Many pharmaceuticals in plants can undergo enzyme-mediated phase I reactions and/or phase II conjugations.[22, 33, 34] For example, acetaminophen and triclosan were conjugated with glucoside, glutathione and other plant components in vegetables.[35-37] Carbadox, lamotrigine, lincomycin, monensin sodium, oxytetracycline, trimethoprim, and tylosin demonstrated greater mass accumulation in roots than in shoots. For caffeine and carbamazepine, the accumulation in shoots was significantly greater than that in roots after 48 hours of exposure (Figure 3.4b and 3.4c). The differences between pharmaceutical accumulation in roots and shoots could result from multiple confounding factors such as transport pathways of pharmaceuticals in lettuce, physicochemical properties of pharmaceuticals, and their affinities to roots, which will be discussed below.

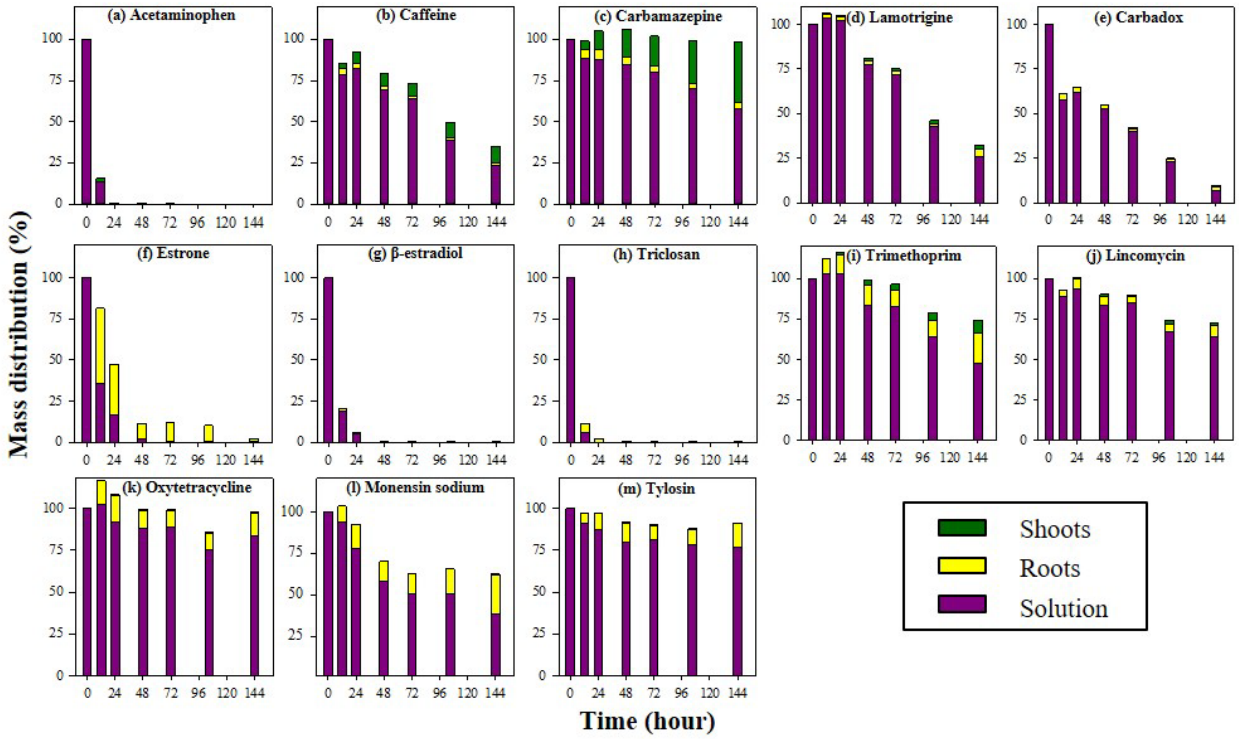

Figure 3.4. Mass distribution of pharmaceuticals in nutrient solution, lettuce roots and shoots as a function of exposure time. The thirteen pharmaceuticals from (a) to (m) were arranged in the order of increasing molecular weight.

Root Concentration Factor and Translocation Factor

The RCF values calculated on the basis of fresh lettuce weight at different sampling time fell within a relatively narrow range during 48 to 144 hours of exposure. Therefore, the RCF values were averaged with the standard deviations presented as error bars in Figure 3.5A. For the readily metabolized pharmaceuticals acetaminophen, β-estradiol and triclosan, the RCF values were calculated using the data obtained during the first 12 hours of exposure. The calculated RCF values follow the order of estrone (358.9 mL g^{-1}) > triclosan (229.2 mL g^{-1}) > acetaminophen (28.6 mL g^{-1}) > monensin sodium (13.1 mL g^{-1}) > trimethoprim (7.4 mL g^{-1}) > β-estradiol (6.2 mL g^{-1}) > tylosin (5.2 mL g^{-1}) > oxytetracycline (4.9 mL g^{-1}) > carbadox (4.1 mL g^{-1}) > lincomycin (2.8 mL g^{-1}) > lamotrigine (2.3 mL g^{-1}) > carbamazepine (2.0 mL g^{-1}) > caffeine (1.7 mL g^{-1}). Statistical analysis revealed no significant difference (one-way ANOVA, $p > 0.05$) in the RCF values at different exposure periods for carbamazepine, lincomycin, monensin sodium, oxytetracycline, and tylosin, indicating the lettuce root uptake of these pharmaceuticals approached a quasi-equilibrium after 48 hours. However, significant differences (one-way ANOVA, $p < 0.05$) in the RCF values were found for caffeine, lamotrigine, carbadox, and trimethoprim, and the RCF values increased with the exposure time, due plausibly to their continual transport from solution to lettuce roots and then to shoots. In this study, the thirteen pharmaceuticals were presented as neutral, cationic, anionic or zwitterionic species in nutrient solution (Table 3.1); log D_{ow} values (in nutrient solution of pH 5.8) ranged from −4.67 to 4.76, and the corresponding log RCF from 0.2 to 2.6 mL g^{-1}. The linear regression between log RCF and log D_{ow} demonstrated a poor relationship with $R^2 = 0.36$ (Figure 3.6A). This result suggests that the uptake of pharmaceuticals by lettuce roots is different from hydrophobic compounds that is governed primarily by partitioning into plant tissues.[38, 39]

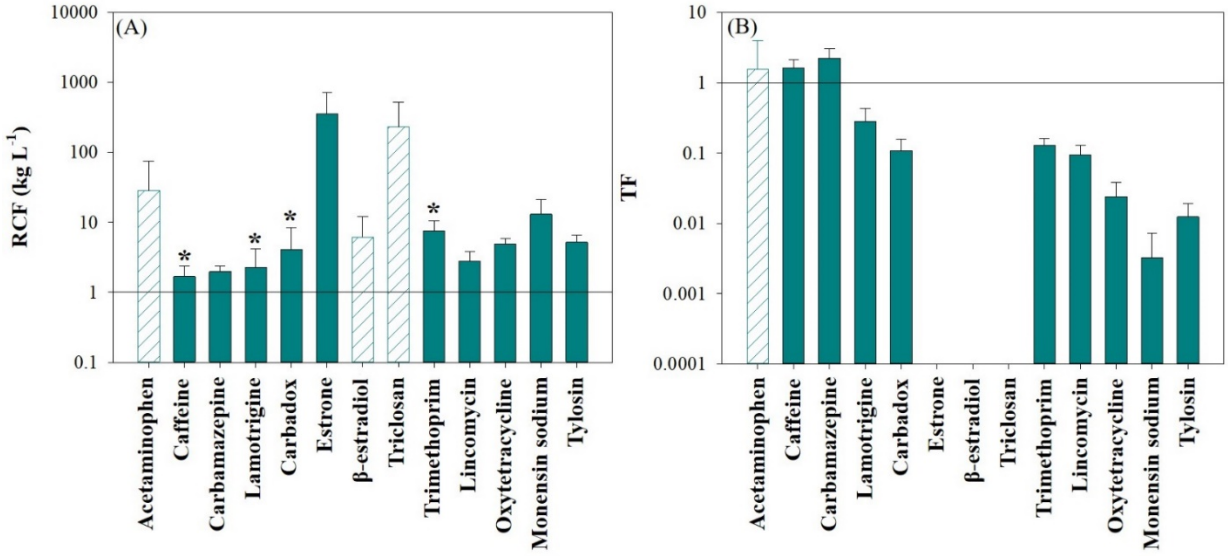

Figure 3.5. (A) Root concentration factors (RCF) and (B) translocation factors (TF) of pharmaceuticals in lettuce on fresh weight basis. Solid bar represents the average value during 48 to 144 hours of pharmaceutical exposure, and the open bar represents the average values of 12 hours of pharmaceutical exposure. Asterisk * indicates the significant difference ($p < 0.05$) among the values at different exposure time.

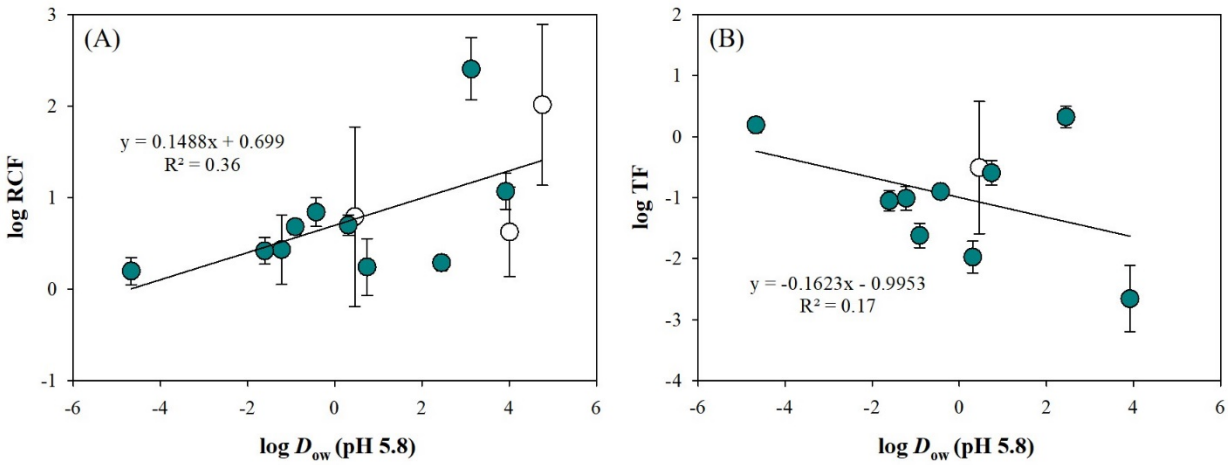

Figure 3.6. Relationships between log D_{ow} values and lettuce root concentration factors (RCF) (A) or translocation factors (TF) (B). Solid circles represent the average values from 48 to 144 hours of exposure, and open circles represent the values at the 12 hours of exposure.

Pharmaceutical transport from roots to shoots was evaluated by TF, and the averaged TF values (during 48−144 hours of uptake) are presented in Figure 3.5B. The TF value for acetaminophen was calculated during the first 12 hours of exposure. The TF values could not be obtained for β-estradiol, estrone and triclosan due to their substantial metabolization in lettuce and minimal amount detected in shoots. The TF values for caffeine and carbamazepine were 1.6 and 2.2, indicating these two pharmaceuticals were readily translocated, and largely accumulated in shoots rather than in roots. For other pharmaceuticals, the TF values were all < 1.0, and ranked as lamotrigine (0.3) > carbadox (0.1) ≈ trimethoprim (0.1) ≈ lincomycin (9.5×10^{-2}) > oxytetracycline (2.4×10^{-2}) > tylosin (1.2×10^{-2}) > monensin sodium (3.2×10^{-3}). Lettuce roots are the major domain for accumulating these pharmaceuticals. Although no apparent relationship was observed between log TF and log D_{ow} (Figure 3.6B), the TF values were found to generally decrease with increasing pharmaceutical molecular weight (MW) (Figure 3.5B and Table 3.1). Among the tested pharmaceuticals, acetaminophen, caffeine, and carbamazepine MW was < 240 g mol^{-1}, and their TF values were > 1.0. The TFs of carbadox, lamotrigine, and trimethoprim (250 g mol^{-1} < MW < 300 g mol^{-1}) ranged from 0.1 to 0.3. For those pharmaceuticals with MW > 400 g mol^{-1}, their TFs were < 0.1. These findings suggested that the MW or molecular size of pharmaceuticals, rather than their lipophilicity, notably influences their transport from lettuce roots to shoots.

Pharmaceutical Sorption to Lettuce Roots

Sorption isotherms of the pharmaceuticals were well fitted with the linear model, from which sorption coefficients (K_p) were estimated. Sorption of pharmaceuticals by dried lettuce roots was categorized into weak sorption (caffeine, carbadox, carbamazepine, and lincomycin) with K_p < 0.05 L g^{-1}, intermediate sorption (lamotrigine, monensin sodium, oxytetracycline, trimethoprim,

and tylosin) with K_p between 0.38–2.22 L g^{-1}, and strong sorption (acetaminophen, β-estradiol, estrone, and triclosan) with K_p > 12.01 L g^{-1} (Figure 3.7). The K_p values of the pharmaceuticals in the strong sorption category could be overestimated because these pharmaceuticals may substantially react with root tissues during the sorption experiment, as supported by their poor mass recoveries in the hydroponic experiment (Figure 3.4).

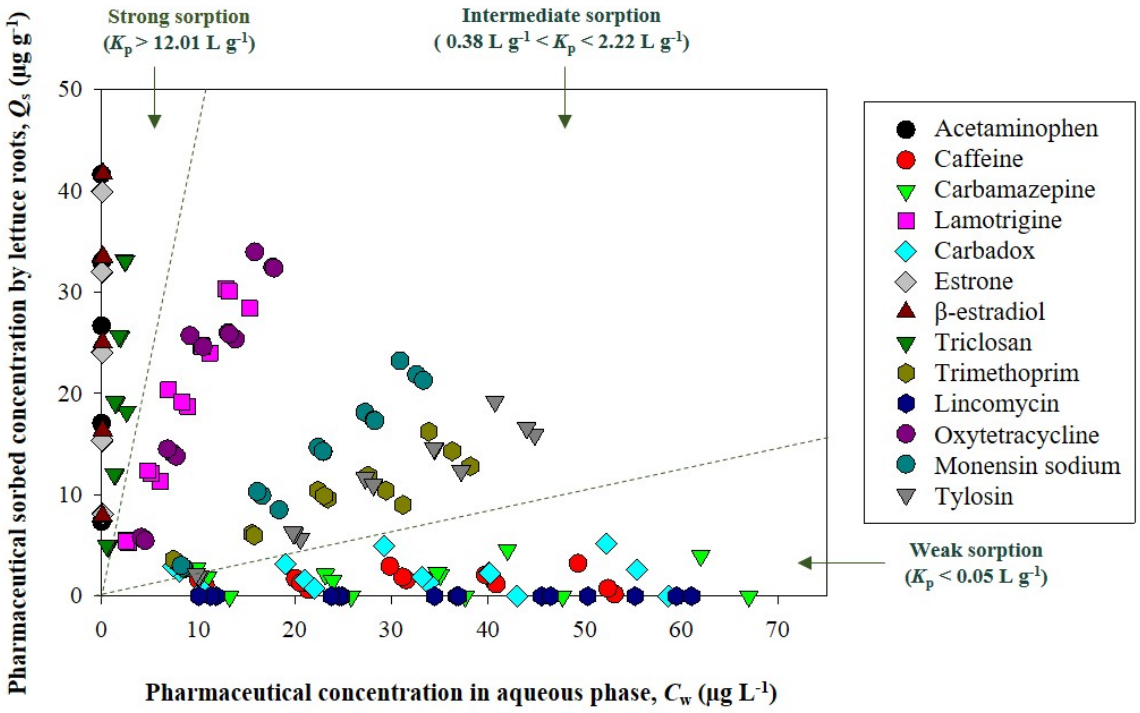

Figure 3.7. Sorption of pharmaceuticals by lettuce roots from nutrient solution. Sorption isotherms were fit to $Q_s = K_p C_w$, where K_p is sorption coefficient (L g^{-1}).

DISCUSSION

This study revealed that many pharmaceuticals could enter lettuce and distribute in lettuce roots and shoots to various degrees. The extent of transport from roots to shoots decreased with increasing MW or molecular size. Poor relationship between log RCF-log D_{ow} or log TF-log D_{ow} indicates that the accumulation and transport of most pharmaceuticals in lettuce are not primarily governed by their lipophilic characteristics, probably due to their high water solubilities. In general, pharmaceuticals enter lettuce roots and transport to shoots with transpiration water flow, in which the molecular size of pharmaceuticals could determine their diffusion into roots, and their affinity to roots could influence their transport to lettuce shoots.

Pharmaceuticals can be transported with water flow into plant roots via symplast and apoplast pathways, and only those pharmaceuticals entered the symplast pathway could be transported upward to plant shoots (Figure 3.8).[20, 21, 23] Hence, the diffusion of pharmaceuticals across cell membranes (especially endodermis impregnated with Casparian strip) is the key process influencing their long distance transport and distribution in lettuce. In general, water and small-sized organic molecules could diffuse through cell membranes. Water molecules can enter the cells by osmosis or through aquaporin,[40, 41] whereas organic compounds (with net neutral charge and MW < 450 g mol^{-1}) could enter the cells by passive diffusion across lipid bilayer membranes.[42, 43] Ionic species of pharmaceuticals could enter cells via integral proteins on cell membranes, which is driven primarily by concentration gradient.[23, 26, 44-46] However, large-sized molecules experience relatively slow diffusion rate through plant cell membranes.[42-45]

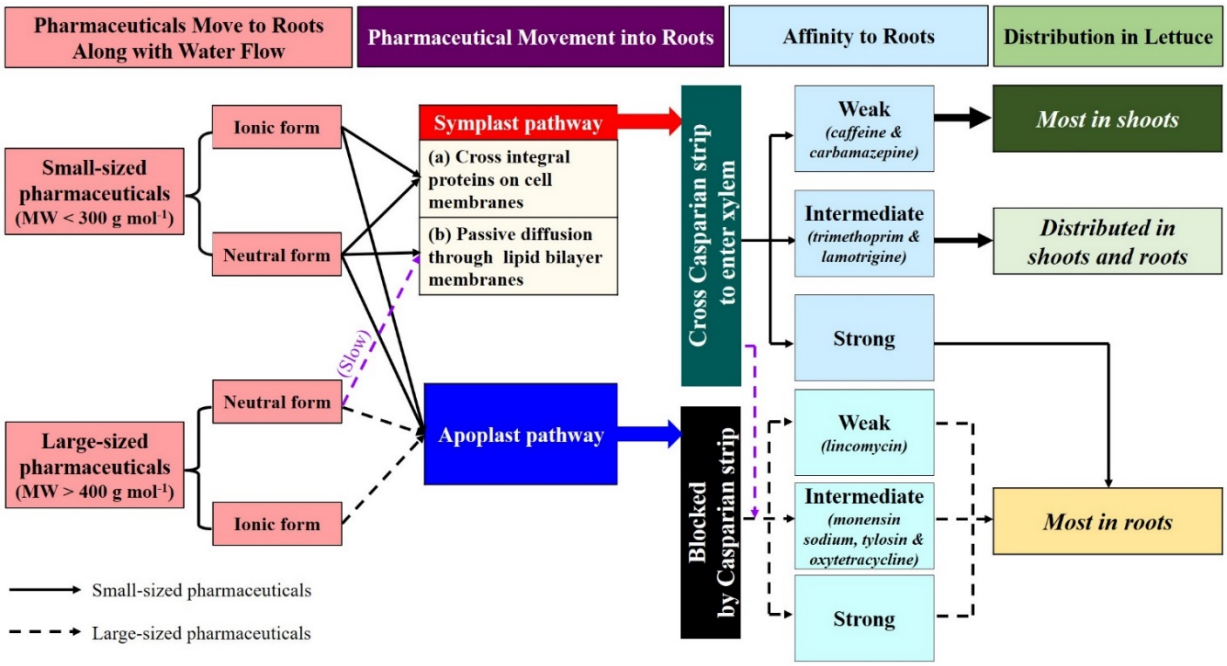

Figure 3.8. Scheme of pharmaceutical movement into lettuce roots, affinity to roots, and distribution in lettuce.

Molecular volume (MV) of pharmaceuticals in this study demonstrates a strong positive linear relationship with their MW ($R^2 = 0.97$) (Table 3.1 and Figure 3.9). Therefore, MW is clearly an appropriate parameter here to describe their molecular size. Caffeine (MW 194 g mol^{-1}, 100% cationic), carbamazepine (MW 236 g mol^{-1}, 100% neutral), lamotrigine (MW 256 g mol^{-1}, 44% cationic + 56% neutral) and trimethoprim (MW 290 g mol^{-1}, 95% cationic + 5% neutral) apparently entered lettuce roots and were transported to shoots with the accumulation in shoots in the order of carbamazepine > caffeine > trimethoprim > lamotrigine (Figure 3.4b, 3.4c, 3.4d, 3.4i). This result suggests that small-sized pharmaceuticals (MW < 300 g mol^{-1}) could enter lettuce roots plausibly by the symplast pathway, and then move upward to the shoots. Although it was reported that ionic species of pharmaceuticals might be restricted from passing through plant cell walls or membranes,[19, 22] caffeine and trimethoprim existing primarily in cationic form in solution still passed through root membranes, and were transported to shoots. Carbadox (MW 262 g mol^{-1}, 100% neutral) was readily metabolized in lettuce; at the 144 hours only 7.2% of the initially applied amount remained in nutrient solution, and 2.2% in roots and 0.6% in shoots (Figure 3.4e). The substantial metabolization of carbadox in lettuce hinders the evaluation of its accumulation and transport in plant. Lincomycin (MW 406 g mol^{-1}, 98% cationic + 2% neutral), monensin sodium (MW 693 g mol^{-1}, 3% neutral + 97% anionic) and tylosin (MW 916 g mol^{-1}, 99% cationic + 1% neutral) showed minimal concentrations in lettuce shoots (< 10 ng g^{-1}). These relatively large-sized molecules (> 0.52 nm^3) might enter lettuce roots through the apoplast pathway, hence being excluded from the entrance into plant cells as evidenced by their limited upward movement to shoots.[43, 47] Oxytetracycline (MW 460 g mol^{-1}, 3% cationic + 97% zwitterion) also has a relatively large size (MV = 0.45 nm^3) and existed predominantly in zwitterionic form, which could pass through cell membranes via passive diffusion but at a slow rate.[48, 49] As a result,

oxytetracycline accumulated mainly in lettuce roots (Figures 3.2k and 3.4k) with low upward transport to shoots attributed to its affinity to lettuce roots (Figure 3.7).

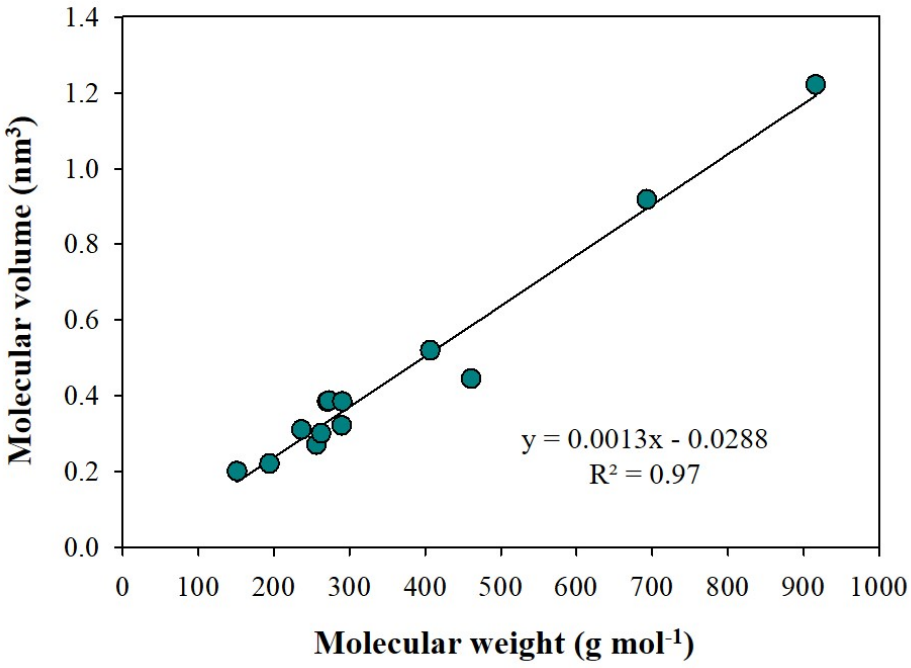

Figure 3.9. Relationship between molecular volume and molecular weight of pharmaceuticals.

Once pharmaceuticals enter lettuce roots along water flow, they would interact with root constituents, which could influence their transport to shoots. Strong interaction of pharmaceuticals with root constituents could render relatively high accumulation in roots and reduce their upward transport to shoots. Caffeine and carbamazepine were weakly affiliated to lettuce roots, and have small MW (Figure 3.7 and Table 3.1). Therefore, they could easily enter xylem and transport to shoots with less accumulation in roots, as evidenced by TF >1 (Figure 3.5). Similar results of higher concentration in leaves than in roots was also observed for these two chemicals in carrots and sweet potatoes.[12] Lamotrigine and trimethoprim exhibited an intermediate affinity to lettuce roots (Figure 3.7); lettuce roots retained more lamotrigine and trimethoprim than those accumulated in shoots (Figures 3.2d, 3.2i and 3.4d and 3.4i). Lincomycin existed predominantly as cation (> 98%) in nutrient solution, and was weakly affiliated with lettuce roots. However, the large molecular size of lincomycin might restrict its entrance into plant cells via the symplast pathway, leading to accumulation mainly in roots, and low upward transport to shoots (Figures 3.2 and 3.5). This was confirmed with another hydroponic experiment which compared lincomycin uptake to lettuce shoots directly (by exposing lettuce shoots to nutrient solution after removal of roots) with the uptake by lettuce whole plants. In the experiment of lincomycin uptake directly by shoots, lincomycin could be taken up and reach to 37.5 ng g^{-1} in lettuce shoots (without roots), while no lincomycin was detected in lettuce shoots in the experiment of uptake through roots. These results indicate that cationic lincomycin could enter lettuce roots predominantly through the apoplast pathway, and its upward movement in xylem was blocked at the endodermis most likely by the Casparian strip. The large-sized molecules monensin sodium and tylosin were retained predominantly in roots with very limited amount transported to shoots, which is, again, attributed to the inhibition by the Casparian strip. Zwitterionic oxytetracycline could slowly enter lettuce via

the symplast pathway; however, the intermediate affinity to lettuce roots might limit its upward transport to shoots (Figures 3.2k and 3.4k). The stronger affinity of oxytetracycline, monensin sodium, and tylosin to root constituents (compared to lincomycin) resulted in the higher accumulation in roots (Figure 3.4j to 3.4m).

Based on the above discussion, the scheme of pharmaceutical uptake and movement in lettuce is summarized in Figure 3.8. Pharmaceuticals of small size (MW < 300 g mol^{-1}) could enter lettuce with water flow via the symplast pathway in which neutral pharmaceutical species could enter cells by passive diffusion, and ionic species via integral transport proteins into root cells. Water flow is the major carrier to distribute pharmaceuticals in roots and shoots. The small-sized pharmaceuticals carried by water flow in the symplast pathway could pass through the Casparian strip to the xylem, followed by the upward transport to shoots. Pharmaceutical sorption by lettuce roots reduces the amount and rate of pharmaceutical upward transport to shoots. These processes collectively cause the accumulation of small-sized pharmaceuticals such as caffeine, carbamazepine, trimethoprim, and lamotrigine in either lettuce roots or shoots, with TF values of the latter two less than the former two due to their stronger affinity to lettuce roots. Large-sized pharmaceuticals (lincomycin, monensin sodium and tylosin) enter lettuce roots mainly via the apoplast pathway, and could be restricted from transporting to lettuce xylem and to shoots due to size exclusion effect.

Application of the Scheme to Other Studies

Several pathways and uptake mechanism have been proposed to describe the transport and accumulation of pharmaceuticals in plants in the literature. However, many of these studies provided the results that were not comparable to other studies or even conflicting. To evaluate whether the proposed scheme of pharmaceutical uptake and movement in lettuce (Figure 3.8)

could be applicable to the uptake of pharmaceuticals by various plants, we selected 17 reported studies (Table 3.4), and examined the fit of our proposed scheme to their results. In this study we emphasized the role of MW of pharmaceuticals in controlling their uptake and movement in plants. The small-sized pharmaceuticals could move upward from roots to stem and leaves described as TF. We calculated the MW values of pharmaceuticals, and their TF values based on reported data. The total was 375 TF values collected for 37 pharmaceuticals and 19 types of plants (Table 3.4). The results showed that for small-sized pharmaceuticals (MW < 300 g mol^{-1}), there were 146 out of 330 TF values > 1, which accounted for > 44% of the data that are available to be evaluated using our proposed scheme. The results of TF values < 1 could be due possibly to sorption of pharmaceuticals by plant roots. However, for most reported studies, no such sorption datasets were reported, hence, the factor of sorption by plant roots could not be evaluated for the applicability of our proposed scheme to other small-sized pharmaceuticals reported in literatures. On the other hand, for the pharmaceutical with MW between 300-400 g mol^{-1}, it is difficult to evaluate the applicability of the scheme because there were not many pharmaceuticals with MW with this ranged tested. In the collected data, 6 out of 22 TF values are > 1, indicating that some pharmaceuticals within 300-400 g mol^{-1} range could efficiently move up to shoots. The size exclusion limits of cell membrane for pharmaceuticals is worthy of further attention and more research efforts. As for the large-sized pharmaceuticals (MW > 400 g mol^{-1}), our proposed scheme correctly elucidates 21 out of 23 TF values (TF < 1), accounting for > 90% of the reported data. The cases of 2 TF values >1 could be due to the extremely long experiment period (> 100 d) during which pharmaceuticals might be metabolized in plant roots resulting in higher concentration in leaves than that in roots. The overall results is that this scheme could well explain 47% of studied small-sized and large-sized pharmaceuticals (i.e., MV < 300 g mol^{-1} and MV > 400 g mol^{-1})

reported in literatures. Further information including pharmaceutical affinity to various plant roots (i.e., especially for small-sized pharmaceuticals) and metabolism in plants is needed for better elucidation these results using our proposed scheme.

This study sheds new light to the uptake pathways and transport characteristics of pharmaceuticals in vegetables (e.g. lettuce), and provides a reasonable interpretation for most results reported in literatures. The mechanistic insights obtained from this study could form the framework for exposure modelling of diverse pharmaceutical compounds and facilitate the development of scientifically informed management strategies to mitigate pharmaceutical accumulation in agricultural food produce.

Table 3.4. Application of proposed scheme of pharmaceutical movement into lettuce for other chemicals taken up by various plants based on molecular weight and translocation factors reported in literatures.

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Benzotriazole	119.12	Pepper	<1[c]	C_{Shoots}/C_{Roots}	*NA*[d]	Lack of sorption data	50
		Rucola	>1[e]	C_{Leaves}/C_{Roots}	Yes		50
		Tomato	<1	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	50
Metformin	129.16	Typha latifolia	0.05	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	51
4-/5-Methylbenzotriazole	133.15	Pepper	<1	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	50
		Tomato	<1	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	50
Acetaminophen	151.16	Cucumber	<1	$C_{Leaves/Stem}/C_{Roots}$	*NA*	Lack of sorption data	11
		Pepper	0.08	$C_{Leaves/Stem}/C_{Roots}$	*NA*	Lack of sorption data	11
Acesulfame	163.15	Cabbage	0.77	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	50
		Carrot	8.55	C_{Leaves}/C_{Roots}	Yes		50
		Parsley	1.10	C_{Leaves}/C_{Roots}	Yes		50
		Potato	>1	C_{Leaves}/C_{Roots}	Yes		50
		Rucola	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	50
Gabapentin	171.24	Carrot	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	50
Gabapentin	171.24	Parsley	0.76	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	50
		Potato	>1	C_{Leaves}/C_{Roots}	Yes		50
		Rucola	>1	C_{Leaves}/C_{Roots}	Yes		50

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
N,N-Diethyl-3-methylbenzamide (DEET)	191.27	Cucumber	0.15	$C_{Leaves/Stem}/C_{Roots}$	*NA*	Lack of sorption data	11
		Lettuce	0.90	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	11
		Pepper	1.73	$C_{Leaves/Stem}/C_{Roots}$	Yes		11
		Spinach	0.36	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	11
Caffeine	194.19	Cabbage	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	50
		Carrot	>1	C_{Leaves}/C_{Roots}	Yes		12
		Carrot (Cool-Humid)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	24
		Carrot (Warm-Dry)	0.12	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	24
		Celery (Mature)	>1	C_{Stem}/C_{Root}	Yes		52
		Cucumber	1.05	$C_{Leaves/Stem}/C_{Roots}$	Yes		11
		Eggplant	>1	C_{Leaves}/C_{Roots}	Yes		50
		Lettuce	2.33	C_{Leaves}/C_{Roots}	Yes		11
		Lettuce	0.32	C_{Leaves}/C_{Roots}	NO		53

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
				(27 day)			
Caffeine	194.19	Lettuce	0.56	C_{Leaves}/C_{Roots} (27 day)	NO		53
		Lettuce	0.40	C_{Leaves}/C_{Roots} (27 day)	NO		53
		Lettuce (Cool-Humid)	0.96	C_{Leaves}/C_{Roots}	*NA*	Could be due to less transpired water	24
		Lettuce (Warm-Dry)	1.22	C_{Leaves}/C_{Roots}	Yes		24
		Pepper	0.31	$C_{Leaves/Stem}/C_{Roots}$	*NA*	Lack of sorption data	11
		Pepper	1.32	C_{Shoots}/C_{Roots}	Yes		50
		Potato	2.04	C_{Leaves}/C_{Roots}	Yes		50
		Spinach	2.90	C_{Leaves}/C_{Roots}	Yes		11
		Sweet Potato	>1	C_{Leaves}/C_{Roots}	Yes		12
		Tomato	1.74	C_{Shoots}/C_{Roots}	Yes		50
		Tomato (Cool-Humid)	2.41	C_{Leaves}/C_{Roots}	Yes		24
		Tomato	0.42	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	24

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
		(Warm-Dry)					
Caffeine	194.19	Zucchini	0.14	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	50
Acridone	195.22	Carrot	>1	C_{Leaves}/C_{Roots}	Yes		50
Crotamiton	203.28	Pea	1.10	C_{Shoots}/C_{Roots}	Yes		21
Cyclophosphamide monohydrate	203.28	Pea	5.50	C_{Shoots}/C_{Roots}	Yes		21
Ibuprofen	206.29	Carrot (Cool-Humid)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	24
		Carrot (Warm-Dry)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	24
		Cucumber	0.08	$C_{Leaves/Stem}/C_{Roots}$	*NA*	Lack of sorption data	11
		Lettuce	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	11
		Lettuce	0.04	C_{Leaves}/C_{Roots} (27 day)	*NA*	Lack of sorption data	53
		Lettuce	0.07	C_{Leaves}/C_{Roots} (27 day)	*NA*	Lack of sorption data	53
		Lettuce	0.05	C_{Leaves}/C_{Roots} (27 day)	*NA*	Lack of sorption data	53

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Ibuprofen	206.29	Lettuce (Cool-Humid)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Lettuce (Warm-Dry)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Pepper	0.01	$C_{Leaves/Stem}/C_{Roots}$	*NA*	Lack of sorption data	[11]
		Spinach	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[11]
		Tomato (Cool-Humid)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Tomato (Warm-Dry)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
Perfluorobutanoate (PFBA)	213.03	Celery	2.87	C_{Shoots}/C_{Roots}	Yes	Lack of sorption data	[54]
		Pea	5.64	C_{Shoots}/C_{Roots}	Yes		[54]
		Radish	4.73	C_{Shoots}/C_{Roots}	Yes		[54]
		Tomato	5.16	C_{Shoots}/C_{Roots}	Yes		[54]
Meprobamate	218.25	Cabbage (Mature)	3.00	C_{Leaves}/C_{Roots}	Yes		[52]
		Carrot	4.68	C_{Leaves}/C_{Roots}	Yes		[24]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
		(Cool-Humid)					
Meprobamate	218.25	Carrot (Warm-Dry)	13.00	C_{Leaves}/C_{Roots}	Yes		24
		Celery (Mature)	<1	C_{Stem}/C_{Roots}	*NA*	Lack of sorption data	52
		Cucumber	3.63	$C_{Leaves/Stem}/C_{Roots}$	Yes		11
		Lettuce	1.58	C_{Leaves}/C_{Roots}	Yes		11
		Lettuce (Cool-Humid)	1.77	C_{Leaves}/C_{Roots}	Yes		24
		Lettuce (Warm-Dry)	2.95	C_{Leaves}/C_{Roots}	Yes		24
		Pepper	16.00	$C_{Leaves/Stem}/C_{Roots}$	Yes		11
		Spinach	1.37	C_{Leaves}/C_{Roots}	Yes		11
		Spinach (Mature)	>1	C_{Leaves}/C_{Roots}	Yes		52
		Tomato (Cool-Humid)	7.55	C_{Leaves}/C_{Roots}	Yes		24
		Tomato	10.72	C_{Leaves}/C_{Roots}	Yes		24

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
		(Warm-Dry)					
Primidone	218.25	Cabbage (Mature)	0.42	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	52
		Carrot (Cool-Humid)	>1	C_{Leaves}/C_{Roots}	Yes		24
		Carrot (Warm-Dry)	>1	C_{Leaves}/C_{Roots}	Yes		24
		Celery (Mature)	1.00	C_{Stem}/C_{Roots}	Yes		52
		Cucumber	2.12	$C_{Leaves/Stem}/C_{Roots}$	Yes		11
		Cucumber (Mature)	<1	C_{Fruit}/C_{Roots}	*NA*	Lack of sorption data	52
		Lettuce	2.26	C_{Leaves}/C_{Roots}	Yes		11
		Lettuce (Cool-Humid)	11.31	C_{Leaves}/C_{Roots}	Yes		24
		Lettuce (Warm-Dry)	4.59	C_{Leaves}/C_{Roots}	Yes		24
		Pepper	12.86	$C_{Leaves/Stem}/C_{Roots}$	Yes		11

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Primidone	218.25	Spinach	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[11]
		Tomato (Cool-Humid)	>1	C_{Leaves}/C_{Roots}	Yes		[24]
		Tomato (Warm-Dry)	3.13	C_{Leaves}/C_{Roots}	Yes		[24]
Nonylphenol	220.35	Collards	0.08	$C_{Stem,\ Original\ leaves,\ and\ New\ Leaves}/C_{Roots}$	*NA*	Lack of sorption data	[55]
		Lettuce	0.03	$C_{Stem,\ Original\ leaves,\ and\ New\ Leaves}/C_{Roots}$	*NA*	Lack of sorption data	[55]
Methyl dihydrojasmonate	226.31	Lettuce	0.72	C_{Leaves}/C_{Roots} (27 day)-0% Biochar	*NA*	Lack of sorption data	[53]
		Lettuce	0.68	C_{Leaves}/C_{Roots} (27 day)-2.5% Biochar	*NA*	Lack of sorption data	[53]
		Lettuce	0.54	C_{Leaves}/C_{Roots} (27 day)-5% Biochar	*NA*	Lack of sorption data	[53]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Bisphenol A	228.29	Collards	0.05	$C_{Stem\text{-}Original\ leaves,\ and\ New\ Leaves}/C_{Roots}$	*NA*	Lack of sorption data	[55]
		Lettuce	0.01	$C_{Stem\text{-}Original\ leaves,\ and\ New\ Leaves}/C_{Roots}$	*NA*	Lack of sorption data	[55]
		Lettuce	0.11	C_{Leaves}/C_{Roots} (27 day)-0% Biochar	*NA*	Lack of sorption data	[53]
		Lettuce	0.17	C_{Leaves}/C_{Roots} (27 day)-2.5% Biochar	*NA*	Lack of sorption data	[53]
		Lettuce	0.14	C_{Leaves}/C_{Roots} (27 day)-5% Biochar	*NA*	Lack of sorption data	[53]
Naproxen	230.26	Cabbage (Mature)	0.84	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[52]
		Carrot (Cool-Humid)	0.04	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Carrot	0.08	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
		(Warm-Dry)					
Naproxen	230.26	Collards	0.51	$C_{Stem,\ Original\ leaves,\ and\ New\ Leaves}/C_{Roots}$	*NA*	Lack of sorption data	[55]
		Cucumber	0.01	$C_{Leaves/Stem}/C_{Roots}$	*NA*	Lack of sorption data	[11]
		Cucumber (Mature)	<1	C_{Fruits}/C_{Roots}	*NA*	Lack of sorption data	[52]
		Lettuce	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[11]
		Lettuce	0.18	$C_{Stem,\ Original\ leaves,\ and\ New\ Leaves}/C_{Roots}$	*NA*	Lack of sorption data	[55]
		Lettuce (Cool-Humid)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Lettuce (Warm-Dry)	0.01	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Pepper	0.08	$C_{Leaves/Stem}/C_{Roots}$	*NA*	Lack of sorption data	[11]
		Spinach	0.05	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[11]
		Tomato (Cool-Humid)	0.02	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Naproxen	230.26	Tomato (Warm-Dry)	0.07	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Tomato (Mature)	1.47	C_{Leaves}/C_{Roots}	Yes		[52]
Diuron	233.09	Carrot (Cool-Humid)	0.08	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Carrot (Warm-Dry)	0.07	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Cucumber	2.05	$C_{Leaves/Stem}/C_{Roots}$	Yes		[11]
		Lettuce	2.08	C_{Leaves}/C_{Roots}	Yes		[11]
		Lettuce (Cool-Humid)	0.14	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Lettuce (Warm-Dry)	0.62	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Pepper	8.70	$C_{Leaves/Stem}/C_{Roots}$	Yes		[11]
		Spinach	2.21	C_{Leaves}/C_{Roots}	Yes		[11]
		Tomato (Cool-Humid)	0.15	C_{Leaves}/C_{Roots}	NO	Could be due to less transpired water	[24]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Diuron	233.09	Tomato (Warm-Dry)	10.14	C_{Leaves}/C_{Roots}	Yes		24
Carbamazepine	236.27	Cabbage	0.72	$C_{Aerials}/C_{Roots}$ (Mean value)	*NA*	Lack of sorption data	32
		Cabbage	0.16	$C_{Aerials}/C_{Roots}$ (Mean value)	*NA*	Lack of sorption data	32
		Cabbage	1.29	C_{Leaves}/C_{Roots}	Yes		50
		Cabbage (Mature)	0.24	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	52
		Carrot	>1	C_{Leaves}/C_{Roots}	Yes		12
		Carrot	4.40	C_{Leaves}/C_{Roots}	Yes		50
		Carrot (Cool-Humid)	2.68	C_{Leaves}/C_{Roots}	Yes		24
		Carrot (Warm-Dry)	6.44	C_{Leaves}/C_{Roots}	Yes		24
		Celery (Mature)	0.67	C_{Stems}/C_{Roots}	*NA*	Lack of sorption data	52
		Collard	>1	C_{Shoots}/C_{Roots}	Yes		56

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Carbamazepine	236.27	Cucumber	2.76	$C_{Leaves/Stem}/C_{Roots}$	Yes		11
		Cucumber	6.66	$C_{Upper\ leaves}/C_{Roots}$	Yes		8
		Cucumber (Mature)	0.32	C_{Fruit}/C_{Roots}	*NA*	Lack of sorption data	52
		Eggplant	0.40	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	50
		Lettuce	2.55	C_{Leaves}/C_{Roots}	Yes		11
		Lettuce	>1	C_{Shoots}/C_{Roots}	Yes		56
		Lettuce	8.08	C_{Leaves}/C_{Roots}	Yes		50
		Lettuce	1.70	C_{Leaves}/C_{Roots} (27 day)	Yes		53
		Lettuce	1.60	C_{Leaves}/C_{Roots} (27 day)	Yes		53
		Lettuce	1.10	C_{Leaves}/C_{Roots} (27 day)	Yes		53
		Lettuce (Cool-Humid)	3.73	C_{Leaves}/C_{Roots}	Yes		24
		Lettuce (Warm-Dry)	4.98	C_{Leaves}/C_{Roots}	Yes		24

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Carbamazepine	236.27	Lettuce (Mature)	1.40	C_{Leaves}/C_{Roots}	Yes		52
		Parsley	2.22	C_{Leaves}/C_{Roots}	Yes		50
		Pea	7.40	C_{Shoots}/C_{Roots}	Yes		21
		Pepper	8.00	$C_{Leaves/Stem}/C_{Roots}$	Yes		11
		Pepper	>1	C_{Shoots}/C_{Roots}	Yes		56
		Pepper	0.76	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	50
		Potato	2.26	C_{Leaves}/C_{Roots}	Yes		50
		Radish	>1	C_{Shoots}/C_{Roots}	Yes		56
		Radish	7.32	C_{Leaves}/C_{Bulbs} (Uf_{soil})	Yes		7
		Tomato	>1	C_{Shoots}/C_{Roots}	Yes		56
		Rucola	1.61	C_{Leaves}/C_{Roots}	Yes		50
		Soybean	1.41	C_{Leaves}/C_{Roots}	Yes		5
		Spinach	0.48	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	11
		Spinach (Mature)	0.36	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	52
		Sweet Potato	>1	C_{Leaves}/C_{Roots}	Yes		12

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Carbamazepine	236.27	Tomato	1.53	C_{Shoots}/C_{Roots}	Yes		[50]
		Tomato (Cool-Humid)	5.65	C_{Leaves}/C_{Roots}	Yes		[24]
		Tomato (Warm-Dry)	16.36	C_{Leaves}/C_{Roots}	Yes		[24]
		Tomato (Mature)	7.40	C_{Leaves}/C_{Roots}	Yes		[52]
		Zucchini	0.61	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[50]
		Cabbage	0.94	$C_{Aerials}/C_{Roots}$ (Mean value)-Soil fortified with high SOM	*NA*	Lack of sorption data	[32]
Salbutamol	239.31	Cabbage	0.04	$C_{Aerials}/C_{Roots}$ (Mean value)-Soil amended with biosolids	*NA*	Lack of sorption data	[32]
Sulfapyridine	249.29	Carrot	>1	C_{Leaves}/C_{Roots}	Yes		[12]
		Sweet Potato	>1	C_{Leaves}/C_{Roots}	Yes		[12]
Tonalide	249.29	Lettuce	0.83	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[53]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
				(27 day)-0% Biochar			
Tonalide	249.29	Lettuce	0.34	C_{Leaves}/C_{Roots} (27 day)-5% Biochar	*NA*	Lack of sorption data	[53]
Gemfibrozil	250.33	Carrot	>1	C_{Leaves}/C_{Roots}	Yes		[12]
		Carrot (Cool-Humid)	0.02	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Carrot (Warm-Dry)	0.03	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Cucumber	<1	$C_{Leaves/Stems}/C_{Roots}$	*NA*	Lack of sorption data	[11]
		Lettuce	0.01	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[11]
		Lettuce (Cool-Humid)	0.01	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Lettuce (Warm-Dry)	0.01	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Parsley	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[50]
		Pepper	0.03	$C_{Leaves/Stems}/C_{Roots}$	*NA*	Lack of sorption data	[11]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Gemfibrozil	250.33	Spinach	0.04	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[11]
		Sweet Potato	>1	C_{Leaves}/C_{Roots}	Yes		[12]
Gemfibrozil	250.33	Tomato (Cool-Humid)	0.03	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Tomato (Warm-Dry)	0.13	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
2-hydroxy carbamazepine	252.27	Potato	>1	C_{Leaves}/C_{Roots}	Yes		[50]
		Rucola	>1	C_{Leaves}/C_{Roots}	Yes		[50]
		Tomato	>1	C_{Shoots}/C_{Roots}	Yes		[50]
		Zucchini	0.16	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[50]
3-hydroxy carbamazepine	252.27	Carrot	>1	C_{Leaves}/C_{Roots}	Yes		[50]
		Eggplant	>1	C_{Leaves}/C_{Roots}	Yes		[50]
		Parsley	>1	C_{Leaves}/C_{Roots}	Yes		[50]
		Potato	>1	C_{Leaves}/C_{Roots}	Yes		[50]
		Rucola	>1	C_{Leaves}/C_{Roots}	Yes		[50]
		Tomato	>1	C_{Shoots}/C_{Roots}	Yes		[50]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
3-hydroxy carbamazepine	252.27	Zucchini	0.11	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[50]
Carbamazepine 10,11-epoxide	252.27	Cabbage	1.45	C_{Leaves}/C_{Roots}	Yes		[50]
		Carrot	6.95	C_{Leaves}/C_{Roots}	Yes		[50]
		Eggplant	2.05	C_{Leaves}/C_{Roots}	Yes		[50]
		Lettuce	8.85	C_{Leaves}/C_{Roots}	Yes		[50]
		Parsley	0.84	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[50]
		Pepper	<1	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	[50]
		Potato	9.79	C_{Leaves}/C_{Roots}	Yes		[50]
		Rucola	1.50	C_{Leaves}/C_{Roots}	Yes		[50]
		Tomato	3.73	C_{Shoots}/C_{Roots}	Yes		[50]
		Zucchini	0.59	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[50]
Dilantin	252.27	Cabbage (Mature)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[52]
		Carrot (Cool-Humid)	3.40	C_{Leaves}/C_{Roots}	Yes		[24]
		Carrot	15.54	C_{Leaves}/C_{Roots}	Yes		[24]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
		(Warm-Dry)					
Dilantin	252.27	Celery (Mature)	0.69	C_{Stems}/C_{Roots}	*NA*	Lack of sorption data	52
		Cucumber	2.13	$C_{Leaves/Stems}/C_{Roots}$	Yes		11
		Cucumber (Mature)	<1	C_{Rruits}/C_{Roots}	*NA*	Lack of sorption data	52
		Lettuce	0.51	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	11
		Lettuce (Cool-Humid)	2.58	C_{Leaves}/C_{Roots}	Yes		24
		Lettuce (Warm-Dry)	1.00	C_{Leaves}/C_{Roots}	Yes		24
		Pepper	7.50	$C_{Leaves/Stems}/C_{Roots}$	Yes		11
		Spinach	2.90	C_{Leaves}/C_{Roots}	Yes		11
		Spinach (Mature)	0.84	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	52
		Tomato (Cool-Humid)	2.41	C_{Leaves}/C_{Roots}	Yes		24
		Tomato	13.05	C_{Leaves}/C_{Roots}	Yes		24

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
		(Warm-Dry)					
Dilantin	252.27	Tomato (Mature)	8.67	C_{Leaves}/C_{Roots}	Yes		[52]
Sulfamethoxazole	253.28	Cabbage	0.37	$C_{Aerials}/C_{Roots}$ (Mean value)	*NA*	Lack of sorption data	[32]
		Carrot	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[12]
		Carrot (Cool-Humid)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Carrot (Warm-Dry)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Cucumber	<1	$C_{Leaves/Stems}/C_{Roots}$	*NA*	Lack of sorption data	[11]
		Lettuce	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[11]
		Lettuce (Cool-Humid)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Lettuce (Warm-Dry)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Pea	0.06	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	[21]
		Pepper	0.08	$C_{Leaves/Stems}/C_{Roots}$	*NA*	Lack of sorption data	[11]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Sulfamethoxazole	253.28	Spinach	0.71	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[11]
		Sweet Potato	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[12]
		Tomato (Cool-Humid)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Tomato (Warm-Dry)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
10,11-dihydro-10-hydroxy carbamazepine	254.28	Eggplant	>1	C_{Leaves}/C_{Roots}	Yes		[50]
		Parsley	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[50]
		Potato	>1	C_{Leaves}/C_{Roots}	Yes		[50]
Ketoprofen	254.28	Pea	1.40	C_{Shoots}/C_{Roots}	Yes		[21]
Diphenhydramine	255.36	Collard	<1	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	[56]
		Lettuce	<1	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	[56]
		Pepper	>1	C_{Shoots}/C_{Roots}	Yes		[56]
		Radish	>1	C_{Shoots}/C_{Roots}	Yes		[56]
		Tomato	>1	C_{Shoots}/C_{Roots}	Yes		[56]
		Soybean	0.24	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[5]
Lamotrigine	256.09	Cabbage	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[50]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Lamotrigine	256.09	Carrot	>1	C_{Leaves}/C_{Roots}	Yes		[50]
		Eggplant	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[50]
		Parsley	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[50]
		Pepper	<1	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	[50]
		Sweet Potato	>1	C_{Leaves}/C_{Roots}	Yes		[12]
		Tomato	<1	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	[50]
		Zucchini	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[50]
		Radish	0.76	C_{Leaves}/C_{Bulbs} (Uf_{soil})	*NA*	Lack of sorption data	[7]
Atenolol	266.34	Cucumber	0.14	$C_{Leaves/Stems}/C_{Roots}$	*NA*	Lack of sorption data	[11]
		Lettuce	0.40	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[11]
		Pepper	1.04	$C_{Leaves/Stems}/C_{Roots}$	Yes		[11]
		Spinach	0.54	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[11]
Metoprolol	267.36	Carrot	>1	C_{Leaves}/C_{Roots}	Yes		[12]
		Sweet Potato	>1	C_{Leaves}/C_{Roots}	Yes		[12]
10,11-dihydro-10,11-trans-dihydroxycarbamazepine	270.28	Carrot	2.74	C_{Leaves}/C_{Roots}	Yes		[50]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
10,11-dihydro-10,11-trans-dihydroxycarbamazepine	270.28	Eggplant	2.14	C_{Leaves}/C_{Roots}	Yes		[50]
		Lettuce	2.17	C_{Leaves}/C_{Roots}	Yes		[50]
		Parsley	0.44	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[50]
		Potato	2.89	C_{Leaves}/C_{Roots}	Yes		[50]
		Tomato	>1	C_{Shoots}/C_{Roots}	Yes		[50]
		Zucchini	0.19	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[50]
Sulfamonomethoxine	280.30	Pea	0.06	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	[21]
Diazepam	284.74	Carrot (Cool-Humid)	0.18	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Carrot (Warm-Dry)	0.52	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Cucumber	0.66	$C_{Leaves/Stems}/C_{Roots}$	*NA*	Lack of sorption data	[11]
		Lettuce	0.30	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[11]
		Lettuce (Cool-Humid)	0.29	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Lettuce	0.50	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
		(Warm-Dry)					
Diazepam	284.74	Pepper	0.90	$C_{Leaves/Stems}/C_{Roots}$	*NA*	Lack of sorption data	[11]
		Spinach	0.03	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[11]
		Tomato (Cool-Humid)	0.71	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Tomato (Warm-Dry)	2.89	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
Tris(2-chloroethyl) phosphate	285.49	Lettuce	1.05	C_{Leaves}/C_{Roots} (27 day)	Yes		[53]
		Lettuce	1.00	C_{Leaves}/C_{Roots} (27 day)	Yes		[53]
		Lettuce	0.72	C_{Leaves}/C_{Roots} (27 day)	*NA*	Lack of sorption data	[53]
Clofibric acid	288.29	Carrot (Cool-Humid)	0.06	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Carrot (Warm-Dry)	0.12	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Lettuce	0.04	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[53]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
				(27 day)			
Clofibric acid	288.29	Lettuce	0.03	C_{Leaves}/C_{Roots} (27 day)	*NA*	Lack of sorption data	[53]
		Lettuce	0.07	C_{Leaves}/C_{Roots} (27 day)	*NA*	Lack of sorption data	[53]
		Lettuce (Cool-Humid)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Lettuce (Warm-Dry)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Tomato (Cool-Humid)	0.18	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Tomato (Warm-Dry)	0.02	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
Triclosan	289.54	Cabbage	0.03	$C_{Aerials}/C_{Roots}$ (Mean value)	*NA*	Lack of sorption data	[32]
		Carrot	<1	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	[57]
		Cucumber	0.03	$C_{Leaves/Stems}/C_{Roots}$	*NA*	Lack of sorption data	[11]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Triclosan	289.54	Lettuce	<1	C_{Leaves}/C_{Roots}	Yes	Could be due to metabolism	11
		Lettuce	0.03	C_{Leaves}/C_{Roots} (27 day)	Yes	Could be due to metabolism	53
		Lettuce	0.07	C_{Leaves}/C_{Roots} (27 day)	Yes	Could be due to metabolism	53
		Lettuce	0.04	C_{Leaves}/C_{Roots} (27 day)	Yes	Could be due to metabolism	53
		Pepper	0.41	$C_{Leaves/Stems}/C_{Roots}$	*NA*	Lack of sorption data	11
		Radish	>1	C_{Shoots}/C_{Roots}	Yes		57
		Radish	0.83	C_{Leaves}/C_{Bulbs} (Uf_{soil})	*NA*	Lack of sorption data	7
		Soybean	0.59	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	5
		Soybean	<1	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	57
		Spinach	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	11
		Cabbage	0.11	$C_{Aerials}/C_{Roots}$	*NA*	Lack of sorption data	32

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined (Mean value)	Applicability	Note	Ref.
Triclosan	289.54	Carrot (Cool-Humid)	0.08	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Carrot (Warm-Dry)	0.37	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Cucumber	0.42	$C_{Leaves/Stems}/C_{Roots}$	*NA*	Lack of sorption data	[11]
		Pea	0.22	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	[21]
		Pepper	0.44	$C_{Leaves/Stems}/C_{Roots}$	*NA*	Lack of sorption data	[11]
		Spinach	0.10	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[11]
		Tomato (Cool-Humid)	0.49	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Tomato (Warm-Dry)	0.20	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Carrot (Cool-Humid)	0.03	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
		Carrot (Warm-Dry)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Triclosan	289.54	Collards	0.13	$C_{Stems,Original\ Leaves,\ and\ New\ Leaves}/C_{Roots}$	*NA*	Lack of sorption data	[55]
		Cucumber	<1	$C_{Leaves/Stems}/C_{Roots}$	*NA*	Lack of sorption data	[11]
		Lettuce	0.07	C_{Leaves}/C_{Roots}	Yes	Could be due to metabolism	[11]
		Lettuce	0.06	$C_{Stems,Original\ Leaves,\ and\ New\ Leaves}/C_{Roots}$	Yes	Could be due to metabolism	[55]
		Lettuce (Cool-Humid)	<1	C_{Leaves}/C_{Roots}	Yes	Could be due to metabolism	[24]
		Lettuce (Warm-Dry)	<1	C_{Leaves}/C_{Roots}	Yes	Could be due to metabolism	[24]
		Pepper	<1	$C_{Leaves/Stems}/C_{Roots}$	*NA*	Lack of sorption data	[11]
		Radish	2.14	C_{Leaves}/C_{Bulbs} (Uf_{soil})	*NA*	Lack of sorption data	[7]
		Spinach	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[11]
		Tomato (Cool-Humid)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Triclosan	289.54	Tomato (Warm-Dry)	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[24]
Hydrochlorothiazide	297.74	Parsley	0.18	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[50]
Perfluorobutanesulfonate (PFBS)	299.09	Celery	0.88	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	[54]
		Pea	4.63	C_{Shoots}/C_{Roots}	Yes		[54]
		Radish	2.66	C_{Shoots}/C_{Roots}	Yes		[54]
		Tomato	5.14	C_{Shoots}/C_{Roots}	Yes		[54]
Fluoxetine	309.33	Cucumber	0.11	$C_{Leaves/Stems}/C_{Roots}$	*NA*	Lack of sorption data	[11]
		Lettuce	0.19	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[11]
		Pepper	3.57	$C_{Leaves/Stems}/C_{Roots}$	*NA*		[11]
		Radish	0.28	C_{Leaves}/C_{Bulbs} (Uf_{soil})	*NA*	Lack of sorption data	[7]
		Soybean	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[5]
		Spinach	0.33	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	[11]
Sulfadimethoxine	310.33	Pea	0.02	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	[21]
Triclocarban	315.58	Collard	<1	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	[56]
		Cucumber	<1	$C_{Leaves/Stems}/C_{Roots}$	*NA*	Lack of sorption data	[11]

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Triclocarban	315.58	Lettuce	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	11
		Lettuce	>1	C_{Shoots}/C_{Roots}	*NA*		56
		Pepper	<1	$C_{Leaves/Stems}/C_{Roots}$	*NA*	Lack of sorption data	11
		Pepper	<1	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	56
		Radish	>1	C_{Shoots}/C_{Roots}	*NA*		56
		Tomato	<1	C_{Shoots}/C_{Roots}	*NA*	Lack of sorption data	56
		Soybean	0.06	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	5
		Spinach	0.01	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	11
Gliclazide	323.41	Pea	5.50	C_{Shoots}/C_{Roots}	*NA*		21
Ciprofloxacin	331.34	Carrot	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	50
		Cabbage	<1	C_{Leaves}/C_{Roots}	*NA*	Lack of sorption data	50
Bezafibrate	361.83	Carrot	>1	C_{Leaves}/C_{Roots}	*NA*		12
		Sweet Potato	>1	C_{Leaves}/C_{Roots}	*NA*		12
Sildenafil	474.59	Carrot	>1	C_{Leaves}/C_{Roots}	NO	Lack of mass balance analysis and sorption study as well as might be metabolized during long period exposure	12
		Sweet Potato	>1	C_{Leaves}/C_{Roots}	NO	Lack of mass balance analysis and sorption	12

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
						study as well as might be metabolized during long period exposure	
Perfluorooctane sulfonate	500.13	Carrot (Cool-Humid)	0.06	C_{Leaves}/C_{Roots}	Yes		24
		Carrot (Warm-Dry)	0.11	C_{Leaves}/C_{Roots}	Yes		24
		Lettuce (Cool-Humid)	0.01	C_{Leaves}/C_{Roots}	Yes		24
		Lettuce (Warm-Dry)	0.02	C_{Leaves}/C_{Roots}	Yes		24
		Tomato (Cool-Humid)	0.05	C_{Leaves}/C_{Roots}	Yes		24
		Tomato (Warm-Dry)	0.19	C_{Leaves}/C_{Roots}	Yes		24
Atorvastatin	558.64	Carrot (Cool-Humid)	<1	C_{Leaves}/C_{Roots}	Yes		24
		Carrot	<1	C_{Leaves}/C_{Roots}	Yes		24

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
		(Warm-Dry)					
Atorvastatin	558.64	Cucumber	0.01	$C_{Leaves/Stems}/C_{Roots}$	Yes		11
		Lettuce	0.05	C_{Leaves}/C_{Roots}	Yes		11
		Lettuce (Cool-Humid)	<1	C_{Leaves}/C_{Roots}	Yes		24
		Lettuce (Warm-Dry)	<1	C_{Leaves}/C_{Roots}	Yes		24
		Pepper	0.01	$C_{Leaves/Stems}/C_{Roots}$	Yes		11
		Spinach	<1	C_{Leaves}/C_{Roots}	Yes		11
		Tomato (Cool-Humid)	<1	C_{Leaves}/C_{Roots}	Yes		24
		Tomato (Warm-Dry)	<1	C_{Leaves}/C_{Roots}	Yes		24
Iopromide	791.11	*Typha latifolia* L.	0.16	C_{Leaves}/C_{Roots} (1 day)	Yes		58
		Typha latifolia L.	0.06	C_{Leaves}/C_{Roots} (3 day)	Yes		58

Table 3.4. (cont'd)

Chemical	MW[a]	Plant	TF[b]	TF Defined	Applicability	Note	Ref.
Iopromide	791.11	*Typha latifolia* L.	0.06	C_{Leaves}/C_{Roots} (7 day)	Yes		[58]
		Typha latifolia L.	0.09	C_{Leaves}/C_{Roots} (14 day)	Yes		[58]
		Typha latifolia L.	0.31	C_{Leaves}/C_{Roots} (28 day)	Yes		[58]

[a]MW: Molecular weight.

[b]TF: Translocation factor.

[c]<1: Obtained either from bar chart (without accurate number) or from no detection in upper plant parts.

[d]NA: Not available.

[e]>1: Obtained either from bar chart (without accurate number) or from no detection in underground plant parts.

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REFERENCES

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CHAPTER IV

INFLUENCE OF TRANSPIRATION STREAM ON UPTAKE AND TRANSPORT OF PHARMACEUTICALS IN LETTUCE

ABSTRACT

Transpiration stream is considered as the major carrier to move pharmaceuticals into and distribute them within plants. However, the relationship between accumulation/transport of pharmaceuticals and transpiration rate in plants remains largely unknown. To address this question, we measured lettuce uptake of thirteen pharmaceuticals from water under different transpiration rates as a function of time. The variation of transpiration rates of lettuce was achieved by applying different concentrations of plant hormone abscisic acid (ABA) in hydroponic nutrient solution. Increasing ABA concentration resulted in decrease of water transpiration in lettuce. Strong positive linear relation was observed between pharmaceutical mass accumulation in shoots and transpired water for caffeine and carbamazepine. Those pharmaceuticals are relatively small in molecular size, and manifested weak affinity to lettuce roots. Lamotrigine and trimethoprim are also small in size, and manifested a relatively strong affinity to lettuce roots. Therefore, their mass accumulation in shoots and roots were positively correlated with transpired water. No apparent relationship was observed for the large-sized pharmaceuticals such as monensin sodium, oxytetracycline and tylosin. Acetaminophen, β-estradiol, estrone, and triclosan were substantially metabolized in lettuce with the total recovered mass < 20% of the initially applied dosages after 48 hours of uptake; this limits the evaluation for the relationship between their transport in lettuce and transpiration stream. Taken together, this study demonstrated that transpiration stream is the major carrier to transport pharmaceuticals in plants. This information is essentially needed to predict pharmaceutical uptake and transport in plants (specifically vegetables) for the risk assessment of food safety and human health.

INTRODUCTION

Accumulation of pharmaceuticals in corps and vegetables originates primarily from the practices of land application of animal manures and biosolids, and crop irrigation with reclaimed water in agricultural production.[1-3] Uptake and distribution of pharmaceuticals in crops (e.g., vegetables) have been investigated with different exposure pathways, e.g., uptake from plant roots vs. irrigation with contaminated water.[4-6] These studies have provided some insights into the uptake processes influenced by the major factors of plant physiological characteristics and physicochemical properties of pharmaceuticals.[7-9]

Pharmaceuticals enter plant roots with water flow via symplast and apoplast pathways.[3, 9] Pharmaceuticals that enter plants in the symplast pathway and pass through Casparian strip at endodermis can be transported by transpiration stream into xylem and then upwards to upper portions.[10, 11] Pharmaceuticals that can directly enter plant cells are relatively small in size (e.g., molecular weight < 300-500 Daltons), and/or are neutrally charged, which facilitate their diffusion through the plant cell membranes.[9, 12-15] Pharmaceutical accumulation in plant roots generally increases with their octanol/water partitioning coefficient (log D_{ow}, normalized to neutral fraction of ionizable pharmaceuticals).[16, 17] Strong affinity to plant roots diminishes the transport of pharmaceuticals from roots to shoots.[2, 3] Many pharmaceuticals contain multiple ionic functional groups, which are dissociated in the solution to form multiple species with varying net charges. It is believed that neutral pharmaceuticals are easier to cross lipid bilayers of plant cell membranes than ionic ones.[6, 8] For instance, among the pharmaceuticals with similar molecular weight, carbamazepine (neutrally charged) was found to be more accumulated in cucumber than cationic lamotrigine and anionic sulfamethoxazole.[18] Negative electrostatic potentials on plant cell membranes attract cationic pharmaceuticals to the proximity of cells, thus limiting their movement

into cells, while the membranes repel anionic pharmaceuticals to approach root cell surfaces.[8, 9, 18] In addition, pH difference between intracellular (pH 7–7.5) and extracellular media (pH 4–6) could also alter the accumulation and movement of ionic pharmaceuticals. For example, lamotrigine (p*K*a = 5.7) exits primarily as neutral species in the cytosol of tomato (pH 7.2), which could diffuse through tonoplasts, and then dissociate into cationic form inside of the vacuoles (pH ~5.5). The cationic lamotrigine is thus trapped in vacuoles of tomato cells (ion trapping).[9, 18]

Recent studies have developed some fundamental understandings on plant uptake of pharmaceuticals;[8, 9, 19] however, little attention was given to elucidate the influence of plant transpiration stream on pharmaceutical transport in plants.[1, 20, 21] Generally, water movement in soils and plants is driven primarily by water potential gradient and vapor concentration in the air.[22] The transpiration rates are distinct among plant species, plant tissues (fruits vs. leaves), and plant growth during different seasons (summer vs. winter) or at different regions (arid vs. humid regions).[18, 20, 23] Greater transpiration rates could lead to a high accumulation of 2,2-bis(chlorophenyl)-1,1,1-trichloroethane (DDT) in pumpkin and zucchini leaves (large size of leaves).[24] Mcfarlane *et al*. demonstrated that the increase of transpiration rate in soybean enhanced the transport of bromacil to shoots.[25] Dodgen *et al.* revealed that more pharmaceuticals were transported to carrot, lettuce, and tomato growing in warm and dry environments than that in cool and humid conditions, which could be attributed to the lower transpiration rates in the latter experimental settings.[21]

The objective of this study was to examine the relationship between transpiration rate and pharmaceutical accumulation and upward transport in lettuce. We hypothesize that the increase in transpiration stream could lead to more uptake and transport of pharmaceuticals in plants, and pharmaceutical molecular size and affinity to plant components could also influence their uptake

and accumulation. To test these hypotheses, lettuce transpiration rate was reduced via adding plant hormone abscisic acid (ABA) in hydroponic solution to decrease the stomatal conductance in lettuce leaves.[26-28] Uptake of multiple classes of pharmaceuticals was measured as a function of exposure time at varying transpiration rates. The relationship between transpired water and the distribution of pharmaceuticals in lettuce was examined to evaluate their accumulation and transport in lettuce in relation to their physicochemical properties.

MATERIALS AND METHODS

Chemicals and Materials

Acetaminophen, β-estradiol, caffeine, carbadox, carbamazepine, estrone, lamotrigine, lincomycin, monensin sodium, oxytetracycline, trimethoprim, triclosan and tylosin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lamotrigine was obtained from Toronto Research Chemicals, Inc. (Toronto, ON, Canada), and triclosan was provided by AK Scientific, Inc. (Union City, CA, USA). Details of selected pharmaceuticals are listed in Table 4.1. Simeton, acquired from Absolute Standards, Inc. (Hamden, CT, USA), was used as internal standard. Acetonitrile, anhydrous sodium sulfate (Na2SO4) (EMD Chemicals, Gibbstown, NJ, USA), disodium ethylenediaminetetraacetate (Na2EDTA), sodium chloride (NaCl, J.T. Baker, Phillipsburg, NJ, USA) and methanol (Sigma-Aldrich, St. Louis, MO, USA) were all used in pharmaceutical extraction following the quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction method described in the Chapter III. Ceramic homogenizers, C18 and primary secondary amine (PSA) from Agilent Technologies (Santa Clara, CA, USA) were also used in the extraction of pharmaceuticals from plants. Ammonium hydroxide (J.T. Baker Chemicals) and formic acid (EMD Chemicals) were added to the mobile phase of liquid chromatography. Waters Oasis hydrophilic–lipophilic balance (HLB) cartridge was obtained from

Waters Corporation (Milford, MA, USA). Water used in the experiments was prepared from a Milli-Q water purification system (Millipore, Billerica, MA, USA). Black Seeded Simpson lettuce (Lactuca sativa) seeds were obtained from Burpee (Warminster, PA, USA). Hydroponic MaxiGro plant nutrient (10−5−14) was purchased from General Hydroponics (Sevastopol, CA, USA). LED Grow Light (GL100LED Full Spectrum 300W LED) was provided by Apollo Horticulture (Rowland Heights, CA, USA).

Hydroponic Uptake Experiment

Lettuce seeds were germinated on moistened paper, and the seedlings grew in a hydroponic nursery with solution pH maintained at 5.8, and electric conductivity (EC) at 0.8 mS cm^{-1}. The nutrient solution was continuously aerated using a fusion air pump. The temperature for lettuce growth was set at 18 ºC, and the lighting period was controlled at 16 hours/day using LED light source with the intensity of 150 μmol/m^2/s. The well-developed lettuce plants were exposed to a mixture of the selected pharmaceuticals (Table 4.1) in nutrient solution (50 μg L^{-1} for each pharmaceutical). The nutrient solution contained the pharmaceutical mixture and ABA at 0, 0.75 or 2.0 mg L^{-1}. The experiments were conducted in triplicate. Lettuce was sampled at 12, 24, 48, 72, 105 and 144 hours for pharmaceutical analysis in roots and shoots, as well as in solution phase. During the experimental period, the transpired water was measured gravimetrically every 24 hours via weighing flasks after taking out the lettuce. The transpired solution was then replenished with freshly prepared nutrient solution free of ABA or pharmaceuticals. After 48 hours of uptake, ABA was depleted due to the uptake by lettuce; additional ABA was supplemented into the nutrient solution to maintain the ABA concentration.

Table 4.1. Physicochemical properties of pharmaceuticals studied.

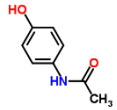
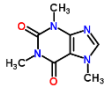
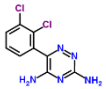
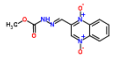
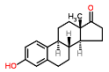
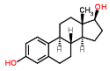
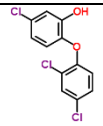
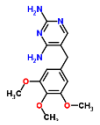
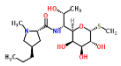
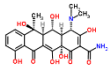
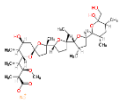
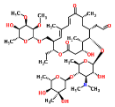
Pharmaceuticals	Molecular weight (g mol^{-1})	Structure[a]	Water Solubility (mg L^{-1})	pK_a	log K_{ow}	log D_{ow}[b] (pH at 5.8)
Acetaminophen	151.16		14000	9.38 (Acid)	0.46	0.46
Caffeine	194.19		21600	10.4 (Base)	–0.07	–4.67
Carbamazepine	236.27		18	2.3 (Base), 13.9 (Acid)	2.45	2.45
Lamotrigine	256.1		170	5.7 (Base)	0.99	0.74
Carbadox	262.22		1755	1.8 (Base), 10.5 (Acid)	–1.22	–1.22
Estrone	270.37		30	10.33 (Acid)	3.13	3.13
β-Estradiol	272.38		3.9	10.33 (Acid)	4.01	4.01

Table 4.1. (cont'd)

Pharmaceuticals	Molecular weight (g mol^{-1})	Structure[a]	Water Solubility (mg L^{-1})	p*K*a	log K_{ow}	log D_{ow}[b] (pH at 5.8)
Triclosan	289.54		10	7.9 (Acid)	4.76	4.76
Trimethoprim	290.32		400	7.12 (Base)	0.91	–0.43
Lincomycin	406.54		927	7.6 (Base)	0.2	-1.61
Oxytetracycline	460.43		313	3.57 (Acid), 7.49 (Acid), 9.44 (Base)	–0.9	–0.91
Monensin sodium	692.87		Slightly soluble	4.3 (Acid)	5.43	3.92
Tylosin	916.10		5	7.73 (Base)	1.63	–0.31

[a]From Chemspider database: http://www.chemspider.com/.

[b] For neutral compounds: log D_{ow} = log K_{ow}; for acidic compounds: log D_{ow} = log K_{ow} + log $\frac{1}{1+10^{(pH-pKa)}}$; for basic compounds: log D_{ow} = log K_{ow} + log $\frac{1}{1+10^{(pka-pH)}}$.[29]

Sample Extraction and Analysis

Extraction and quantification of pharmaceuticals in nutrient solution, lettuce roots and shoots were described in details in the Chapter III. Pharmaceutical concentration in nutrient solution and lettuce plants system was analyzed by a Shimadzu Prominence high-performance liquid chromatography (Columbia, MD, USA) coupled to a Scie x 4500 QTrap mass spectrometer (LC-MS/MS) (Foster City, CA, USA). Pharmaceuticals in nutrient solution were extracted using HLB solid-phase-extraction (SPE) cartridges preconditioned with 3.0 mL methanol and 5.0 mL water. Twenty milliliter of nutrient solution sample (spiked with 150 mg L^{-1} Na_2EDTA) was passed through the preconditioned cartridge, which was then washed with 5.0 mL of water, and eluted with 5.0 mL of methanol. For the extraction of pharmaceuticals from plant, lettuce roots (100 mg) and shoots (250 mg) were first placed in 50-mL polypropylene centrifuge tubes containing 1.0 mL of 300 mg L^{-1} Na_2EDTA solution and then vortexed for 1 min. Two ceramic homogenizers and 1.75 mL of methanol, along with 2.0 g of Na_2SO_4 and 0.5 g of NaCl, were added into the tubes, and vortexed for 1.5 min. The tubes were centrifuged at 5050 g for 10 min, and the supernatant (in methanol) were collected. After that, 3.25 mL of acetonitrile was added to the tube, and vortexed for 1.5 min. The tubes were centrifuged at 5050 g for 10 min, and the supernatants were combined with the methanol extracts. The combined extract (1.2 mL) was cleaned up by dispersive solid phase extraction (d-SPE) sorbents of 12.5 mg of C18, 12.5 mg of PSA and 225 mg of Na_2SO_4. The mixtures of extracts and d-SPE were vortexed for 1 min, and centrifuged at 9240 g for 10 min. The supernatants were collected for LC-MS/MS analysis.

To measure the target pharmaceutical concentration in samples, an Agilent Eclipse Plus C18 column (50 mm × 2.1 mm, particle size 5μm, Santa Clara, CA, USA) was used for separation in the LC-MS/MS. The flow rate was 0.35 mL/min, and the sample injection volume was 10 μL. The

pharmaceuticals were analyzed at both positive and negative ion modes in the QTrap mass spectrometer. At the positive ion mode, the binary mobile phase consisted of water with 0.3% formic acid (Phase A) and acetonitrile/methanol with 0.3% formic acid (65/35, v/v) (Phase B). The gradient program started with 2 min of pre-equilibration with 100% of Phase A, and the Phase B increased to 40% during 0.1–1.0 min then increased to 70% between 1.0–2.0 min, and to 80% between 2.0–3.0 min, then to 100% between 3.0–3.5 min, and the Phase B was held at 100% until 7.2 min. In the QTrap mass spectrometer, the ionspray voltage was 5000 V, temperature was set at 700 °C, curtain gas pressure was 20 psi and entrance potential was 10 V. At the negative ion mode, the binary mobile phase consisted of water with 0.005 % NH_4OH, and phase B was the mixture of acetonitrile/methanol (9/1, v/v). The gradient program was also started with 2 min of pre-equilibration with 100% of Phase A, and Phase B increased to 5% between 0.1–2.0 min, then to 100% between 2.0–10.0 min, and held it until 12.0 min. In the QTrap mass spectrometer, the ionspray voltage was –4500 V, temperature was at 700 °C, curtain gas pressure was 40 psi, and entrance potential was −10 V. The multiple reaction monitoring (MRM) transitions used for pharmaceutical quantification and qualification are listed in Table 4.2. The statistical analyses were conducted using one-way ANOVA with post-hoc Tukey test (OriginPro 2016).

Table 4.2. Multiple reaction monitoring (MRM) transitions and mass spectrometer parameters for the analysis of pharmaceuticals.

Chemicals	MRM transitions (m/z)	DP[b] (V)	CE[c] (V)	CXP[d] (V)
Positive ionization mode				
Acetaminophen	**151.9 → 110**	**60**	**20**	**8**
	151.9 → 93	60	30	6
Caffeine	**195.0 → 138**	**60**	**30**	**10**
	195.0 → 110	60	30	6
Carbamazepine	**237.0 → 193.7**	**80**	**30**	**10**
	237.0 → 192	80	30	12
Lamotrigine	**255.9 → 210.7**	**86**	**43**	**14**
	255.9 → 165.7	91	37	13
Carbadox	**262.9 → 230.9**	**60**	**20**	**12**
	262.9 → 144.8	60	30	8
Trimethoprim	**291.0 → 261**	**80**	**30**	**12**
	291.0 → 230	100	30	12
Lincomycin	**407.1 → 126**	**60**	**30**	**8**
	407.1 → 359.1	80	30	6
Oxytetracycline	**461.0 → 426.1**	**60**	**30**	**8**
	461.0 → 283.1	60	50	8
Monensin sodium	**694.2 → 676.3**	**120**	**50**	**6**
	694.2 → 480	120	70	6
Tylosin	**916.3 → 173.8**	**100**	**40**	**10**
	916.3 → 83	60	100	4

Table 4.2. (cont'd)

Chemicals	MRM transitions (m/z)	DP[b] (V)	CE[c] (V)	CXP[d] (V)
Negative ionization mode				
Estrone	**269.1 → 154**	**−105**	**−48**	**−19**
	269.1 → 143	−105	−66	−15
β-Estradiol	**271.0 → 182.9**	**−40**	**−50**	**−12**
	271.0 → 144.8	−40	−50	−4
Triclosan	**286.9 → 35**	**−40**	**−30**	**−6**
	289.0 → 35	−40	−20	−8

[a] The product ion coupled with precursor ion used to quantify (in bold) and qualify. Pharmaceuticals.
[b] Declustering potential.
[c] Collision energy.
[d] Collision cell exit potential.

RESULTS AND DISCUSSION

Water Transpiration in Lettuce

To evaluate the effects of transpiration stream on uptake and transport of pharmaceuticals in lettuce, a range of transpiration stream rates through the lettuce were achieved by adding ABA to the hydroponic solution. The transpired water on the basis of lettuce shoot (dry weight) increased to various extent with uptake time for both levels of ABA applications and the ABA-free controls (Figure 4.1). Compared to the ABA-free controls, the transpiration rates decreased in the presence of ABA, and higher ABA concentration (2.00 mg L^{-1}) caused more reduction in transpiration than that at lower ABA level (0.75 mg L^{-1}). The presence of ABA in nutrient solution could effectively diminish lettuce stomata conductance, and hence reduce the transpired water through plants.[30, 31] The range of transpired water on lettuce shoots (dry weight) varied from 16.9 to 104.0 g g^{-1} during the experimental periods. After 24 hours of uptake, a significant difference in transpiration rates was found between the solution amended with ABA and the ABA-free samples ($p < 0.05$) (Figure 4.1). After 144 hours of experimental period, the transpired water on the dry weight of lettuce shoots in the ABA-free controls was approximately 1.2 and 1.5 times greater than the lettuce amended with 0.75 and 2.00 mg L^{-1} of ABA. The transpiration rate during 144 hours of uptake was estimated at 0.72, 0.61, and 0.48 g of H_2O per gram of dry shoot weight per hour for the lettuce growing in 0, 0.75 and 2.00 mg L^{-1} of ABA solution. The significant difference between the ABA-treated lettuce and the ABA-free controls warrants the execution of the following experiments to evaluate the effects of transpired water on pharmaceutical uptake and transport in lettuce.

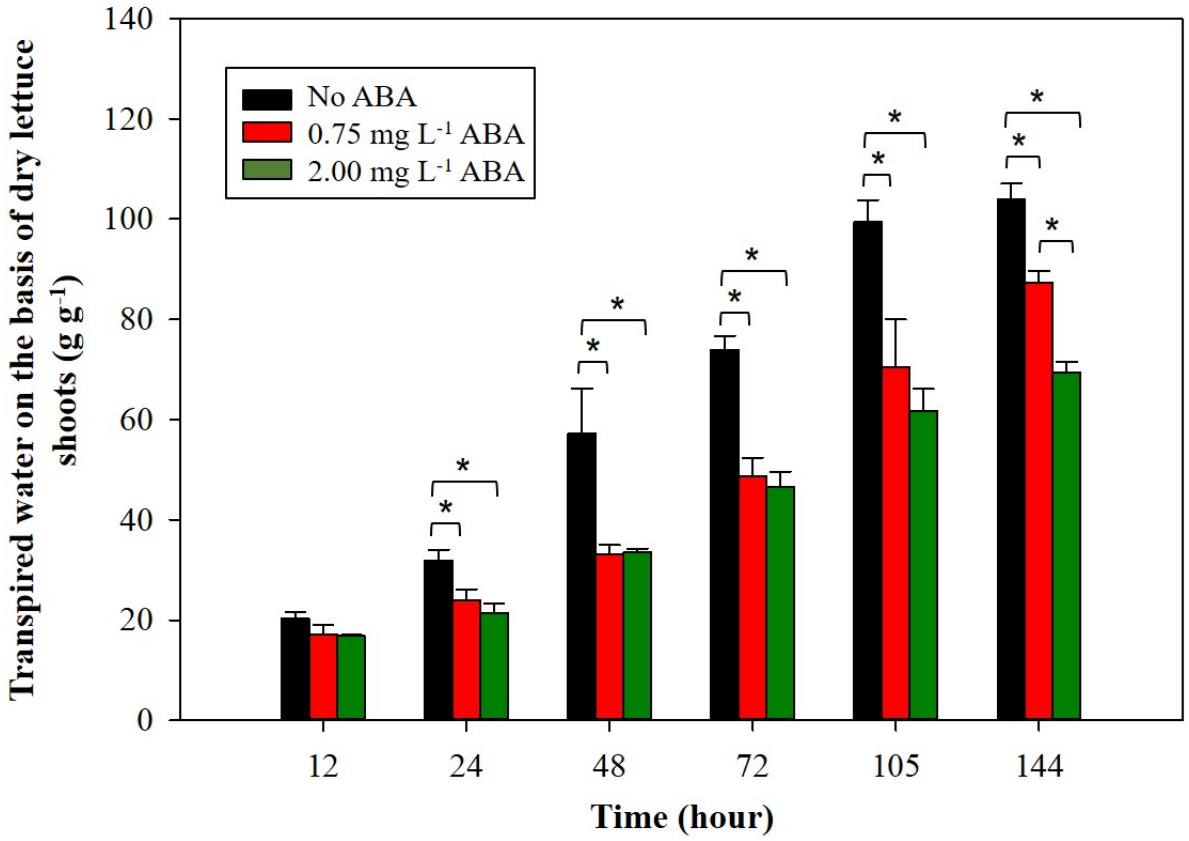


Figure 4.1. Transpired water on the basis of lettuce shoot (dry weight) as a function of uptake time. Asterisk * indicates significant difference between two treatments ($p < 0.05$).

Mass Distribution of Pharmaceuticals

The uptake kinetics of pharmaceuticals by lettuce with different transpiration rates, and their distributions in hydroponic solution, lettuce roots and shoots are shown in Figures 4.2-4.4. Carbamazepine, caffeine, lamotrigine, and trimethoprim entered lettuce, and distributed in the roots and shoots with the amount increasing with exposure time (Figure 4.2). During the 144 hours of exposure period, the overall mass recoveries including those present in solution and in lettuce were 93% for carbamazepine, 44% for caffeine, 43% for lamotrigine, and 75% trimethoprim. Among these recovered pharmaceutical masses, 3% and 25% of carbamazepine, 1% and 7% of caffeine, 2% and 1% of lamotrigine, and 12% and 6% of trimethoprim were distributed in lettuce roots and shoots, respectively. The presence of ABA in nutrient solution caused the decrease in their accumulation in lettuce shoots. Compared to the ABA-free controls, the presence of 0.75 mg L^{-1} of ABA in nutrient solution resulted in 15% of decrease for carbamazepine, 3% of decrease for caffeine, 1% of decrease for lamotrigine, and 2% of decrease for trimethoprim in shoots; the presence of 2.0 mg L^{-1} of ABA resulted in 22% of decrease for carbamazepine, 5% of decrease for caffeine, 2% of decrease for lamotrigine, and 4% of decrease for trimethoprim in shoots. Caffeine, carbamazepine, lamotrigine, and trimethoprim were readily transported into lettuce roots and moved upward to shoots, which could be attributed primarily to their relatively small molecular size as indicated by their molecular weight (e.g. < 300 Daltons). Small molecular size (e.g., molecular weight < 300-500 Daltons) facilitates the transport of organic chemicals with water flow through cell membranes via passive diffusion.[13-15] In contrast to caffeine and carbamazepine, lamotrigine and trimethoprim demonstrated more accumulation in lettuce roots than that in shoots (Figure 4.2), which could be due to their relatively strong affinity to lettuce roots than caffeine and carbamazepine. The measurement of sorption of lamotrigine and

trimethoprim by lettuce roots was approximately 5 times greater than that of caffeine and carbamazepine (Figure 4.5). Therefore, lettuce roots could retain more lamotrigine and trimethoprim than caffeine and carbamazepine when they transported within lettuce with transpiration flow, which could result in more accumulation in lettuce roots (Figure 4.2).

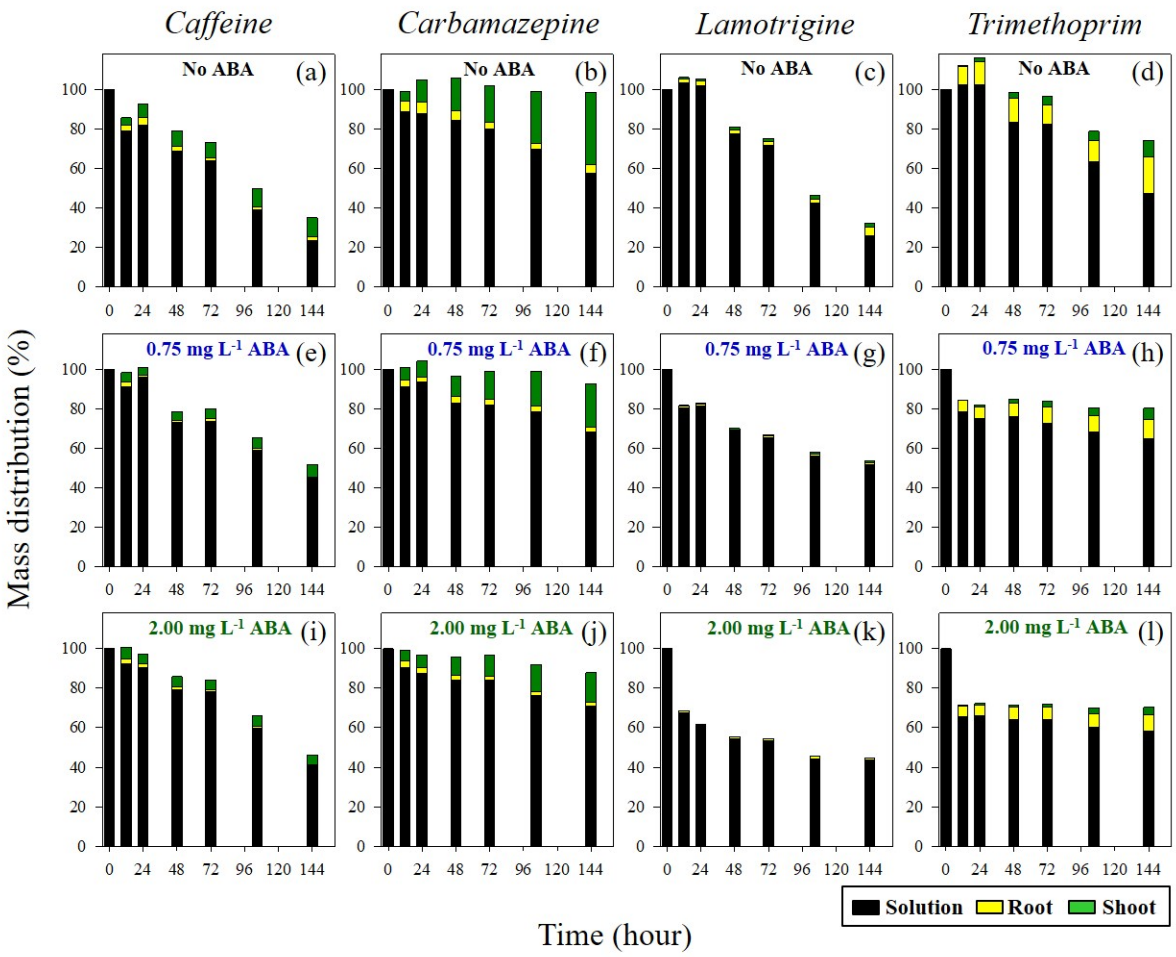


Figure 4.2. Mass distribution of caffeine, carbamazepine, lamotrigine, and trimethoprim in nutrient solution, lettuce roots and shoots as a function of uptake time in the presence of 0, 0.75, and 2.00 mg L^{-1} of abscisic acid (ABA) in the nutrient solution.

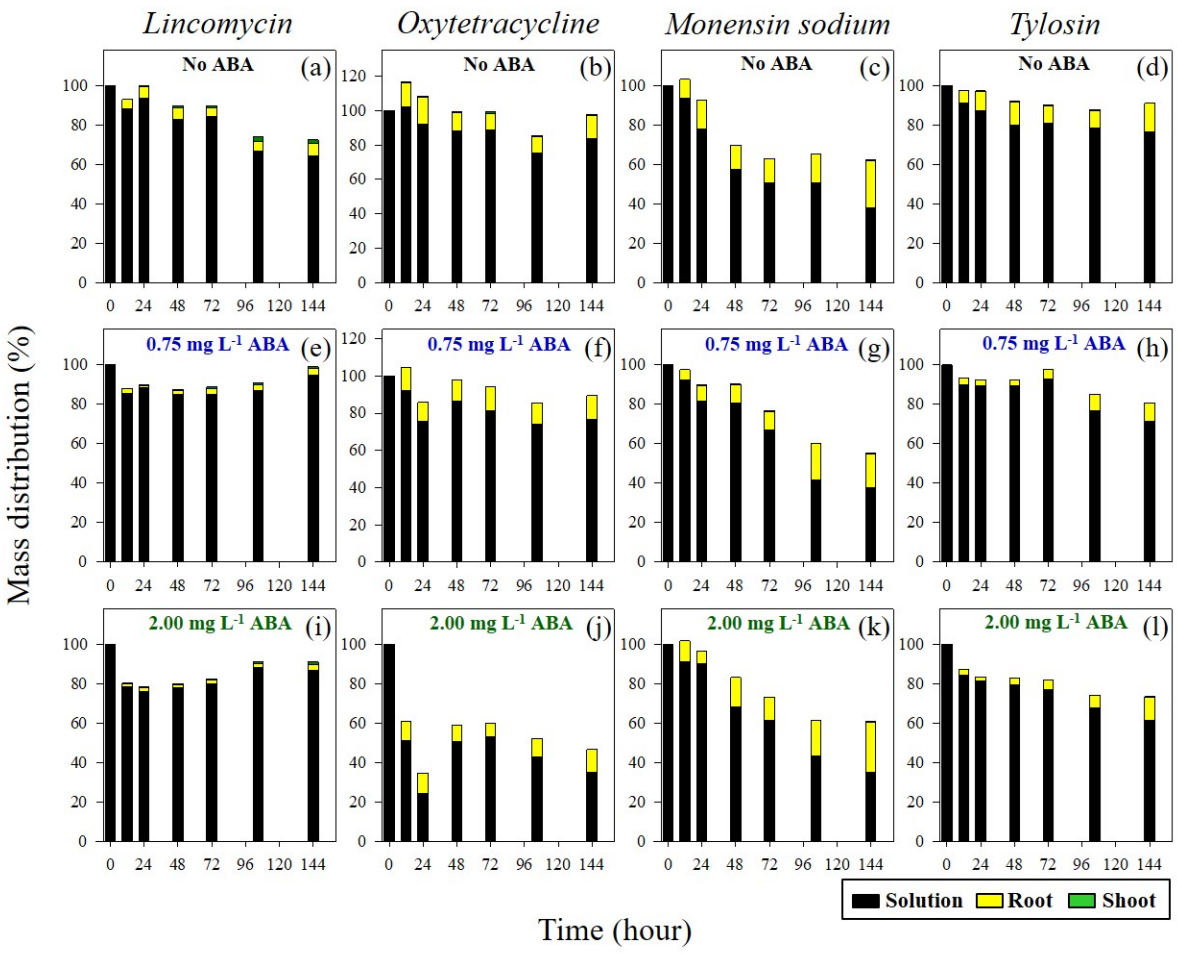


Figure 4.3. Mass distribution of lincomycin, oxytetracycline, monensin sodium, and tylosin in nutrient solution, lettuce roots and shoots as a function of uptake time in the presence of 0, 0.75, and 2.00 mg L^{-1} of abscisic acid (ABA) in nutrient solution.

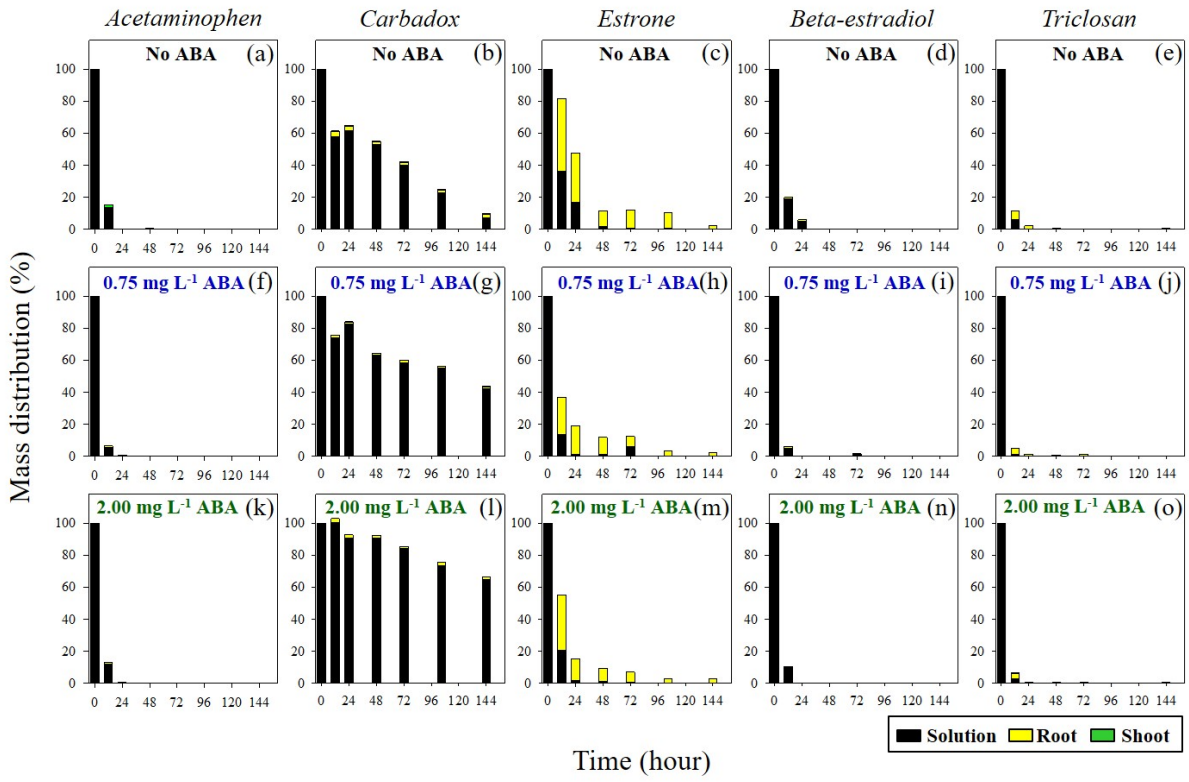


Figure 4.4. Mass distribution of acetaminophen, carbadox, estrone, β-estradiol, and triclosan in nutrient solution, lettuce roots and shoots as a function of uptake time in the presence of 0, 0.75, and 2.00 mg L^{-1} of abscisic acid (ABA) in nutrient solution.

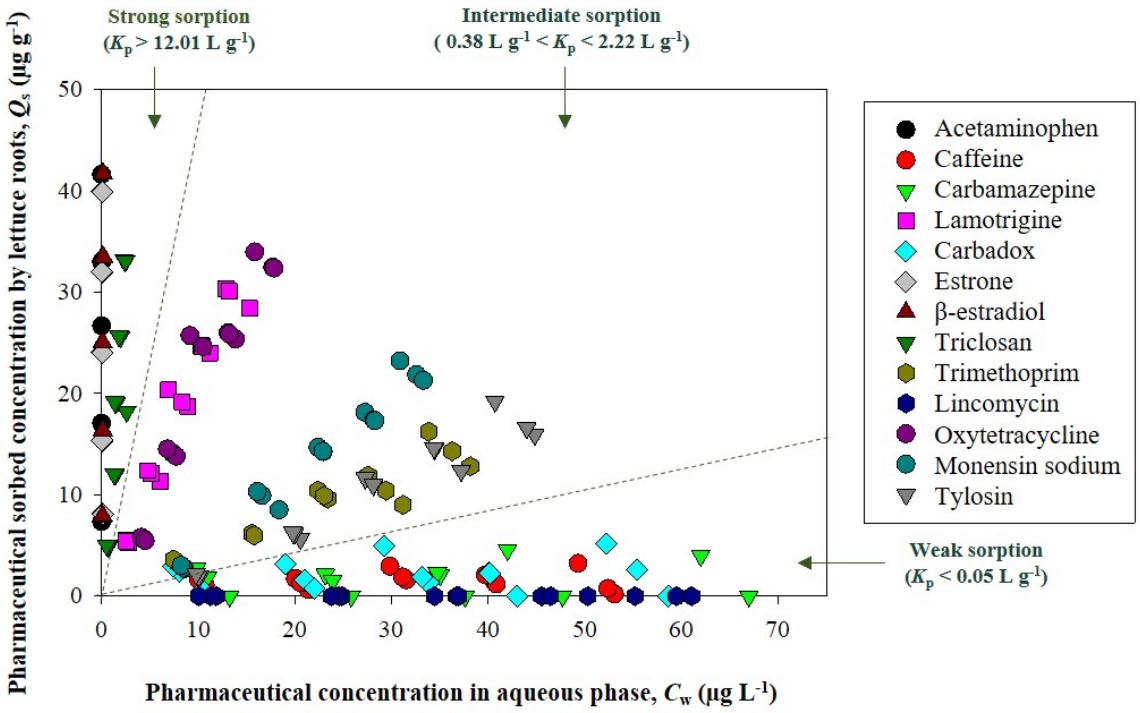


Figure 4.5. Sorption of pharmaceuticals by lettuce roots from nutrient solution. Sorption isotherms were fit to $Q_s = K_p C_w$, where K_p is sorption coefficient (L g^{-1}) (Adapted from Chapter III).

Lincomycin, monensin sodium, oxytetracycline, and tylosin were primarily accumulated in lettuce roots, and < 3% of the originally applied pharmaceuticals was accumulated in shoots (Figure 4.3). After 144 hours of uptake, the total mass recoveries were 87% for lincomycin, 78% for monensin sodium, 59% for oxytetracycline, and 82% for tylosin. ABA amendment in nutrient solution did not result in the apparent decrease in pharmaceutical mass accumulated in roots (Figure 4.3). It was noted that the molecular size of lincomycin, monensin sodium, oxytetracycline, and tylosin are relatively large with molecular weight > 400 Daltons. Plant cell membranes could limit the intercellular transport of these large-sized molecules, and their upward transport to aerial parts could be inhibited by the Casparian strip at endodermis.[9, 13] As a result, greater accumulation in lettuce roots was observed for these pharmaceuticals with minimal amount transported to the shoots (Figure 4.3).

Many pharmaceuticals could be extensively metabolized and form multiple products in plants. About 80% of the initially applied estrone was lost during the first 48 hours of exposure, and the remaining amount continued to decrease down to < 3% after 144 hours (Figure 4.4c, 4.4h, and 4.4m). Carbadox demonstrated substantial metabolism during the experimental period (~10% left after 144 hours of uptake) in the absence of ABA (Figure 4.4b); however, the presence of ABA might cause the reduced uptake of carbadox, hence less metabolism of carbadox in lettuce, as indicated by ~67% of carbadox remaining in the system (the presence of 2.0 mg L^{-1} of ABA) (Figure 4.4l). Acetaminophen, β-estradiol, and triclosan manifested even more rapid metabolic reactions with < 10% of the initially applied amount in the system during the first 24 hours of exposure (Figure 4.4). The rapid metabolism of these pharmaceuticals in lettuce led to minimal concentration or even below detection limits in roots and shoots. Therefore, carbamazepine, caffeine, lamotrigine, trimethoprim, lincomycin, monensin sodium, oxytetracycline, and tylosin

are selected to scrutinize the influence of transpiration stream on transport of these compounds in lettuce.

Effects of Transpiration Stream on Pharmaceutical Accumulation and Transport

Water flow is considered as the major carrier to transport pharmaceuticals from soil water to roots, and then distribute the chemicals in various portions of plants. The ABA amendment in the hydroponic solution reduced the transpiration rate in lettuce. To gain the insight into the relationship between uptake and transport of pharmaceuticals and transpiration stream in lettuce, pharmaceutical mass distributed in roots and shoots is plotted against transpired water for all data collected at different sampling time in the absence and presence of ABA (Figure 4.6). The use of pharmaceutical mass instead of concentration in lettuce could minimize the impact of dilution effects due to the increasing biomass from plant growth with time.

For the small-sized pharmaceuticals the accumulated mass of caffeine, carbamazepine, lamotrigine, and trimethoprim in shoots all demonstrated moderate to excellent linear relationship with the amount of water transpired (Figure 4.6a–4.6d). In contrast, the accumulated mass in lettuce roots and water transpiration had no apparent relationship for caffeine and carbamazepine, and moderate relationship for lamotrigine and trimethoprim (Figure 4.6e–4.6h). These results confirmed that the small-sized pharmaceuticals in water were transported with transpiration stream upward to shoots. During the transport process, strong interaction between pharmaceuticals and lettuce roots could retard the accumulation of pharmaceuticals in shoots. The stronger affinity of lamotrigine and trimethoprim to lettuce roots, compared to caffeine and carbamazepine (Figure 4.5), resulted in a moderate relationship between the accumulated mass in shoots and transpired water, whereas this relationship was more apparent for caffeine and carbamazepine (Figure 4.6). Caffeine and carbamazepine were weakly sorbed by roots, which cause little retention by lettuce

roots, and hence no apparent relationship between their accumulation in roots and transpired water (Figure 4.6e and 4.6f). The measured caffeine and carbamazepine in roots could be primarily from those in root water. The slope of the linear relationship refers to pharmaceutical mass transfer to roots or shoots on the basis of per unit of transpired water. It is noted that the slopes of mass transfer to roots vs. to shoots were similar for lamotrigine (0.0016 vs 0.0016) and trimethoprim (0.0067 vs 0.007) (Figure 4.6c, 4.6g, 4.6d, and 4.6h). These results indicate that sorption of lamotrigine and trimethoprim by root components could contribute to the retention of pharmaceuticals in the hydroponic system. The slopes of mass transfer to shoots was much greater than that to roots for caffeine and carbamazepine (Figure 4.6a, 4.6e, 4.6b, and 4.6f), indicating minimal accumulation in lettuce roots compared to shoots, and further implying transpiration flow as a carrier to move these pharmaceuticals to shoots.

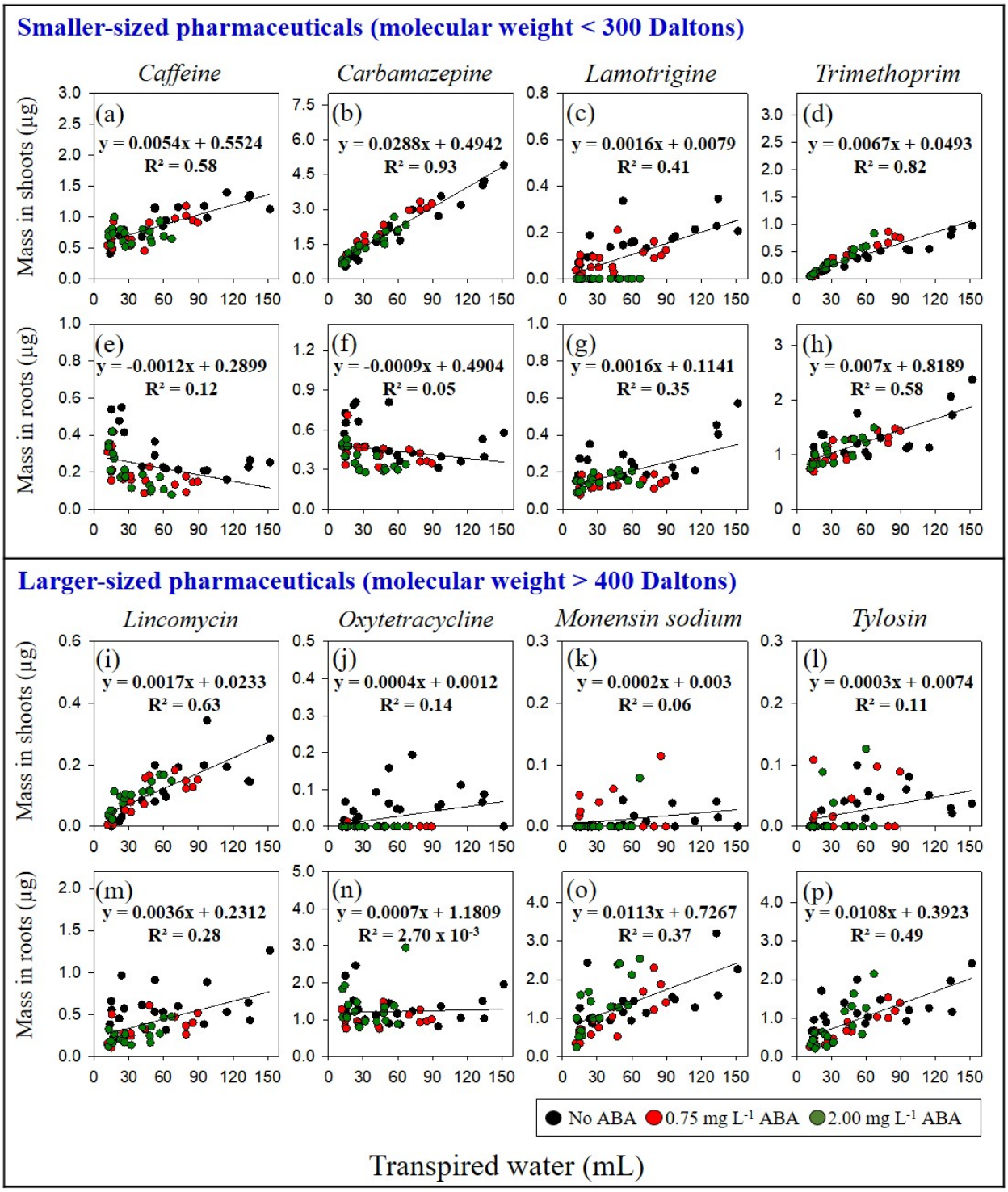


Figure 4.6. Relationship between mass accumulation in lettuce shoots and roots and transpiration water for caffeine, carbamazepine, lamotrigine, lincomycin, monensin sodium, oxytetracycline, trimethoprim, and tylosin in the absence of ABA, and in the presence of 0.75 and 2.00 mg L^{-1} of ABA in the nutrient solution.

In contrast to the small-sized pharmaceuticals, the accumulation of large-sized pharmaceuticals (monensin sodium, oxytetracycline and tylosin) in shoots demonstrated weak relationship with transpired water, except for lincomycin that demonstrated a strong relationship (Figure 4.6i to 4.6l). In general, the amount of large-sized pharmaceutical mass in lettuce shoots was less than that of small-sized molecules (Figure 4.6a to 4.6d). The relationship between the mass in roots and transpired water were apparent different, i.e. weak for oxytetracycline, moderate for lincomycin, monensin sodium and tylosin (Figure 4.6m to 4.6p). This could be attributed to their affinity to lettuce roots and charged speciation in solution. Lincomycin had a very weak affinity to lettuce roots, whereas monensin sodium, oxytetracycline, and tylosin manifested relatively great sorption to lettuce root components (Figure 4.5). In the hydroponic solution (pH 5.8), lincomycin existed primarily as cationic (98%) and as neutral (2%) species, monensin sodium as anionic (97%) and neutral (3%) species, oxytetracycline as zwitterionic (97%) and cationic (3%) species, and tylosin as cationic (99%) and neutral (1%) species. Large-sized pharmaceuticals, especially ionic species, are generally excluded from cell membranes while neutral pharmaceuticals could still cross the lipid bilayers of cell membranes but at a slow rate.[13] Thus, transpiration stream could carry neutral species of lincomycin into roots , and then to shoots without considerable retention by roots. Monensin sodium and tylosin also had a small portion of neutral species; however, their molecular size are much larger, and they were highly affiliated with lettuce roots. Both factors could limit their transport to shoots with transpiration stream. The zwitterionic oxytetracycline might be able to cross the lipid bilayers of cell membranes, but could be at a slow rate due to its large molecular size.[32, 33] The affinity to lettuce roots (Figure 4.5) reduced the transport of oxytetracycline to shoots with transpiration stream. Despite the large-sized pharmaceuticals were excluded from cell membranes, they could still enter lettuce roots in the apoplast pathway, and interact with root

components from transpired water. Thus, the mass of lincomycin, monensin sodium, and tylosin in lettuce roots was somewhat positively related to the amount of transpired water (Figure 4.6m, 4.6o, and 4.6p). No significant difference in oxytetracycline mass accumulated in lettuce roots was observed among all samples collected with and without ABA amendment ($p = 0.33$) (Figure 4.6n).

In summary, this study revealed the relationship between pharmaceutical accumulation and transport in lettuce and the rates of transpiration stream, which indicates that transpired water is the major carrier to transport small-sized pharmaceuticals to lettuce shoots. The strong affinity of pharmaceuticals to lettuce roots decreases their transfer from roots to shoots. Transpiration stream plays a minor role in the transport of large-sized pharmaceuticals to shoots. Thus, they are primarily accumulated in lettuce roots. These results regarding the influence of transpiration stream on pharmaceutical uptake and transport in plants could be applied to predictive models for evaluating the potential risks associated with agricultural production. The reduction in transpiration rate could mitigate accumulation and transport of pharmaceuticals in aerial parts of agricultural produce.

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CHAPTER V

METABOLIC DEMETHYLATION AND OXIDATION OF CAFFEINE IN LETTUCE

ABSTRACT

Pharmaceuticals could be metabolized in crops. Caffeine is one of the most consumed central nervous system stimulant drug and extensively presented in popular beverages. Therefore, it was widely disseminated in the environment and frequently detected in agriculture produce. To date, little is known about the metabolism of caffeine in lettuce. In this study, we examined the uptake and metabolism of caffeine in lettuce using a hydroponic system. Lettuce samples were collected after 10, 24, 48, 72, 105, and 144 hours of exposure. After sample extraction, caffeine distribution in hydroponic solution, lettuce roots and shoots was quantified using a liquid chromatography-QTRAP tandem mass spectrometry (LC-QTrap-MS/MS). Caffeine was primarily translocated to lettuce shoots and less accumulated in roots. However, its total mass distributed in the hydroponic solution and lettuce continued to decrease with uptake time. There was > 50% of applied caffeine lost in the system after 144 h, suggesting caffeine was likely metabolized in lettuce after being taken up. Eight caffeine metabolites were further characterized and tentatively identified using the LC-QTrap-MS/MS under the mode of enhanced mass scan with information dependent acquisition criteria followed by enhanced product ion (EMS-IDA-EPI) scan using MasterView™ Software analysis. The apparent caffeine metabolism in lettuce was demethylation, including the loss of one, two and three methyl functional groups. The contribution of demethylation accounts for 20% of caffeine initially added to the system after 144 h, which was further confirmed with reference standards. Other metabolism of caffeine in lettuce included oxidation, hydroxylation, and 1st demethylation followed by hydroxylation, and their amount increased with uptake time. These results indicate that it is important to understand the metabolism of pharmaceuticals in plants because metabolites may have similar and even higher bioactivity relative to that of the parent compound. The established workflow including kinetic study and LC-QTrap-MS/MS analysis

could be applied to identify the metabolites of other types of pharmaceuticals and agrochemicals in agricultural crops.

INTRODUCTION

Land application of biosolids and crop irrigation with reclaimed water in agricultural production are the major routes for dissemination of pharmaceuticals in agroecosystems. Pharmaceuticals present in soil and water can be accumulated in crops (e.g., vegetables), and then transfer through the food chain due to the dietary exposure by animals and humans. During the past several years major research efforts have been dedicated to investigate the uptake and accumulation of pharmaceuticals by crops from soil and water.[1-5] The accumulation of pharmaceuticals (e.g., caffeine, carbamazepine, and naproxen) were detected in the edible parts of celery, lettuce, and cabbage with the concentrations up to 0.17 μg kg^{-1}, when irrigated with reclaimed water containing caffeine, carbamazepine, and naproxen at the concentrations of 11, 4.2, and 0.43 ng L^{-1}.[4]

Many pharmaceuticals are metabolized in plants, and their intermediate or end products might still contain the bioactive functional moieties.[3, 6] For example, carbamazepine was readily metabolized into 10,11-epoxycarbamazepine, 10,11-dihydroxycarbamazepine, 2-hydroxycarbamazepine, 3-hydroxycarbamazepine in tomato, cucumber, sweet potato and carrot.[3, 6, 7] Among these products, 10,11-epoxide-carbamazepine demonstrated even higher toxic potency than the parent compound carbamazepine.[8] Long-term consumption of carbamazepine-contaminated crops/vegetables could lead to detrimental impacts to human health.[9] Acetaminophen could be conjugated to glutathione and glycoside when the oxidative stress increased (i.e., increase of peroxidase and ascorbate peroxidase activity) in horseradish hairy root cultures.[10] Triclosan was primarily conjugated with saccharides, disaccharides, malonic acid, and sulfate in carrot cells and in carrot plants.[11] Ibuprofen underwent transformative and conjugated reactions mediated by cytochrome P450 monooxygenase in *P. australis*.[12] Considering that plants

function as “green liver” in the natural system,[13, 14] many xenobiotic compounds including pharmaceuticals could be metabolized within plants via phase I reactions such as oxidation, reduction, or hydrolysis (e.g., formation of 10,11-epoxycarbamazepine from carbamazepine), and phase II conjugations with malonic acid, glucose, glutathione, and cysteine (e.g., formation of 4-hydroxyl-glutatuionyl-diclofenac from diclofenac).[9, 15, 16] The products formed from phase I and phase II reactions generally manifested increased hydrophilicity, facilitating the sequestration of metabolites in vacuole and apoplast.[6, 15] Currently, it still remains largely unclear on the mechanism and pathways of pharmaceutical metabolisms in plants.[6, 17, 18]

Caffeine is the most commonly administered pharmaceutical to humans for stimulating the central nervous system, and is also the most frequent ingredient in coffee, teas, cocoa, and soft drinks.[19-21] Withdraw of caffeine from the long-term human consumption causes headache, fatigue, and anxiety.[22, 23] The large amount of annual consumption of caffeine, as well as improper disposal of unused/expired caffeine-containing medicine/drinks, has resulted in its widespread dissemination in wastewater treatment plants (WWTPs), [24, 25] surface water and groundwater.[26-28] For example, in the effluents from WWTPs of the European Union, caffeine concentration could reach up to 3002 ng L^{-1}.[26] In the U.S., caffeine concentration reached 41.2 ng L^{-1} in surface water of Biscayne Bay, Florida.[28] Irrigation with reclaimed water or contaminated surface/groundwater could lead to the accumulation of caffeine in agricultural products, and propagate its dissemination along with the food chain. For example, irrigation with reclaimed water could cause the accumulation of caffeine in carrot and celery with concentration of 0.43 and 0.17 μg kg^{-1} (dry weight).[4] The study in Chapter III found that in hydroponic experiments over 60 % of the initially fortified caffeine was lost in lettuce after 144 hours of exposure, indicating caffeine was intensively metabolized in lettuce. Pierattini et al. reported that in *Populus alba.* L. Villafranca, exogenous

caffeine underwent demethylation reactions losing one $–CH_3$ and forming theobromine and theophylline.[29] In many plants such as coffee and tea plants, endogenous caffeine could be biosynthesized from xanthosine to methylxanthosine, methylxanthine and theobromine.[30, 31] In mammals caffeine primarily undergoes phase I demethylation reactions in animal livers by losing one $–CH_3$ group and forming theobromine, paraxanthine and theophylline.[19, 32] To our knowledge, little is known about the metabolism processes of caffeine in lettuce or other vegetables, even though lettuce is one of the most common ready-to-eat fresh vegetables with average daily intake of 0.23 g/kg/day.[33] Therefore, the accumulation and metabolism of caffeine in fresh vegetables is key information for accessing the potential risks interplayed with environmental quality, food safety and human health.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with various or hybrid mass analyzers (e.g., triple quadrupole, Orbitrap, linear ion trap and time-of-flight) has become the major tools employed to identify metabolites derived from pharmaceuticals in different environmental and plant matrices.[15, 34-37] High-resolution time-of-flight (TOF) mass spectrometry provides accurate mass for the determination of metabolites. Ion trap mass spectrometry could achieve more enriched fragment ions for structure elucidation and quantification. Triple quadrupole mass spectrometry could quantify trace amount of pharmaceuticals using multiple reaction monitoring (MRM) scan as long as the corresponding authentic standards are available.[34, 36]

This study aimed to investigate the uptake and distribution of caffeine in a leafy vegetable (lettuce), and examine the metabolism of exogenous caffeine in the plant. A liquid chromatography coupled to a QTrap tandem mass spectrometry (LC-QTrap-MS/MS) was used to obtain the fragmentation patterns of metabolites for elucidating their chemical structures, and to quantify

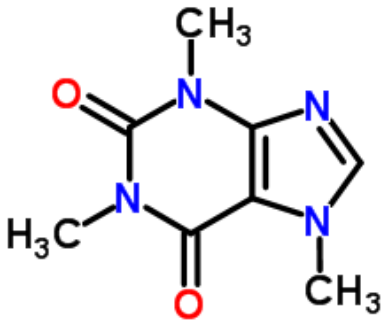
caffeine and its metabolites using MRM mode. The substantial loss of caffeine in lettuce from the study in Chapter III indicates that caffeine could undergo extensive metabolism in lettuce. In this study, the identification of caffeine metabolites was performed by comparing the mass spectra of lettuce extracts from lettuce exposure to caffeine and its caffeine-free controls. This would effectively eliminate the impacts of endogenously formed caffeine in lettuce (if any) and improve the confidence of identified unknown metabolites.[38] Kinetic uptake of caffeine was conducted in a hydroponic system for further elucidation of the evolution of formed metabolites. These results provide the needed information for evaluating the uptake, translocation and metabolism of caffeine in lettuce. The analytical workflow established in this study could be expanded to investigate the metabolism of other pharmaceuticals in crops.

MATERIALS AND METHODS

Chemicals and Materials

Caffeine, 3-methylxanthine, 7-methylxanthine, theobromine, theophylline, paraxanthine and xanthine were purchased from Sigma-Aldrich (St. Louis, MO, USA). The physicochemical properties of caffeine are summarized in Table 5.1. Methanol (HPLC grade) and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and anhydrous sodium sulfate (Na_2SO_4) were purchased from EMD Chemicals (Gibbstown, NJ, USA). Ceramic homogenizers, C18, primary secondary amine (PSA) powders, and reserpine were purchased from Agilent Technologies (Santa Clara, CA, USA). Disodium ethylenediaminetetraacetate (Na_2EDTA) and sodium chloride (NaCl) were obtained from J.T. Baker (Phillipsburg, NJ, USA). Waters Oasis hydrophilic–lipophilic balance (HLB) cartridge was purchased from Waters Corporation (Milford, MA, US). Water used in this study was generated from a Milli-Q water purification system (Millipore, Billerica, MA, USA).

Table 5.1. Physicochemical properties of caffeine.

Properties	Caffeine
Molecular structure	
Formula	$C_8H_{10}N_4O_2$
Molecular weight	194.19
Water solubility[b] (mg L^{-1})	21600
pK_a[b]	10.4 (base)
log K_{ow}[b]	−0.07
log D_{ow}[c] (pH at 5.8)	−4.67

[a]From Chemspider database: http://www.chemspider.com/.
[b]From TOXNET database: http://toxnet.nlm.nih.gov/index.html.
[c]For basic compound: log D_{ow} = log K_{ow} + log $\frac{1}{1+10^{(pka-pH)}}$.[39]

Hydroponic Experiment

Black Seeded Simpson lettuce (*Lactuca sativa*) seeds were germinated on moistened paper tissues, and the seedlings were then transferred to a hydroponic system. The nutrient solution was prepared using MaxiGrow plant nutrient (10-5-14) (General Hydroponics, Sevastopol, CA, USA) with pH at 5.8 and electrical conductivity (EC) of 0.4 mS cm^{-1}. The pH and EC values were measured every 24 hours to keep the optimized growing conditions by adjusting pH between 5.6−5.8 and gradually increasing EC from 0.4 to 0.8 mS cm^{-1} before lettuce reached the stage of maturity. Lettuce seedlings were exposed to LED light for 16 hours per day at an intensity of 150 μmol m^{-2} s^{-1} (Apollo Horticulture Full Spectrum 300W, Rowland Heights, CA, USA). The nutrient solution was aerated using a fusion pump, and the ambient temperature was kept at 18ºC.

After 22 days of growth, lettuce developed well with matured roots, and reached 35−40 cm in height and 8.0−10.5 g of biomass (fresh weight), which was then transferred into an Erlenmeyer flask containing 210 mL of nutrient solution (pH = 5.8, EC = 0.8 mS cm^{-1}). The nutrient solution contained 575 μg L^{-1} of caffeine. The experiment was carried out in triplicate. Experimental controls included lettuce exposed to caffeine-free nutrient solution and nutrient solution containing caffeine but without lettuce. These experiments were performed under the same conditions described above. All flasks were wrapped with aluminum foil to prevent potential photodegradation of pharmaceuticals (if any). During the experimental period, the pH and EC values of nutrient solution were daily adjusted to pH 5.6−5.8 and EC of 0.8 mS cm^{-1}. To compensate the water lost by transpiration, the same amount of freshly prepared nutrient solution free of caffeine was replenished into the flask every day. At 10, 24, 48, 72, 105 and 144 hours of exposure, three flasks with lettuce and the corresponding controls were sacrificed for sampling lettuce and nutrient solution. The lettuce samples were rinsed with deionic water, separated into

roots and shoots, freeze-dried, and ground to powders prior to the extraction and analysis of caffeine.

Dried lettuce roots (100 mg) or shoots (250 mg) were placed in polypropylene centrifuge tubes, sequentially extracted with 1.0 mL of 300 mg L^{-1} of Na_2EDTA, 1.75 mL of methanol, and 3.25 mL of acetonitrile in the presence of two pieces of ceramic homogenizers, 0.2 g of Na_2SO_4 and 0.5 g of NaCl. The extracts were separated from lettuce tissues by centrifuge at 9240 *g* for 10 min; the supernatants were combined, and cleaned up using dispersive solid phase extraction (d-SPE) sorbents (12.5 mg of C18, 12.5 mg of primary secondary amine (PSA), and 225 mg of Na_2SO_4). Caffeine in the nutrient solution was extracted using Waters Oasis hydrophilic–lipophilic-balanced (HLB) solid phase extraction cartridge. The HLB cartridge was preconditioned using 3.0 mL of methanol and 5.0 mL of water. Nutrient solution (20 mL) was passed through the preconditioned HLB cartridge, and caffeine retained by the cartridge was eluted with 5.0 mL of methanol. Caffeine in extracts were analyzed using a Shimadzu 20A liquid chromatography System (Columbia, MD, USA) coupled to a Sciex 4500 QTrap mass spectrometer (Foster City, CA, USA). The averaged extraction recoveries of caffeine from nutrient solution, lettuce roots and shoots were measured at 116.3%, 91.8%, and 106.2%.

Caffeine Analysis by LC-QTrap-MS/MS

Caffeine in nutrient solution, lettuce roots and shoots was analyzed using a Shimadzu Prominence high-performance liquid chromatography (Columbia, MD, USA) coupled to a Sciex 4500 QTrap tandem mass spectrometer (Foster City, CA, USA) (LC-QTrap-MS/MS) under positive ion mode. Multiple reaction monitoring (MRM) mode was used with the transitions of m/z 195.0→110.0 for qualification and m/z 195.0→138.0 for quantification. The Turbo IonSpray source voltage was 5000 V, temperature was at 500 ºC, curtain gas pressure was 25 psi, collision

gas was set as medium, ion source gas 1 and gas 2 pressure were both 60 psi. An Agilent SB-C18 column (100 mm × 3 mm, particle size 3.5 μm, Santa Clara, CA, USA) was used in the LC-QTrap-MS/MS for separation. The binary mobile phases were phase A water containing 0.2 mM ammonium acetate and phase B acetonitrile. The programming gradient of mobile phase was set up as: 2 min of pre-equilibration with 100% phase A; 0−6 min 40% phase B; 6−8 min increased to 100% phase B and held it until 8.5 min. The flow rate was 0.35 mL min^{-1}, and the injection volume was 10 μL. The retention time for caffeine was 5.84 min. All the samples were quantified using matrix-matched standard curves. The instrumental parameters for the analysis are provided in Table 5.2.

Table 5.2. Parameters of LC-QTrap-MS/MS for caffeine analysis.

Precursor ion (m/z)	Product ion[a] (m/z)	DP[b] (V)	CE[c] (V)	CXP[d] (V)
195.0	**138.0**	60	30	10
195.0	110.0	60	30	6

[a]The precurse/product ion transitions used to quantify (in bold) and qualify caffeine.
[b]Declustering potential.
[c]Collision energy.
[d]Collision cell exit potential.

Identification of Caffeine Metabolites using LC-QTrap-MS/MS

Caffeine metabolites were identified by LC-QTrap-MS/MS using the combination of enhanced mass scan, information dependent acquisition and enhanced product ion scan (EMS-IDA-EPI). Before scanning samples, reserpine was injected to calibrate the linear ion trap of LC-QTrap-MS/MS. The LC conditions (column, mobile phase composition and gradient) used were the same to the settings for the analysis of caffeine. In the QTrap tandem mass spectrometry, the voltage of Turbo IonSpray source was 5000 V at positive ion mode, and −4500 V at negative ion mode. The source temperature was set at 500 ºC, curtain gas pressure at 25 psi, collision gas was set as High, and pressure of ion source gas 1 and 2 at 60 psi for both positive and negative ion mode. The EMS scan for MS spectrum ranged from m/z 50 to 600 at a scan rate of 10,000 Da/s. The declustering potential (DP), entrance potential (EP), and collision energy (CE) were 60, 10, and 10 V at the positive ion mode, and −10, −10, and −10 V at the negative ion mode. The threshold of IDA was set up at 1000 cps to trigger EPI scan to obtain MS/MS spectrum. This threshold setting for IDA was to eliminate many minor peaks derived from the complicated plant matrices. The EPI scan had the same scanning rate, and DP and EP voltages to those in the EMS scan. The CE was set up particularly at 35 V for positive ion mode, and −35 V for negative ion mode, and the collision energy spread (CES) was set at 15 V. This setup allowed to acquire the averaged MS/MS spectrum with CE at 20, 35 and 50 V under positive ion mode, and at −20, −35 and −50 V at negative ion mode.

Non-target Screening of Caffeine Metabolites in Lettuce

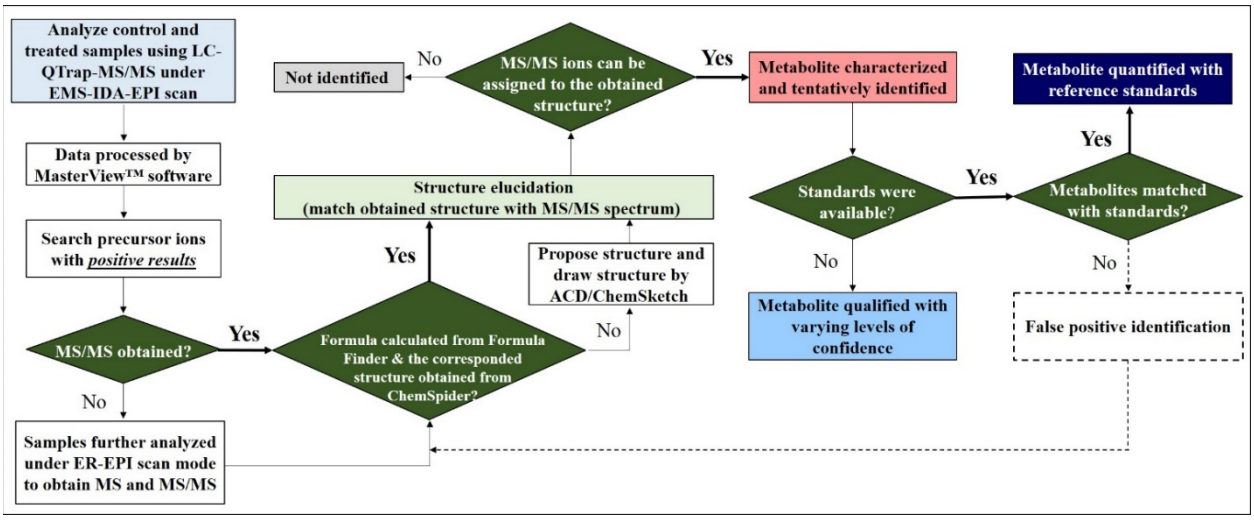
LC-QTrap-MS/MS was used to identify metabolites under both positive and negative ion mode and by the combinations of enhanced mass scan (EMS) as the survey scan, information dependent acquisition (IDA) criteria, and enhanced product ion (EPI) scan to obtain MS/MS defragment

patterns. This approach was applied to analyze the samples with caffeine-fortified and caffeine-free lettuce after 144 h of uptake. The working flowchart for identification of caffeine metabolites in lettuce using LC-QTrap-MS/MS is shown in Scheme 5.1. The data obtained from LC-QTrap-MS/MS were processed using the MasterView™ software in the PeakView® 2.2 package (SCIEX, Foster City, CA, USA). In the MasterView™, the default threshold of the ratio of precursor ion in sample to that in control was set as 3, i.e., the precursor ions with the intensity > 3X of the control was considered as the positive results. The positive precursors were further analyzed to obtain their MS and MS/MS spectra, and the supplemental enhanced resolution followed by enhanced product ion (ER-EPI) scan was used for the precursor ions of interest (without the obtained MS/MS spectra). The MS and MS/MS spectra were linked to the Formula Finder in MasterView™ to obtain the corresponding molecular formulas. The selected formulas were matched with the chemicals provided from the ChemSpider database to obtain the corresponding structures. These structures were examined whether they contained the similar moieties of the parent compound e.g., xanthine structure in the metabolites from caffeine. If no appropriate chemical structure available from the ChemSpider database, ChemSketch software (ACD/Labs, Toronto, Ontario, Canada was used to design the possible structures that were imported to MasterView™, and analyzed for defragment patterns using the MS and MS/MS spectra. Once most fragments from the spectra matched well with the assigned structures, the metabolites could be tentatively identified. The example of identification of caffeine was presented in Figure 5.1. Those tentatively identified metabolites were further confirmed using the authentic standards (if available). The LC-QTrap-MS/MS used in this study is classified as a low-resolution mass spectrometry, false positive results could occur to those tentatively identified metabolites when the mass spectra or LC retention time (RT) did not match with authentic standards. In general, those tentatively identified metabolites

without the confirmation by authentic standards could be ranked with the confidence at varying levels.[40]

For the tentatively identified metabolites with authentic standards, they were further confirmed in the lettuce shoot samples (144 h) analyzed under the optimized MRM mode developed using the authentic standards with quantification/qualification transition pairs and matched RT. All the samples (nutrient solution, lettuce roots, and shoots) collected through 0 to 144 hours of exposure were also analyzed under the MRM mode for the metabolites using matrix-matched standard curves. For the tentatively identified metabolites without authentic standards, their primary fragments were selected to establish MRM transitions. Then the samples were analyzed, and the IDA and EPI were further used to confirm the metabolites. The peak areas of metabolites were integrated at the specific RT acquired from the positive results, and normalized to the caffeine-free control samples.

Scheme 5.1. Flowchart of identification of caffeine metabolites in lettuce using LC-QTrap-MS/MS.



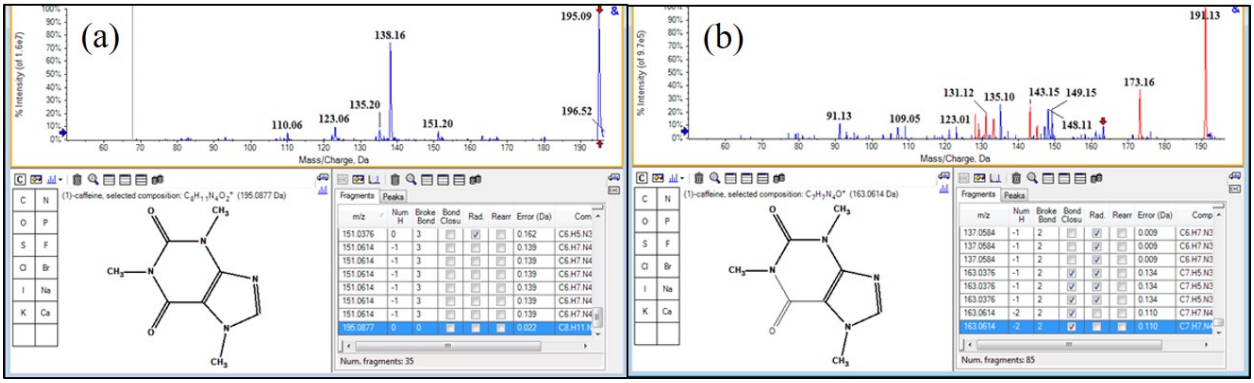


Figure 5.1. Caffeine identification through structure elucidation with (a) tentatively identified chemical with the fragments in MS/MS spectrum (highlighted in blue) assigned to caffeine structure, and (b) not identified chemicals with many fragments highlighted in red not assigned to chemical structure.

RESULTS AND DISCUSSION

Uptake of Caffeine by Lettuce

Our previous results indicated that more than 85% of caffeine taken up by lettuce could be metabolized in plants in hydroponic experiments at an initial caffeine concentration of 50 μg L^{-1}. To further elucidate the metabolism of caffeine in lettuce, in this study a higher caffeine concentration (i.e., 575 μg L^{-1}) was applied in the hydroponic solution, which was needed for the detection, identification and quantification of the formed unknown metabolites. The application of the high concentration of caffeine in nutrient solution did not render the apparent adverse effects on lettuce growth (Figure 5.2). The mass distributions of caffeine in hydroponic solution, lettuce roots, and shoots are plotted against the uptake periods (Figure 5.3). It is apparent that the amount of caffeine in hydroponic solution gradually decreased with time to 12.9% of the initially applied amount after 144 hours of exposure. At the same time, the mass distribution of caffeine in lettuce shoots increased from 4.6% (12 h) to 31.3% (144 h), and the amount in lettuce roots remained relatively low within the range of 1.6% to 3.3% during the experimental period. This could be attributed to relatively small size of caffeine molecules and its minimal affinity to lettuce roots, which facilitates the entrance of caffeine to lettuce roots and translocation to shoots along with the transpiration stream as described in the Chapter III.

It is noteworthy that the total recovered mass of caffeine kept decreasing with uptake time (Figure 5.3). The total recoveries of caffeine in the hydroponic solution-lettuce system were 97.2%, 91.4%, 71.8%, 73.6%, 68.8%, and 45.9% of the initial amount of caffeine after 10, 24, 48, 72, 105 and 144 hours of uptake. Photodegradation of caffeine and other losses were negligible based on the fact that the measured caffeine concentration was within 97 to 105% of the initial amount in the lettuce-free controls during 144 hours of uptake. In this study, the extraction recoveries of

caffeine from nutrient solution, lettuce roots, and shoots were all > 90%. After 144 hour of exposure, caffeine not present in nutrient solution was assumed to all enter lettuce, which was equivalent to ~87.1% of the initial amount, and the metabolism occurred only within the plant. Therefore, it is estimated that approximately 62.1% of caffeine absorbed in lettuce was metabolized most likely in shoots. The extracts of lettuce shoots collected at 144 hours of exposure were therefore used for identifying metabolites.

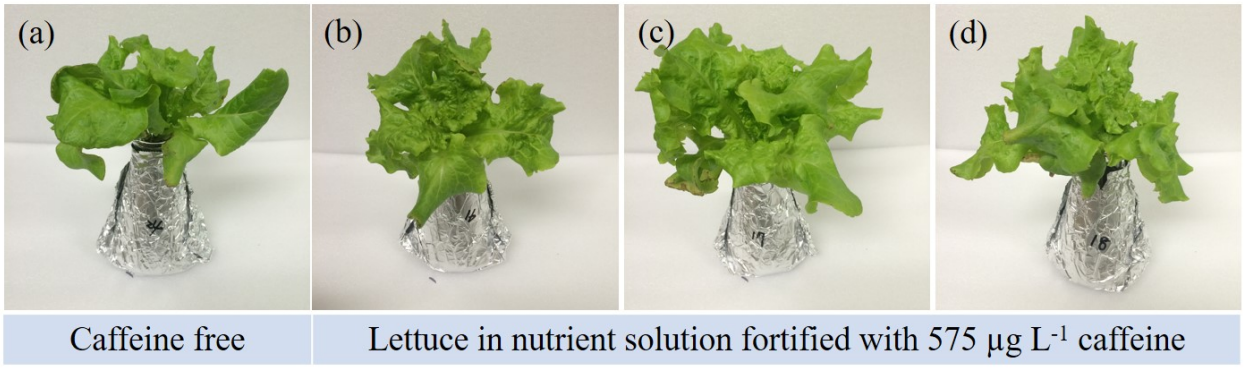


Figure 5.2. Lettuce exposed to nutrient solution (a) without caffeine (controls) and (b to d) in the presence of 575 µg L^{-1} of caffeine after 144 hours of uptake.

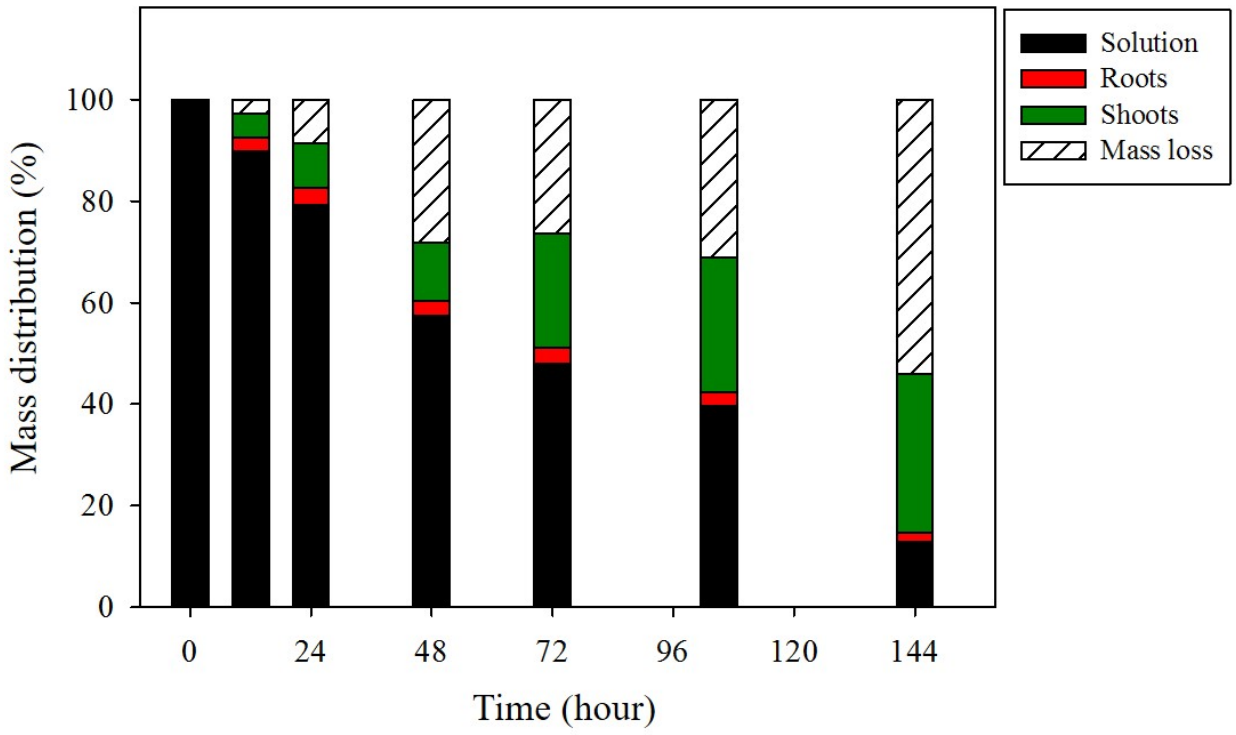
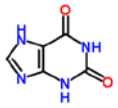
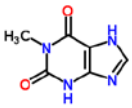
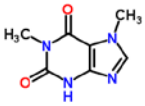
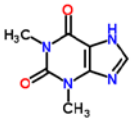
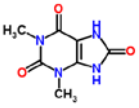
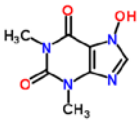
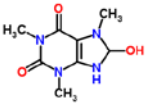


Figure 5.3. Caffeine mass distribution in nutrient solution, lettuce roots and shoots as a function of uptake time.

Caffeine Metabolism in Lettuce

The caffeine metabolites in lettuce were identified using the optimized EMS-IDA-EPI scan in LC-QTrap-MS/MS. EMS-IDA-EPI scan is commonly used to identify metabolites in the non-target screening.[41] In this study, eight metabolites derived from caffeine were tentatively identified, which were xanthine, methylxanthine, theobromine, paraxanthine, theophylline, 1,3-dimethyluric acid, 1,3,7-trimethyluric acid (M210), and 8-hydroxy-1,3,7-trimethyl-3,7,8,9-tetrahydro-1H-purine-2,6-dione (M212) (Table 5.3). Among these eight metabolites, the authentic reference standards were commercially available for xanthine, 3-methylxanthine, 7-methylxanthine, theobromine, paraxanthine, theophylline, and 1,3-dimethyluric acid. These standards were used to further confirm the metabolites present in lettuce shoots using the European Union Guideline 2002/657/EC.[42] To do so, two pairs of precursor and product transitions were selected for each authentic reference standard to identify the metabolite, which could reach the identification points (IP) assigned as 4. The IP value of 4 meets with the minimum requirement of IP $\geq$ 3 for veterinary drugs and organic contaminants. The response ratio of two transitions, MS/MS spectra and RT obtained from lettuce samples were compared with reference standards. The results revealed that the six metabolites except 1,3-dimethyluric acid were unambiguously identified by their fingerprints, and confirmed their presence in lettuce shoots.

Table 5.3. Tentatively identified caffeine metabolites.

Metabolite	Reaction	Formula	Precursor ion (MH^+, m/z)	Structure[a]	RT[b] (min)
Xanthine	3rd Demethylation	$C_5H_4N_4O_2$	152.95		1.1
Methylxanthine	2nd Demethylation	$C_6H_6N_4O_2$	166.97		4.3
Theobromine	1st Demethylation	$C_7H_8N_4O_2$	180.98		4.3
Paraxanthine					
Theophylline					
1,3-dimethyluric acid[c]	1st Demethylation followed by oxidation	$C_7H_8N_4O_3$	197.07		4.1
7-Hydroxy-1,3-dimethyl-3,7-dihydro-1H-purine-2,6-dione (M196)[d]	1st Demethylation followed by hydroxylation	$C_7H_8N_4O_3$	197.07		4.1
1,3,7-Trimethyluric acid (M210)	Oxidation	$C_8H_{10}N_4O_3$	211.08		5.4
8-Hydroxy-1,3,7-trimethyl-3,7,8,9-tetrahydro-1H-purine-2,6-dione (M212)	Hydroxylation	$C_8H_{12}N_4O_3$	213.09		5.9

[a]From Chemspider database: http://www.chemspider.com/.
[b]Retention time acquired from EMS-IDA-EPI scan type.
[c]False positive identification.
[d]Tentatively identified followed by the false positive identification of 1,3-dimethyluric acid.

A false positive result occurred when matching the RT with the reference standard 1,3-dimethyluric acid (precursor ion m/z 197.07, $C_7H_8N_4O_3$). The RT for two major transitions m/z 197.0→140.0 and m/z 197.0→179.0 of 1,3-dimethyluric acid was at 4.3 min. However, the RT for those transitions in lettuce shoot samples was at 5.8 min. Instead, another chemical 7-hydroxy-1,3-dimethyl-3,7-dihydro-1H-purine-2,6-dione (M196) with the same formula and precursor ion obtained from ChemSpider database matched well with the MS/MS spectrum with all major fragments assigned to the structure of M196 (Figure 5.4). M196 was formed by the substitution of 7–CH_3 with –OH functional group. The major fragments of m/z 182, 181, 169, 167, and 153 resulted from the loss of one –CH_3, one –O, two CH_2, one –O and one –CH_2, and one –O and two –CH_2 (Figure 5.4). M210 could be formed by oxidation at C–8 of caffeine. The major fragments of m/z 196, 181, 167, and 153 were associated with the loss of one –CH_3, one –O and one –CH_2, one –O and two –CH_2, and one –O and three –CH_2. M212 could be formed by hydroxylation at C-8, and the its major fragments of m/z 195, 185, 167 were formed by the loss of one –H_2O, two –CH_2, and one –OH and two methyl groups (–CH_2 and –CH_3). Importantly, these metabolites all contained the core fragments of caffeine with the fragment of m/z 167 which could be formed by the loss of two –CH_2 functional groups from caffeine. The identification confidence of the metabolites M196, M210, and M212 (without available reference standards) could be annotated as level 3-putatively characterized compound classes according to the minimum reporting standards documented by Sumner et al. in 2007.[40] In the document, four levels of identification confidence were proposed for metabolite identifications. The level 1-identified compounds referred to chemicals should be identified by reference standards. The level 2-putatively annotated compounds referred to chemicals should be identified through physicochemical properties and/or through matching commercial database. The level 3-putatively characterized compound referred

to chemicals were identified with spectral similar to known compounds. The level 4-unknown compounds referred to chemicals could only be differentiated based on spectral data. In the present study, the metabolites of caffeine were identified through matching MS and MS/MS spectra with known structure obtained from ChemSpider database.

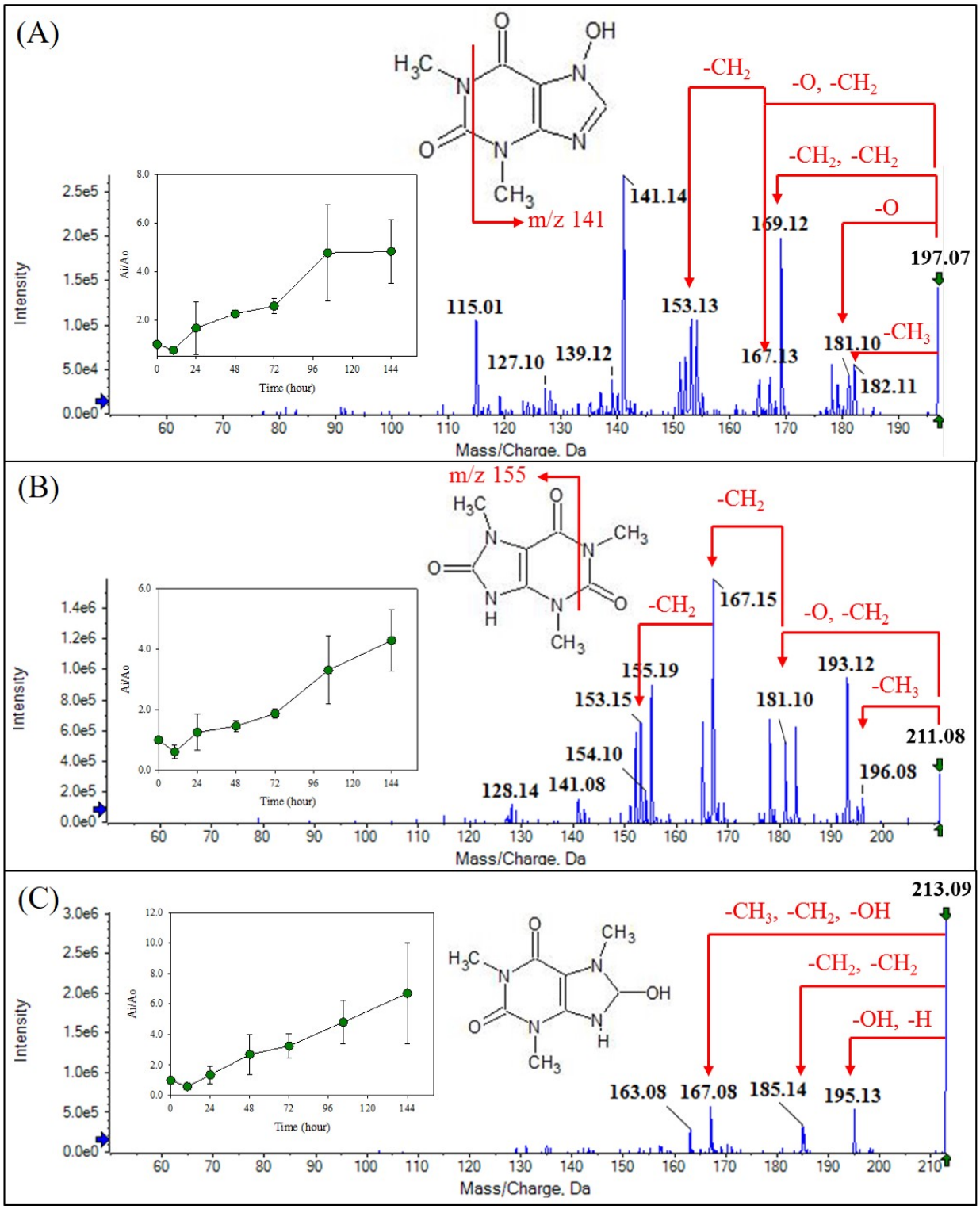


Figure 5.4. MS/MS spectra of caffeine metabolites (without reference standards) (A) 7-Hydroxy-1,3-dimethyl-3,7-dihydro-1H-purine-2,6-dione (M196), (B) 1,3,7-trimethyluric acid (M210), and (C) 8-Hydroxy-1,3,7-trimethyl-3,7,8,9-tetrahydro-1H-purine-2,6-dione (M212), and their integrated areas relative to caffeine-free controls (Ai/Ao) as a function of uptake time.

The analysis of caffeine metabolism in lettuce showed that caffeine primarily metabolized via demethylation and oxidation/hydroxylation reactions (Figure 5.5), and these reactions primarily occurred in lettuce shoots. At 144 hour of exposure, the demethylation metabolites in the nutrient solution, lettuce roots, and shoots was quantified, and accounted for 0.4%, 1.3%, and 17.1% of the initially applied caffeine. In addition, the oxidation/hydroxylation metabolites were positively found only in lettuce shoot samples with the response > 3 times of that in control, and not detected in lettuce roots or nutrient solution. The stepwise demethylation reactions are the major metabolism pathway (Figure 5.5). Caffeine lost one $–CH_3$ functional group forming theobromine (1–N demethylation), paraxanthine (3–N demethylation), and theophylline (7–N demethylation), then lost the second $–CH_3$ functional group forming 3-methylxanthine and 7-methylxanthine. These two compounds could lose the third $–CH_3$ forming xanthine. The demethylation reaction of caffeine with the loss of one $–CH_3$ functional group (N-demethylation) is common in human and animals where this reaction is primarily mediated by hepatic cytochrome P450s (CYP) 1A2 and 2E1.[43-45] In plants especially in coffee and tea, caffeine derived from purine nucleotides mediated by xanthosine methyltrasferase and theobromine synthase followed by caffeine synthase.[46, 47] Meanwhile, in coffee and tea plants caffeine could be transformed to theobromine and theophylline, then to 3-methylxanthine followed by xanthine, and eventually to CO_2 and NH_3,[30, 48, 49]

In addition to demethylation, caffeine and its metabolites could undergo oxidation/hydroxylation reactions forming 7-hydroxy-1,3-dimethyl-3,7-dihydro-1H-purine-2,6-dione (M196) via 7–N demethylation followed by 7–N hydroxylation, 1,3,7-trimethyluric acid (M210) via oxidation at C-8, and 8-hydroxy-1,3,7-trimethyl-3,7,8,9-tetrahydro-1H-purine-2,6-dione (M212) via hydroxylation at C-8 (Figure 5.5). The oxidation and hydroxylation of caffeine in lettuce were also observed in mammals, which are mediated by P450 enzymes.[50-52] In humans,

the oxidation of caffeine was mediated by CYP1A2, and hydroxylation was mediated by CYP3A4 in marmosets.[50] Caffeine underwent oxidation at C-8 position was also observed in *Camellia assamica* var. kucha and Coffea *liberica*.[30, 53] In this study the identified metabolites were primarily derived from the type of phase I reactions. Caffeine conjugates with glucose and glutathione were not found when the precursor ions were scanned for glycoside conjugate m/z 357 ($[caffeine]^+$ + 162) and glutathione conjugates m/z 500 ($[caffeine]^+$ + 305) among the positive results.

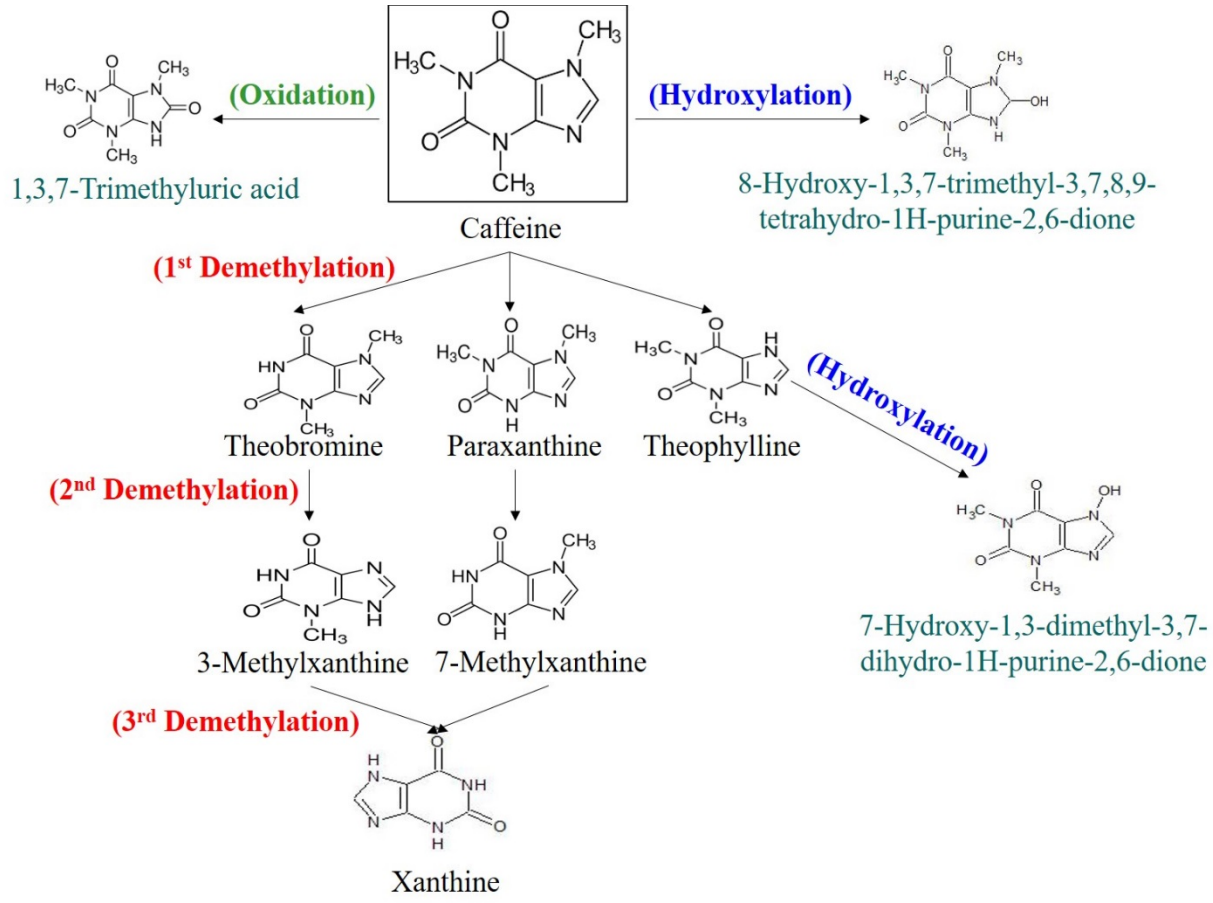


Figure 5.5. Proposed metabolism pathways of caffeine in lettuce.

Kinetics of Caffeine Metabolism in Lettuce

To elucidate the kinetics of caffeine metabolism in lettuce, caffeine and its metabolites present in hydroponic solution, lettuce roots and shoots were identified and analyzed by LC-QTrap-MS/MS under MRM scan mode. Caffeine and its metabolites with authentic standards (including xanthine, 3-methylxanthine, 7-methylxanthine, theobromine, paraxanthine, and theophylline) were quantified using the matrix-matched standard curves for the samples collected during 0 to 144 hours of uptake (Figure 5.6). The optimized LC-QTrap-MS/MS parameters for the analysis of caffeine metabolites are provided in Table 5.4. Among the metabolites 3-methylxanthine and 7-methylxanthine demonstrated the same RT at 4.48 min, and very similar defragment patterns. Paraxanthine and theophylline (loss of one $–CH_3$ from caffeine) also had very similar RT and defragments. It is difficult to separate these two groups of similar metabolites for quantification. Thus, these metabolites were quantified as sum of 3-methylxanthine/7- methylxanthine, and paraxanthine/theophylline. For the metabolites M196, M210 and M212 without authentic reference standards, their precursors and the most abundant product ion transitions were paired, and the corresponding integrated areas were calculated relative to the background of the caffeine-free controls, as a semi-quantification of these metabolites.

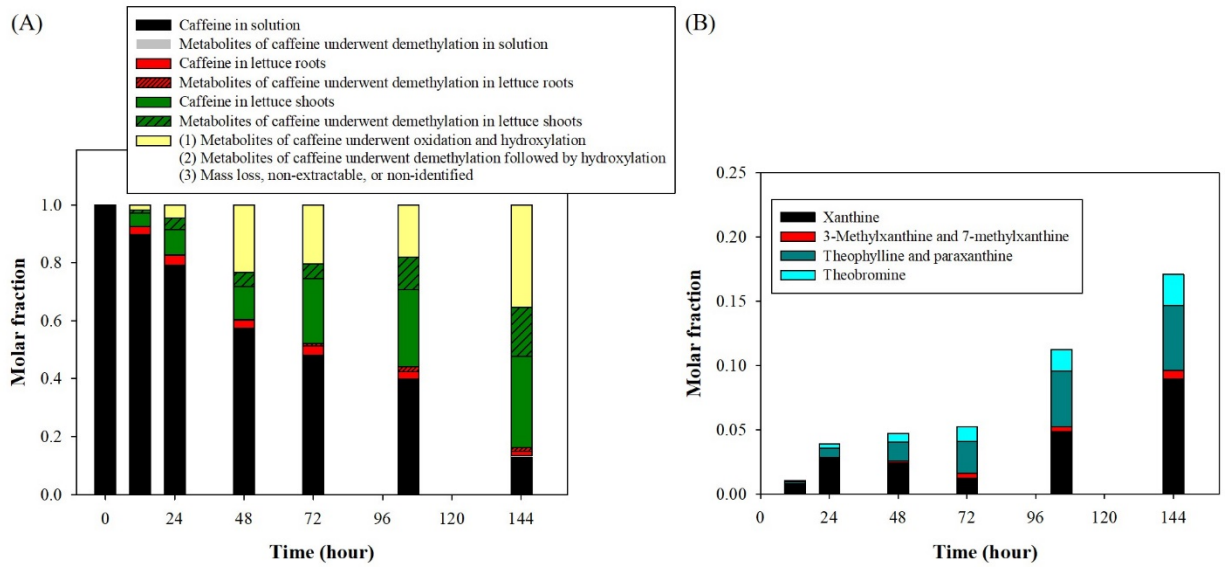


Figure 5.6. (A) Distributions of caffeine and its metabolites in hydroponic solution, lettuce roots and shoots on molar basis as a function of uptake time. (B) Distributions of caffeine demethylation metabolites in lettuce shoots as a function of uptake time.

Table 5.4. Transition ion pairs of multiple reaction monitoring (MRM) at positive ion mode and parameters of QTrap-MS/MS for analysis of caffeine metabolites.

Chemicals	Precursor ion (m/z)	Product ion[a] (m/z)	DP[b] (V)	CE[c] (V)	CXP[d] (V)	RT[e] (min)
Xanthine	153.1	**109.9**	6	25	8	1.30
		135.9	6	21	8	
3-Methylxanthine/ 7-Methylxanthine	166.9	**123.9**	6	23	8	4.48
		96.0	6	27	8	
Theobromine	180.9	**137.8**	71	25	8	4.48
		162.9	71	25	10	
Paraxanthine/ Theophylline	180.9	**123.9**	36	27	8	5.19
		95.8	36	31	6	

[a]The product ion coupled with precursor used to quantify (in bold) and qualify pharmaceutical.
[b]Declustering potential.
[c]Collision energy.
[d]Collision cell exit potential.
[e]Retention time (RT) confirmed with reference standards by analysis using MRM scan mode.

The overall mass distributions of caffeine and its demethylation metabolites on molar basis in hydroponic solution, lettuce roots and shoots are shown in Figure 5.6A. The molar fractions of caffeine decreased with time in nutrient solution, and minimal amount of the demethylation metabolites was present (e.g., $< 4.1 \times 10^{-3}$). The molar fractions of caffeine and its demethylation metabolites in lettuce roots remained within a narrow range between 0.03 and 0.04. In lettuce shoots caffeine and demethylation metabolites increased with exposure periods, and to 0.49 at 144 hours. These results revealed that demethylation reactions occurred primarily in lettuce shoots, and to a much less extent in roots where the amount of demethylation metabolites was approximately 7.6% of those in shoots. The total molar fractions of caffeine and its demethylation metabolites (including xanthine, 3-methylxanthine/7-methylxanthine, theobromine, and paraxanthine/theophylline) in nutrient solution, lettuce roots and shoots varied as a function of exposure periods, which accounted for 98.3%, 95.5%, 76.6%, 79.7%, 82.0%, and 64.7% of the initially applied caffeine at 10, 24, 48, 72, 105, and 144 hours of uptake (Figure 5.6A). At 144 hours of uptake, caffeine and its demethylation metabolites were 12.9% and 0.4% in solution, 1.6% and 1.3% in lettuce roots, and 31.3% and 17.1% in shoots. The remaining unquantified/unidentified fractions was 35.3% of the initially applied caffeine, which could be attributed to the products formed by oxidation, hydroxylation, and conjugation reactions (if any). In addition, the measured oxidative and hydroxylated metabolites M196, M210, and M212 increased approximately 4 to 6 times during the 144 hours of exposure (insert panels in Figure 5.4), which is consistent with the increase in the unquantified/unidentified fractions with uptake time proceeding (Figure 5.6A)

Among the demethylation metabolites in lettuce shoots, xanthine, 3-methylxanthine/7-methylxanthine, theobromine, and paraxanthine/theophylline were all detected. Xanthine and the

metabolites with the loss of one $–CH_3$ were the predominant products (Figure 5.6B). Xanthine was found in all shoot samples, and 3- or 7-methylxanthine was not detected in the samples collected at 10 hours of exposure. These results indicate that demethylation reaction is a relatively rapid process, and the reaction could be even faster during the loss of the second $–CH_3$ group. In general, the molar fraction of each demethylation metabolite increased with uptake time, and the total molar fractions increased from 0.01 to 0.17 during 10 to 144 hours of uptake. At 144 h of uptake, the demethylation metabolites in lettuce shoots accounted for 17.1% of the initially applied caffeine. Among these metabolites, the sum of molar fractions of caffeine metabolites with the loss of one $–CH_3$ was 0.09, and xanthine fraction (loss of three$–CH_3$ groups) was 0.07. In contrast, the molar fractions of 3-, and 7-methylxanthine (loss of two $–CH_3$ groups) were very small i.e. < 0.007. These results again indicated that the transformation rate from 3- and 7-methylxanthine to xanthine was very rapid, and/or they easily underwent other reactions, e.g., oxidation or hydroxylation. In lettuce roots, only very small amount of xanthine and theobromine was detected, and 3-, and 7-methylxanthine, paraxanthine and theophylline were all below the limits of detection. The total molar fractions of xanthine and theobromine in lettuce roots accounted for < 1.3% of the initially applied caffeine at 144 hours of exposure. In hydroponic solution negligible amounts of xanthine and paraxanthine/theophylline were found (< 0.5% of the initially applied caffeine). None of 3-, 7-methylxanthine or theobromine was detected in the hydroponic solution. Lettuce shoots were the major domains for accumulation and metabolism of caffeine after it entered the lettuce. Pierattini et al.[29] also found that higher concentration of exogenous theobromine and theophylline in the leaves of *Populus alba* than that in stems or roots. Minimal amount of metabolites in lettuce roots could be due to that fact that caffeine is readily translocated to shoots where it could be extensively metabolized.

Implications

Caffeine and many other pharmaceuticals such as carbamazepine, salbutamol, and trimethoprim are commonly detected in agriculture produce,[1, 4, 54] which poses potential risks to animal and human health via dietary consumption. These pharmaceuticals could be metabolized in vegetables; the formed metabolites could still maintain the bioactive moieties and function in a similar manner as the parent compounds. For example, theobromine and theophylline are the bioactive stimulants same as caffeine, which are also commonly used in the therapy of acute and chronic asthma.[19, 20] Risk assessment associated with the single parent compounds without the consideration of metabolites could underestimate the risks to ecosystem and human health. This study provides a sound workflow for non-target screening of metabolites using LC-QTrap-MS/MS operating under EMS-IDA-EPI scan mode. Although LC-QTrap-MS/MS is not a high-resolution mass spectrometry, the linear ion trap could provide more enriched and high abundant fragments for identification and quantification of trace-level metabolites in plants with complicated matrices. This enhanced sensitivity facilitates the elucidation of chemical structures of metabolites, and quantification of low concentration of these compounds in environmental samples. Therefore, the analytical workflow established in this study to examine the metabolism of caffeine in lettuce could be further expanded to investigate the metabolism of other pharmaceuticals in environmental matrices and in plant and animal biota.

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CHAPTER VI

FUTURE RESEARCH

This research work highlighted that the molecular size of pharmaceuticals and their sorption affinity to plant roots played important roles in their accumulation and transport in lettuce. Small-sized pharmaceuticals could enter lettuce roots and easily transport upward to lettuce shoots; the strong affinity to lettuce roots reduced their transport to shoots. More transport of small-sized pharmaceuticals from lettuce roots to shoots was observed with increasing transpiration stream. In contrast, large-sized pharmaceuticals mainly accumulated in roots regardless of their affinity to lettuce roots. In plants, some pharmaceuticals could be further metabolized. The metabolites could still maintain the bioactive moieties and pose risks to animal and human health after consumption.

Based on previous studies and the findings in this study, transpiration water flow, plant physiological characteristics, pharmaceutical physicochemical properties, and interaction between pharmaceuticals and plant tissues as well as transformation of pharmaceuticals in plants collectively influence pharmaceutical accumulation in the different parts of a plant. To better understand the synergistic effects of a mixture of pharmaceuticals on food production and human health through long-term consumption of pharmaceutical-contaminated crops, more research is needed to provide the information needed for developing an appropriate risk assessment framework. According to the present study, the following research is suggested as the next-step studies:

Uptake Mechanism

Pharmaceutical size and their interaction with plant roots are two major factors determining the extent of pharmaceutical accumulation and transport in plants. It still needs to better clarify the size exclusion limit of cell membrane for pharmaceuticals (in neutral or ionic form) to enter cells through the symplast pathway, the ways (passive or active transport) for pharmaceuticals to cross phospholipid bilayer membranes, and the types of integral proteins involved in pharmaceutical

movement into cells. More data on sorption of multiple classes of pharmaceuticals by various types plant roots could be beneficial to evaluating the potential of accumulation in plants. Moreover, future experiments on plant uptake of pharmaceuticals at a range of pH in hydroponic nutrient solution could help explain the various extent and pathways for accumulation of ionizable pharmaceuticals in plants. These experiments could be extended to plant uptake of pharmaceuticals from soil pots in greenhouse conditions, and to field scales with soils amended with biosolids, or irrigated with reclaimed water. These results could be compared to examine whether the results from hydroponic studies could help elucidate the uptake of pharmaceuticals from soils in pot experiments and at large field scales.

Transformation of Pharmaceuticals in Plants

Pharmaceuticals accumulated in plants can be further metabolized. However, the information regarding to their extent of transformation in plants and toxicity to plants and humans is very limited due to the relatively complex plant matrices that interfere with instrument analysis. Development of effective and accurate analytical methodology to identify and quantify metabolites is necessary for the comprehensive risk assessment. The studies on pharmaceutical metabolism in whole plants are very labor intensive. Many previous studies used cell cultures or hairy roots to simulate the metabolism of pharmaceuticals in plants;[1, 2] these studies may not provide a direct and convincing evidence to describe the transformation processes of pharmaceuticals during plant growth. Innovative and sound methods are need for fast screening of metabolites derived from multiple classes of pharmaceuticals in plants. This will facilitate the studies on pharmaceutical metabolism in whole plants, and could be used to develop predictive model to describe pharmaceutical fate in plants, and to improve the evaluation of the risks associated with human exposure.

Potential Phytotoxicity from Pharmaceuticals

Several previous studies showed the phytotoxicity of pharmaceuticals to plants e.g., damage to plant appearance or changes in hormone levels.[3] However, this type of information is extremely scarce, and has been variable under different experimental conditions. Changes in plant hormone levels influence intercellular communications of plants and alter the formation and growth of plant organs. The next-step study is to investigate what types of pharmaceuticals, and whether or to what extent pharmaceuticals could alter hormone levels in plants, and what the morphological effects on plant development are. If this relationship is established, the alteration of hormone levels in plants could be used as an indicator for preventing the negative impacts from land application of biosolids and crop irrigation with reclaimed water.

Development of Predictive Models for Plants Growing in Various Seasons and Regions

Increase in transpiration rates could enhance the accumulation and transport of pharmaceuticals in plants, especially for small-sized pharmaceuticals. Plant transpiration rate varies during different seasons and in different regions. Plants have higher transpiration rates in semi-arid or arid regions than those in humid regions, which could lead to different uptake of pharmaceuticals. Considering the major factors influencing pharmaceutical uptake by plants e.g., molecular size, affinity to roots, ionic speciation, the predictive models for plant uptake of pharmaceuticals should incorporate these factors and the environment conditions including the effects of varying weather on transpiration stream.

Plant uptake of pharmaceuticals in a hydroponic system is an ideal setting to evaluate the major uptake mechanism and investigate the metabolism. Ranking the factors that influence pharmaceutical uptake and understanding the extent of metabolism in plants are the key points to effectively mitigate pharmaceutical accumulation in crops. Integration of the results from

hydroponic, soil pots, and field studies could provide more comprehensive datasets for developing the predictive models that could help more accurately assess the risks to food safety and human health, as a result from pharmaceutical accumulation in agricultural crops.

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