JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. 2018, 66, 7907−7915

pubs.acs.org/JAFC

Metabolic Demethylation and Oxidation of Caffeine during Uptake by Lettuce

Ya-Hui Chuang,[†] Cheng-Hua Liu,[†,‡] Raymond Hammerschmidt,[†] Wei Zhang,[†,‡] Stephen A. Boyd,[†] and Hui Li*[,†]

[†]Department of Plant, Soil and Microbial Sciences and [‡]Environmental Science and Policy Program, Michigan State University, East Lansing, Michigan 48824, United States

Supporting Information

ABSTRACT: Pharmaceuticals can be metabolized after being taken up by plants. The metabolites could manifest similar or equivalent bioactivity to the parent compound, promoting the critical need to understand the metabolism in plants. Caffeine has been frequently detected in agriculture produce; however, little attention is given to its metabolites in vegetables. This study examined uptake and metabolism of caffeine in lettuce in a hydroponic system. Caffeine and its metabolites in aqueous solution and lettuce were identified and quantified using a liquid chromatography coupled to a QTrap tandem mass spectrometry instrument. After 144 h, over 50% of applied caffeine dissipated in the hydroponic lettuce system, and eight caffeine metabolites were identified primarily in the shoots. Caffeine underwent demethylation reactions, which were confirmed with authentic standards, and the total amount accounted for 20% of the initially applied caffeine. Other metabolism pathways included oxidation and hydroxylation, and the amount of metabolites increased over uptake time.

KEYWORDS: *pharmaceuticals, caffeine, uptake, vegetable, metabolism, LC-QTrap-MS/MS*

INTRODUCTION

The practices of land application of biosolids and irrigation with reclaimed water in agricultural production are the major routes to disseminate pharmaceuticals in agroecosystems. Pharmaceuticals generally cannot be completely removed from conventional wastewater treatments and are frequently found in biosolids and reclaimed water.[1,2] Pharmaceuticals present in soil and water can enter field crops and vegetables, which serve as the starting point in the food chain to animals and humans. During the past several years major research efforts have been dedicated to the investigation of pharmaceutical uptake by crops and vegetables from soil and water.[1,3−6] Accumulation of pharmaceuticals, e.g., caffeine, carbamazepine, and naproxen, has been detected in the edible parts of celery, lettuce, and cabbage with a concentration up to 0.17 μg kg^{-1} when irrigated with reclaimed water containing caffeine, carbamazepine, and naproxen at concentrations of 11, 4.2, and 0.43 ng L^{-1}, respectively.[6]

Many pharmaceuticals are metabolized in plants, and their intermediate or end products still contain bioactive functional moieties.[5,7] For example, carbamazepine was readily metabolized to 10,11-epoxycarbamazepine, 10,11-dihydroxycarbamazepine, 2-hydroxycarbamazepine, and 3-hydroxycarbamazepine in tomato, cucumber, sweet potato, and carrot.[5,7,8] Among these products, 10,11-epoxycarbamazepine was found to be the major metabolite of carbamazepine in sweet potato and carrot leaves[8] and demonstrated a toxic potency even higher than that of the parent compound carbamazepine.[9] Long-term consumption of carbamazepine-contaminated crops/vegetables could lead to negative impacts on human health. For example, the genotoxic 10,11-epoxycarbamazepine may potentially destruct DNA and cause associated mutations.[8,10] Wu et al. (2013)[11] showed that acetaminophen accumulated only in vegetable roots and was not present in leaves of lettuce, spinach, cucumber, and pepper; this could mitigate the potential risk to humans via dietary consumption. Acetaminophen could be conjugated to glutathione and glycoside when the oxidative stress increased in horseradish hairy root cultures.[12] Triclosan could conjugate with saccharides, disaccharides, malonic acid, and sulfate in carrot.[13] Ibuprofen underwent transformative and conjugative reactions mediated by cytochrome P450 monooxygenase in *Phragmites australis*.[14] Considering that plants function as a "green liver" in the natural system,[15,16] many xenobiotic compounds including pharmaceuticals can 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 conjugation with malonic acid, glucose, glutathione, or cysteine (e.g., diclofenac conjugation with glutathione).[10,17,18] The products formed from phase I and II reactions generally manifest increased hydrophilicity, which facilitates the sequestration of metabolites in vacuole and apoplast.[7,17] Currently, the mechanism and transformation pathways of most pharmaceuticals in plants still remain largely unclear[7,19,20] and need further study.

Caffeine is one of the most commonly administered pharmaceuticals to humans for stimulating the central nervous system and is an ingredient in coffee, tea, cocoa, and many soft drinks.[21−23] Human withdrawal from the long-term consumption of caffeine can cause headaches, fatigue, and anxiety.[24,25] The large amount of consumption of caffeine, as well as

Received: April 28, 2018
Revised: June 23, 2018
Accepted: June 29, 2018
Published: June 29, 2018

DOI: 10.1021/acs.jafc.8b02235
J. Agric. Food Chem. 2018, 66, 7907−7915

improper disposal of unused/expired caffeine-containing medicine/drinks, has resulted in its widespread dissemination in wastewater treatment plants (WWTPs),[26,27] surface water, and groundwater.[28−30] For example, the caffeine concentration reached 3002 ng L^{-1} in effluents from WWTPs[28] and 41.2 ng L^{-1} in surface water at Biscayne Bay, Florida, USA.[30] Irrigation with reclaimed water or contaminated surface/groundwater can lead to an accumulation of caffeine in agricultural products and propagate its dissemination along the food chain. It has been documented that irrigation with reclaimed water could cause the accumulation of caffeine in cucumber and tomato fruits with a concentration >1 ng g^{-1} (dry weight).[5] Pierattini et al. (2016) reported that in *Populus alba*. L. Villafranca, exogenous caffeine underwent demethylation reactions by losing one $-CH_3$ and forming theobromine and theophylline.[31] In coffee and tea plants, endogenous caffeine could be biosynthesized from xanthosine to methylxanthosine, methylxanthine, and theobromine.[32,33] In mammals caffeine usually undergoes demethylation reactions in livers by losing one $-CH_3$ group and forming theobromine, paraxanthine, and theophylline.[21,34] Little is known about the metabolism processes of caffeine in lettuce and other vegetables, even though lettuce is one of the most common ready-to-eat fresh vegetables, e.g., average daily intake of 0.23 g/kg/day.[35] It has been found that caffeine intake could affect fertility rate, sleep quality (especial for children), bladder symptoms, and interaction with other prescribed drugs.[36] Therefore, understanding the accumulation and metabolism of caffeine in fresh vegetables is the key 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 (i.e., triple quadrupole, Orbitrap, linear ion trap, and time-of-flight) has become the major tool employed to identify metabolites derived from pharmaceuticals in environmental and plant matrices.[17,37−40] High-resolution time-of-flight (TOF) mass spectrometry provides accurate mass information for determination of metabolites. Ion trap mass spectrometry can achieve more enriched fragment ions for structure elucidation and quantification of metabolites. Triple quadrupole mass spectrometry can quantify trace amounts of metabolites using the scan mode of multiple reaction monitoring (MRM) as long as the corresponding authentic standards are provided.[37,39]

This study aimed to investigate uptake and distribution of caffeine in the leafy vegetable lettuce and to 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 fragment patterns of metabolites for the elucidation of their chemical structures and to quantify caffeine and the metabolites using MRM mode. The identification of caffeine metabolites was performed by comparing the mass spectra of the extracts from the lettuce exposed to caffeine with the caffeine-free controls. This approach could effectively eliminate the impacts of endogenously formed caffeine in lettuce (if any) and improve the confidence of identified metabolites.[41] Kinetic uptake of caffeine was conducted in a hydroponic system to further elucidate the evolution of formed metabolites. The results provide the information for evaluating uptake, translocation, and metabolism of caffeine in lettuce. The analytical workflow established in this study could be extended to investigate the metabolism of other pharmaceuticals in plants.

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 S1. The reagents and materials used for sample extraction and analysis are provided in the Supporting Information (SI).

Hydroponic Experiment. Black Seeded Simpson lettuce (*Lactuca sativa*) seeds were germinated on moist 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 an electrical conductivity (EC) of 0.4 mS cm^{-1}. The pH and EC values were measured every 24 h to keep the optimized growing conditions by adjusting pH between 5.6 and 5.8 and gradually increasing EC from 0.4 to 0.8 mS cm^{-1} before the lettuce reached the stage of maturity. Lettuce seedlings were exposed to LED light for 16 h per day at an intensity of 150 μmol m^{-2} s^{-1} (Apollo Horticulture Full Spectrum 300 W, Rowland Heights, CA, USA). The nutrient solution was aerated using a fusion pump, and the ambient temperature was kept at 18 °C.

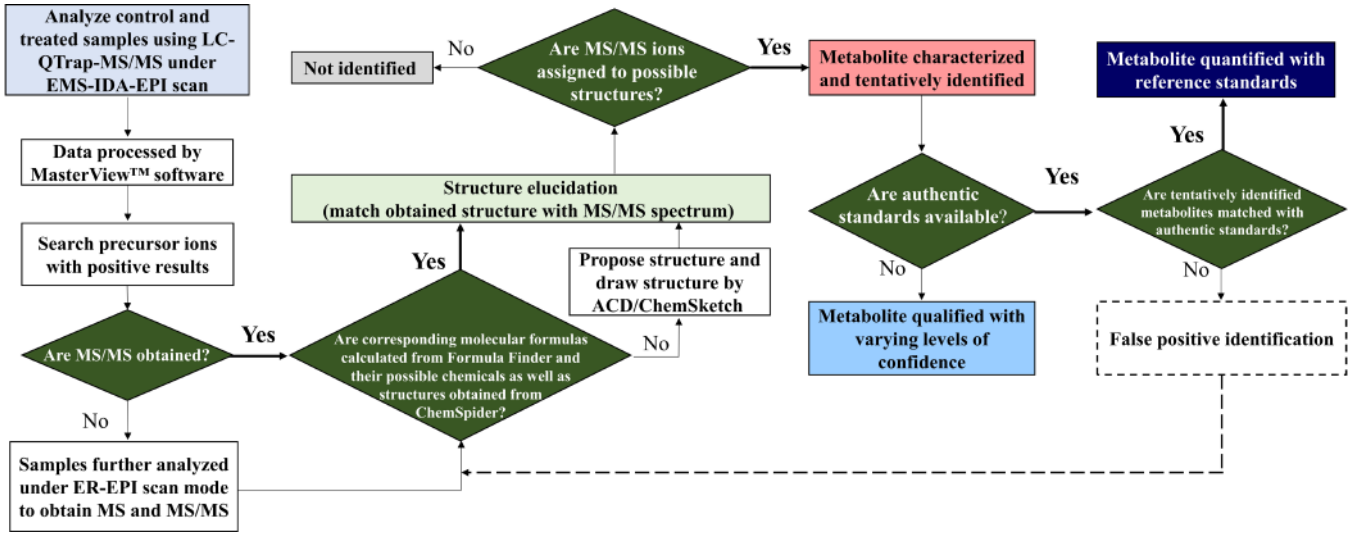
After 22 days of growth, the lettuce was well-developed with matured roots and reached 25−30 cm in height and 8.0−10.5 g of biomass (fresh weight). The lettuce was then transferred into an Erlenmeyer flask containing 210 mL of nutrient solution (pH = 5.8, EC = 0.8 mS cm^{-1}) with a caffeine concentration of 575.0 μg L^{-1}. 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 adjusted to 5.6−5.8 and 0.8 mS cm^{-1}, respectively, daily. To compensate for water loss 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 h of exposure, three flasks with lettuce and the corresponding controls were sacrificed for sampling lettuce and nutrient solution. The lettuce samples were rinsed with deionized water, separated into roots and shoots, freeze-dried, and ground to powders prior to the extraction for 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} disodium ethylenediaminetetraacetate (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 centrifugation 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, and 225 mg of Na_2SO_4). Caffeine in the nutrient solution was extracted using a Waters Oasis hydrophilic−lipophilic-balanced (HLB) cartridge. The HLB cartridge was preconditioned using 3.0 mL of methanol and 5.0 mL of water. Nutrient solution (20.0 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 chromatographic system (Columbia, MD, USA) coupled to a SCIEX 4500 QTrap tandem mass spectrometer (Foster City, CA, USA). The averaged extraction recoveries of caffeine from nutrient solution, lettuce roots, and lettuce shoots were measured at 116.3%, 91.8%, and 106.2%, respectively. The details of caffeine analysis by LC-QTrap-MS/MS are provided in the SI and Table S2.

Non-Target Screening of Caffeine Metabolites in Lettuce. LC-QTrap-MS/MS was used to identify metabolites under both positive and negative ionization modes, along with the combination of an enhanced mass scan (EMS) as the survey scan, information dependent acquisition (IDA) criteria, and an enhanced product ion (EPI) scan to obtain the MS/MS fragment patterns. This approach was

DOI: 10.1021/acs.jafc.8b02235
J. Agric. Food Chem. 2018, 66, 7907−7915

Scheme 1. Flowchart of Identification of Caffeine Metabolites using LC-QTrap-MS/MS



applied to analyze the samples from caffeine-fortified and caffeine-free lettuce after 144 h of uptake. The details of the analysis by LC-QTrap-MS/MS for identifying caffeine metabolites are provided in the SI. The working flowchart for identification of caffeine metabolites is shown in Scheme 1. The data obtained from LC-QTrap-MS/MS were processed using MasterView software operated within the PeakView 2.2 package (SCIEX, Foster City, CA, USA). In the MasterView, the default threshold of the ratio of precursor ion in the caffeine-fortified lettuce extract to that in the caffeine-free control was set as 3; that is, the intensity of precursor ion >3 times that in the control was considered as the positive result. The mass spectra of the precursors and their corresponding EPI-triggered MS/MS spectra were then linked to the Formula Finder in MasterView to obtain the related molecular formulas. If the MS/MS spectra of precursor ions could not be obtained from the EMS-IDA-EPI scan, a supplemental enhanced resolution combined with enhanced product ion (ER-EPI) scan was then targeted to the precursor ions of interest to obtain the MS/MS spectra, from which the corresponding molecular formulas were calculated. The calculated formulas were used to search possible chemicals and obtain their structures in the ChemSpider database. The structures were examined for similar moieties to the parent compound, e.g., xanthine structure in the metabolites. If the appropriate chemical structure could not be obtain from the ChemSpider database, ChemSketch (ACD/Labs, Toronto, Ontario, Canada) was used to construct the possible structures which were then imported to MasterView to elucidate the fragment patterns of the MS and MS/MS spectra. When the fragments of mass spectra were assigned to possible structures, the metabolites were considered to be tentatively identified, exemplified with the identification of caffeine in Figure S1. The tentatively identified metabolites were further confirmed using authentic standards if available. The LC-QTrap-MS/MS used in this study is classified as a low-resolution mass spectrometry. False positive result could occur to those tentatively identified metabolites when the mass spectra or LC retention time (RT) did not match with the authentic standards. In general, these tentatively identified metabolites, without confirmation by authentic standards, could be ranked at different confidence levels.[42]

For the tentatively identified metabolites with the authentic standards, they were further confirmed using the optimized MRM mode with quantification/qualification transitions and RT. All of the samples (aqueous solution, lettuce roots, and lettuce shoots) collected were analyzed under the MRM mode for the metabolites using the matrix-matched standard curves. For the tentatively identified metabolites without the authentic standards, their primary fragments with the highest abundance were selected to establish MRM transitions as semi-quantitative approach, and IDA and EPI were further used to confirm the metabolites. The peak areas of the metabolites were integrated at the specific RT acquired from the positive results and normalized to the corresponding area of the caffeine-free controls.

RESULTS AND DISCUSSION

Uptake of Caffeine by Lettuce. Our preliminary study indicated that more than 60% of caffeine taken up by lettuce was metabolized in lettuce in the hydroponic experiment with the initial caffeine concentration of 50 μg L^{-1}. To better elucidate the metabolism of caffeine in lettuce, a higher caffeine concentration (575 μg L^{-1}) was dissolved in the aqueous solution, which facilitates the detection, identification, and quantification of the formed metabolites. The application of the higher concentration of caffeine did not render the apparent adverse effects on lettuce growth (Figure S2). The mass distribution of caffeine in aqueous solution, lettuce roots, and lettuce shoots are plotted against the uptake periods (Figure 1). It is apparent

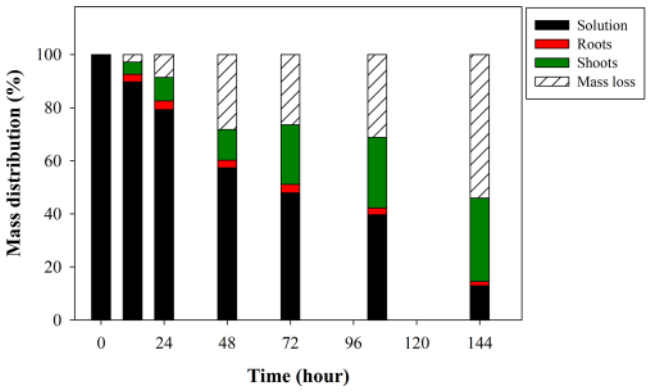


Figure 1. Mass distribution of caffeine in aqueous solution, lettuce roots, and lettuce shoots as a function of uptake time.

that the amount of caffeine in the solution gradually decreased with time and reduced to 12.9 ± 7.7% of the initially applied dosage after 144 h of exposure. At the same time, the mass fractions of caffeine in lettuce shoots increased from 4.6 ± 0.3% (10 h) to 31.3 ± 6.1% (144 h), and the fractions in lettuce roots remained relatively low within the range of 1.6–3.3% during the experimental period. This could be attributed to the relatively small size of the caffeine molecule and its minimal affinity to lettuce roots. Both factors facilitate the

DOI: 10.1021/acs.jafc.8b02235
J. Agric. Food Chem. 2018, 66, 7907−7915

entrance of caffeine to lettuce roots and translocation to shoots with the transpiration stream, resulting in a limited amount of caffeine retained by roots.

The total amount of recovered caffeine decreased with uptake time (Figure 1) and were 97.2 ± 3.6%, 91.4 ± 4.9%, 71.8 ± 8.3%, 73.6 ± 7.3%, 68.8 ± 17.1%, and 45.9 ± 14.3% of the initial amount after 10, 24, 48, 72, 105, and 144 h of uptake, respectively. Photodegradation of caffeine and other losses were negligible based on the observation that in the lettuce-free controls the caffeine concentration remained within the range of 97−105% of the initial dosage during 144 h of uptake. The extraction efficiencies of caffeine from aqueous solution, lettuce roots, and lettuce shoots were all >90%. After the 144 h of exposure, caffeine not present in the aqueous solution was assumed to enter the lettuce, which was equivalent to ∼87.1% of the initial dosage, and the metabolism occurred only in lettuce. It was estimated that approximately 62.1% of caffeine absorbed in lettuce was metabolized most likely in shoots. The extracts of lettuce shoots collected at 144 h of exposure were thus used to identify the potential metabolites.

Caffeine Metabolism in Lettuce. The caffeine metabolites in lettuce were identified using the optimized EMS-IDA-EPI scan in LC-QTrap-MS/MS. The EMS-IDA-EPI scan is commonly used to identify metabolites in non-target screening.[43] 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 S3). Among these eight metabolites, the commercially available authentic standards xanthine, 3-methylxanthine, 7-methylxanthine, theobromine, paraxanthine, theophylline, and 1,3-dimethyluric acid were used to confirm the metabolites in lettuce shoots based on the European Union Guideline 2002/657/EC.[44] To do so, two pairs of precursor and product transitions obtained from the authentic standards were selected to confirm the metabolite. This operation achieves the identification points (IP) assigned as 4, which meets the minimum requirement of IP value ≥ 3 for the confirmation of veterinary drugs and organic contaminants. The comparison of response ratio of the two transitions, MS/MS spectra, and RT between the extracts of lettuce samples and authentic standards revealed that the six metabolites (excluding 1,3-dimethyluric acid) were unambiguously identified by their fingerprints and were confirmed to be formed in lettuce shoots.

A false positive result occurred when matching the RT with the authentic standard 1,3-dimethyluric acid (precursor ion m/z 197.07, $C_7H_8N_4O_3$). The RT of 1,3-dimethyluric acid (the two major transitions m/z 197.0 → 140.0 and m/z 197.0 → 179.0) was at 4.3 min. The unknown metabolite in lettuce shoot extracts had the same two transitions, but the RT was 5.8 min. Instead, 7-hydroxy-1,3-dimethyl-3,7-dihydro-1H-purine-2,6-dione (M196) with the same molecular formula and precursor ion obtained from the ChemSpider database matched well with the MS/MS spectrum assigned to M196 (Figure 2A). M196 metabolite was formed by substituting the $-CH_3$ with an −OH functional group at the 7-N position of caffeine. 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 2A). M210 metabolite was formed by an oxidative reaction occurring at the C-8 position 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 metabolite was formed by hydroxylation at C-8, and the major fragments of m/z 195, 185, and 167 were formed by the loss of one $-H_2O$, two $-CH_2$, and one −OH and two methyl groups ($-CH_2$ and $-CH_3$). These three metabolites all contained the core structure of two conjugated rings of caffeine (Figure 2), which further confirms that these metabolites are derivatives from caffeine. The identification confidence of the metabolites M196, M210, and M212 (without the authentic standards) could be annotated as the class of level 3 of putatively characterized compounds according to the minimum reporting standards documented by Sumner et al. (2007).[42] In the document, four identification confidence levels are proposed to classify the identified metabolites. Level 1 of identified compounds refers to the chemicals matched with authentic standards. Level 2 of putatively annotated compounds refers to the chemicals which are identified through physicochemical properties and/or match with a published spectral database. Level 3 of putatively characterized compounds refers to the chemicals identified with spectral similarity to the chemical class. Level 4 refers to unknown compounds which could be differentiated and quantified using the spectral data. In this study, these three caffeine metabolites were identified via matching MS and MS/MS spectra to the known structures in the ChemSpider database.

The analysis and identification of caffeine metabolites indicate that caffeine in lettuce was metabolized primarily via demethylation and oxidation/hydroxylation reactions (Figure 3). These reactions occurred mainly in lettuce shoots. After 144 h of exposure, the demethylation metabolites in aqueous solution, lettuce roots, and lettuce shoots accounted for 0.4 ± 0.1%, 1.3 ± 0.5%, and 17.1 ± 10.8%, respectively, of the initially applied caffeine. The three oxidation/hydroxylation metabolites (shown in Figure 2) were found only in lettuce shoots. Stepwise demethylation is the major metabolism pathway (Figure 3). Caffeine lost one $-CH_3$ functional group, forming theobromine (1−N demethylation), paraxanthine (3−N demethylation), and theophylline (7−N demethylation), and then lost the second $-CH_3$ functional group, forming 3-methylxanthine and 7-methylxanthine. These two compounds could lose the third $-CH_3$ to produce xanthine. The demethylation reaction of caffeine with the loss of one $-CH_3$ functional group (N-demethylation) commonly occurs in humans and animals where this reaction is mediated primarily by hepatic cytochrome P450s (CYP) 1A2 and 2E1.[45−47] In coffee and tea plants, caffeine is derived from purine nucleotides mediated by xanthosine methyltrasferase and theobromine synthase followed by caffeine synthase.[48,49] Meanwhile, caffeine in plants could be also transformed to theobromine and theophylline, then to 3-methylxanthine and xanthine, and eventually to CO_2 and NH_3.[32,50,51]

In addition to demethylation, caffeine and its metabolites could undergo oxidation/hydroxylation reactions forming M196 metabolite via demethylation and hydroxylation at the N-7 position, M210 via oxidation at the C-8 position, and M212 via hydroxylation at the C-8 position (Figure 3). The oxidation and hydroxylation of caffeine also occur in mammals, which are mediated by P450 enzymes.[52−54] In humans, the oxidation of caffeine is mediated by CYP1A2, and hydroxylation is mediated by CYP3A4 in marmosets.[52] Caffeine that underwent oxidation at the C-8 position was also found in *Camellia assamica* var. kucha and *Coffea liberica*.[32,55] In this study the identified metabolites were derived primarily from

DOI: 10.1021/acs.jafc.8b02235
J. Agric. Food Chem. 2018, 66, 7907−7915

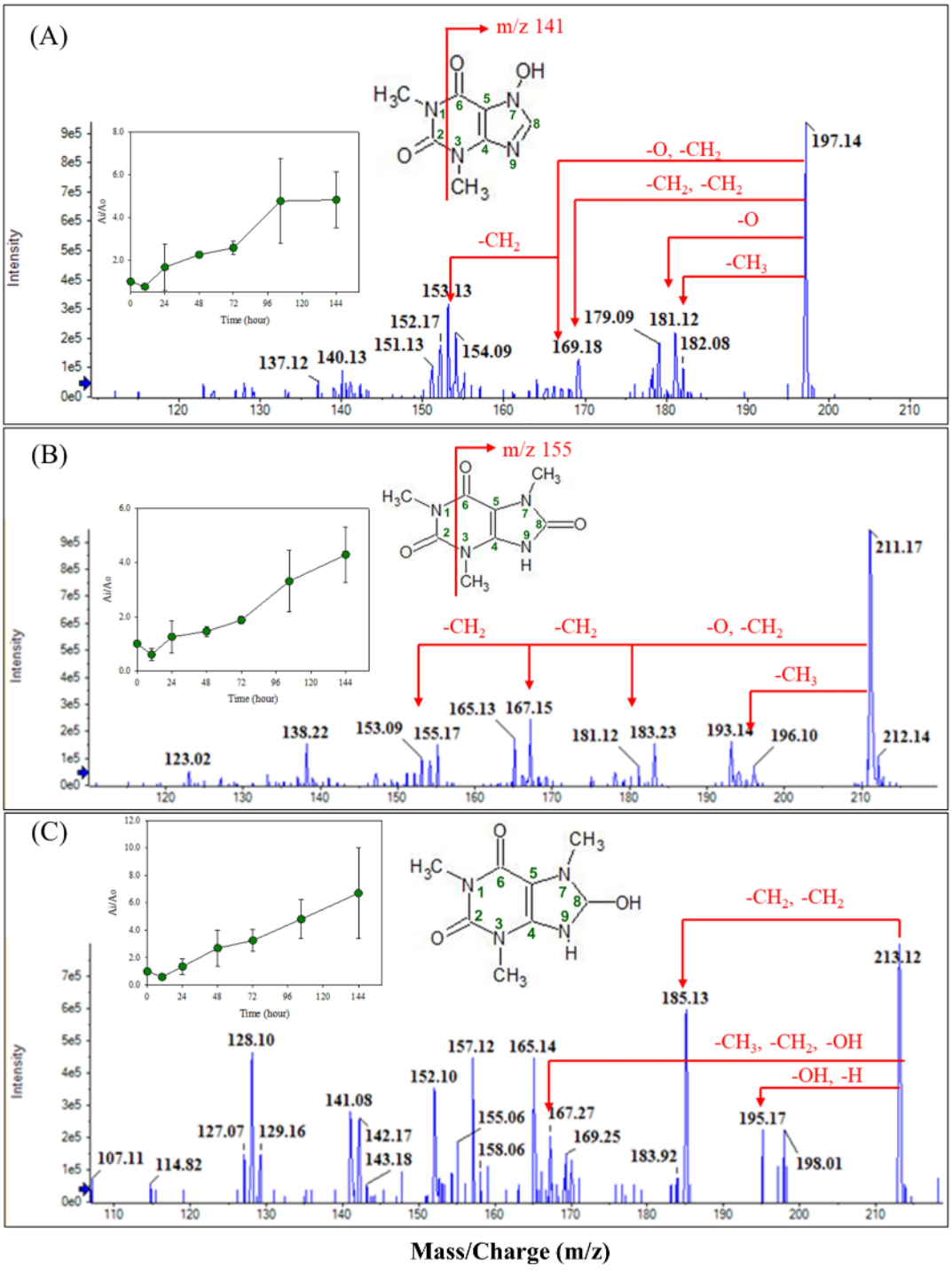


Figure 2. MS/MS spectra of caffeine metabolites (without authentic standards): (A) 7-Hydroxy-1,3-dimethyl-3,7-dihydro-1H-purine-2,6-dione (M196), (B) 1,3,7-trimethyluric acid (M210), and (C) 8-hydroxy-1,3,7-trimethyl-3,7,8,9-tetrahydro-1H-purine-2,6-dione (M212), and their integrated areas relative to caffeine-free controls (A_i/A_0) as a function of uptake time (inserted panels).

the phase I reactions. To check if the phase II conjugative products were formed, the m/z values of 357 for the caffeine-glycoside conjugate (195 [caffeine]$^+$ + 162) and m/z 500 for the caffeine-glutathione conjugate (195 [caffeine]$^+$ + 305) were scanned in the lettuce extracts. These caffeine conjugates were not found to be present in the lettuce.

Kinetics of Caffeine Metabolism in Lettuce. To elucidate the kinetic metabolism in lettuce, caffeine and its identified metabolites present in aqueous solution, lettuce roots, and lettuce shoots were quantified 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, and the optimized instrumental parameters are provided in Table S4. Among the metabolites, 3- and 7-methylxanthine manifested the same RT at 4.48 min and very similar fragment patterns. The same phenomena were also observed for paraxanthine and theophylline (products with the loss of one $-CH_3$ from caffeine) (Table S4). It is difficult to quantify these metabolites

DOI: 10.1021/acs.jafc.8b02235
J. Agric. Food Chem. 2018, 66, 7907−7915

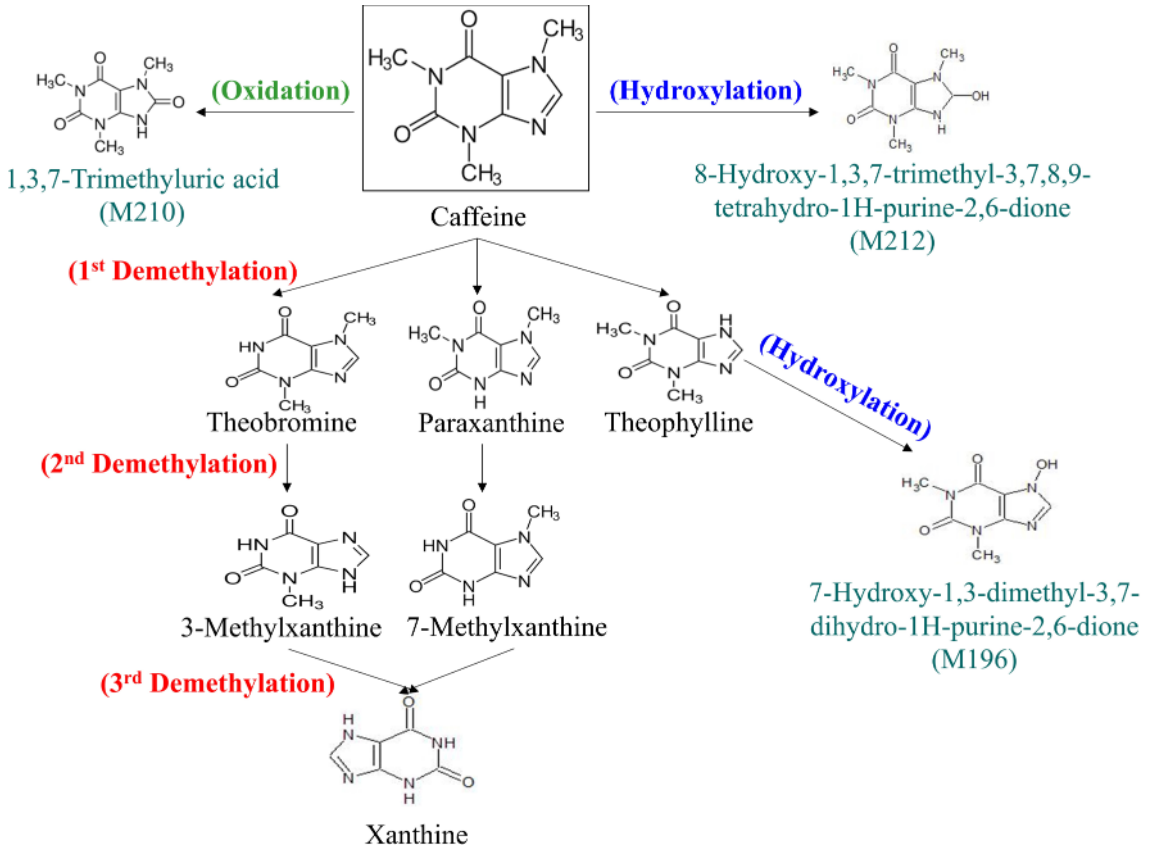


Figure 3. Proposed metabolism pathways of caffeine in lettuce.

individually; therefore, we quantified these metabolites as a group of methylxanthine (the sum of 3- and 7- methylxanthine) and the sum of paraxanthine and theophylline. For the metabolites M196, M210, and M212 without authentic standards, the transitions of the precursors and the most abundant product ions were paired, and the corresponding areas were integrated relative to the background of the caffeine-free controls, which served as a semi-quantification for these metabolites (insert panels in Figure 2).

The mass distribution of caffeine and its demethylation metabolites on the basis of molar fractions in the aqueous solution, lettuce roots, and lettuce shoots is presented in Figure 4A. The molar fractions of caffeine in the aqueous solution decreased with time, and the minimal amount of demethylation metabolites was present ($<4 \times 10^{-3}$). The molar fractions of caffeine and its demethylation metabolites in lettuce roots remained within a narrow range between 0.02 and 0.04. In lettuce shoots caffeine and its demethylation metabolites increased with exposure time and reached 0.47 ± 0.10 at 144 h. These results revealed that demethylation reactions occurred predominantly in lettuce shoots. In lettuce roots the accumulation of demethylation metabolites was approximately 7.6% of those found in shoots. The total molar fractions of caffeine and its demethylation metabolites (including xanthine, 3- and 7-methylxanthine, theobromine, and paraxanthine/theophylline) in the aqueous solution, lettuce roots, and lettuce shoots varied as a function of uptake time, which accounted for 98.2 ± 3.8%, 95.1 ± 2.7%, 76.2 ± 7.7%, 79.2 ± 7.3%, 80.8 ± 18.9%, and 63.0 ± 15.1% of the initially applied caffeine at 10, 24, 48, 72, 105, and 144 h of uptake, respectively (Figure 4A). At 144 h, caffeine and its demethylation metabolites were 12.9 ± 7.7% and 0.4 ± 0.1% in the solution, 1.6 ± 0.6% and 1.3 ± 0.5% in lettuce roots, and 31.3 ± 6.1% and 17.1 ± 10.8% in lettuce shoots. The remaining 37.0% of the initially applied caffeine could be considered as unquantified, unextractable, and unidentified fractions. In addition, the measured oxidative and hydroxylated metabolites M196, M210, and M212 increased approximately 4 to 6 times during the 144 h of exposure (inserted panels in Figure 2), which is consistent with the increase in the unquantified, unextractable, and unidentified fractions as the uptake time proceeded (Figure 4A).

In lettuce shoots, xanthine, 3- and 7-methylxanthine, theobromine, and paraxanthine/theophylline were all detected, and xanthine and those metabolites with the loss of one $-CH_3$ were the predominant products (Figure 4B). Xanthine was found in all shoot samples; methylxanthine was detected in lettuce after 24 h of exposure. These results indicate that the demethylation reaction is a relatively rapid process. The total molar fractions of demethylation products increased from 0.01 to 0.17 during 10 to 144 h of uptake. At 144 h, the sum of molar fractions of the caffeine metabolites with the loss of one $-CH_3$ was 0.07, and the xanthine fraction (with the loss of three $-CH_3$ groups) was 0.09. The molar fractions of methylxanthine, i.e., loss of two $-CH_3$ groups, were <0.007, which was 10 times less than those of the other two groups of metabolites. These results indicated that the transformation rate from 3- and 7-methylxanthine to xanthine was very rapid and/or that they could undergo other reactions, e.g., oxidation or hydroxylation (Figure 3). In lettuce roots, only a very small amount of xanthine and theobromine was present, and methylxanthine, paraxanthine, or theophylline was all below the limit of detection. The total molar fractions of xanthine and

DOI: 10.1021/acs.jafc.8b02235
J. Agric. Food Chem. 2018, 66, 7907−7915

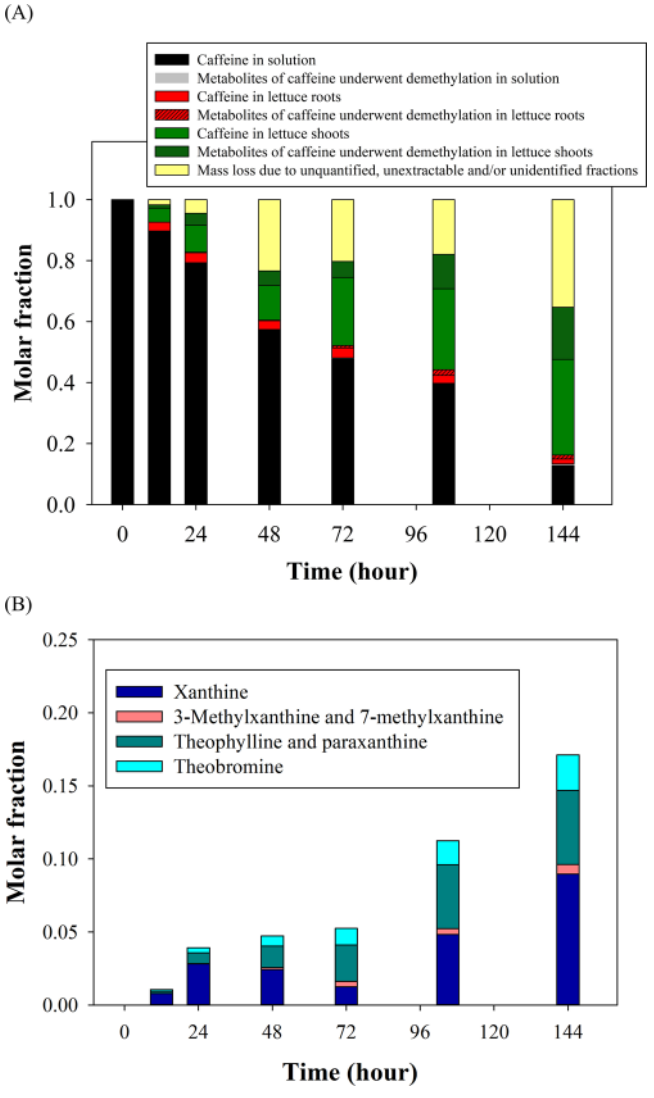


Figure 4. (A) Distribution of caffeine and its metabolites in the aqueous solution, lettuce roots, and lettuce shoots on a molar basis as a function of uptake time. (B) Distribution of caffeine demethylation metabolites in lettuce shoots as a function of uptake time.

theobromine in lettuce roots accounted for <1.3% of the initially applied caffeine. In the solution phase a negligible amount of xanthine and paraxanthine/theophylline was found (<0.5% of the initially applied caffeine), and 3- and 7-methylxanthine or theobromine was not detected. Thus, lettuce shoots were considered as the major domain for accumulation and metabolism of caffeine. Pierattini et al.[31] reported a concentration of exogenous theobromine and theophylline in *Populus alba* leaves that was relatively higher than those in stems or roots. The minimal amount of metabolites in lettuce roots could be attributed to the fact that caffeine is readily translocated to shoots where it was extensively metabolized.

Implications. Caffeine and many other pharmaceuticals, such as carbamazepine, salbutamol, and trimethoprim are frequently detected in agriculture produce,[3,6,56] which poses potential risks to animal and human health by dietary consumption. These pharmaceuticals could be metabolized in vegetables, and the formed metabolites might still retain the bioactive moieties and maintain functionality in a similar manner to the parent compounds. For example, theobromine and theophylline are bioactive stimulants similar to caffeine and are also commonly used in the therapy of acute and chronic asthma.[21,22] Caffeine metabolism could be attenuated or accelerated in field grown lettuce, compared to the hydroponically grown lettuce described in this study. The risk assessment of pharmaceuticals without considering their metabolites could underestimate the potency to ecosystem and human health. This study provides a sound workflow for non-target screening of metabolites using LC-QTrap-MS/MS operated under EMS-IDA-EPI scan mode. Although LC-QTrap-MS/MS is not a high-resolution mass spectrometry, the linear ion trap can enrich the abundancy of ionic fragments, which could improve the quantification sensitivity for trace levels of metabolites in plants. The enhanced sensitivity also facilitates the elucidation of chemical structures of metabolites. The analytical workflow established in this study could be extended to investigate the metabolism of other pharmaceuticals in the environment and in plants and animals as well.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b02235.

Additional description of materials and methods, supplementary tables, and supplementary figures (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: lihui@msu.edu. Tel: 517-353-0151. Fax: 517-355-0270.

ORCID

Cheng-Hua Liu: 0000-0001-5864-8107

Wei Zhang: 0000-0002-2937-1732

Hui Li: 0000-0003-3298-5265

Funding

This study was supported in part by the Agriculture and Food Research Initiative competitive grants program (2016-67017-24514) from the USDA National Institute of Food and Agriculture and Michigan AgBioResearch GREEEN Projects.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

EMS, enhanced mass scan; EPI, enhanced product ion; IDA, information dependent acquisition; IP, identification point; LC-QTrap-MS/MS, liquid chromatography coupled to QTrap tandem mass spectrometry; MRM, multiple reaction monitoring; RT, retention time.

■ REFERENCES

(1) Miller, E. L.; Nason, S. L.; Karthikeyan, K. G.; Pedersen, J. A. Root uptake of pharmaceuticals and personal care product ingredients. *Environ. Sci. Technol.* **2016**, *50*, 525−541.

(2) Wu, X. Q.; Dodgen, L. K.; Conkle, J. L.; Gan, J. Plant uptake of pharmaceutical and personal care products from recycled water and biosolids: a review. *Sci. Total Environ.* **2015**, *536*, 655−666.

(3) Wu, C.; Spongberg, A. L.; Witter, J. D.; Fang, M.; Czajkowski, K. P. Uptake of pharmaceutical and personal care products by soybean plants from soils applied with biosolids and irrigated with contaminated water. *Environ. Sci. Technol.* **2010**, *44*, 6157−6161.

(4) Carter, L. J.; Harris, E.; Williams, M.; Ryan, J. J.; Kookana, R. S.; Boxall, A. B. Fate and uptake of pharmaceuticals in soil-plant systems. *J. Agric. Food Chem.* **2014**, *62*, 816−825.

DOI: 10.1021/acs.jafc.8b02235
J. Agric. Food Chem. 2018, 66, 7907−7915

(5) Goldstein, M.; Shenker, M.; Chefetz, B. Insights into the uptake processes of wastewater-borne pharmaceuticals by vegetables. *Environ. Sci. Technol.* **2014**, *48*, 5593−5600.
(6) Wu, X.; Conkle, J. L.; Ernst, F.; Gan, J. Treated wastewater irrigation: uptake of pharmaceutical and personal care products by common vegetables under field conditions. *Environ. Sci. Technol.* **2014**, *48*, 11286−11293.
(7) Riemenschneider, C.; Seiwert, B.; Moeder, M.; Schwarz, D.; Reemtsma, T. Extensive Transformation of the Pharmaceutical Carbamazepine Following Uptake into Intact Tomato Plants. *Environ. Sci. Technol.* **2017**, *51*, 6100−6109.
(8) Malchi, T.; Maor, Y.; Tadmor, G.; Shenker, M.; Chefetz, B. Irrigation of root vegetables with treated wastewater: evaluating uptake of pharmaceuticals and the associated human health risks. *Environ. Sci. Technol.* **2014**, *48*, 9325−9333.
(9) Tomson, T.; Almkvist, O.; Nilsson, B. Y.; Svensson, J. O.; Bertilsson, L. Carbamazepine-10,11-epoxide in epilepsy. A pilot study. *Arch. Neurol.* **1990**, *47*, 888−892.
(10) Riemenschneider, C.; Al-Raggad, M.; Moeder, M.; Seiwert, B.; Salameh, E.; Reemtsma, T. Pharmaceuticals, their metabolites, and other polar pollutants in field-grown vegetables irrigated with treated municipal wastewater. *J. Agric. Food Chem.* **2016**, *64*, 5784−5792.
(11) Wu, X. Q.; Ernst, F.; Conkle, J. L.; Gan, J. Comparative uptake and translocation of pharmaceutical and personal care products (PPCPs) by common vegetables. *Environ. Int.* **2013**, *60*, 15−22.
(12) Bartha, B.; Huber, C.; Harpaintner, R.; Schroder, P. Effects of acetaminophen in Brassica juncea L. Czern.: investigation of uptake, translocation, detoxification, and the induced defense pathways. *Environ. Sci. Pollut. Res.* **2010**, *17*, 1553−1562.
(13) Macherius, A.; Eggen, T.; Lorenz, W.; Moeder, M.; Ondruschka, J.; Reemtsma, T. Metabolization of the bacteriostatic agent triclosan in edible plants and its consequences for plant uptake assessment. *Environ. Sci. Technol.* **2012**, *46*, 10797−10804.
(14) He, Y. J.; Langenhoff, A. A. M.; Sutton, N. B.; Rijnaarts, H. H. M.; Blokland, M. H.; Chen, F. R.; Huber, C.; Schroder, P. Metabolism of ibuprofen by Phragmites australis: Uptake and phytodegradation. *Environ. Sci. Technol.* **2017**, *51*, 4576−4584.
(15) Sandermann, H., Jr. Higher plant metabolism of xenobiotics: the 'green liver' concept. *Pharmacogenetics* **1994**, *4*, 225−241.
(16) Sandermann, H.; Altman, A.; Ziv, M.; Izhar, S. Plant metabolism of organic xenobiotics. Status and prospects of the 'green liver' concept. *Curr. Plant Sci. Biotechnol. Agric.* **1999**, *36*, 321−328.
(17) Bartha, B.; Huber, C.; Schroder, P. Uptake and metabolism of diclofenac in Typha latifolia - How plants cope with human pharmaceutical pollution. *Plant Sci.* **2014**, *227*, 12−20.
(18) Huber, C.; Bartha, B.; Harpaintner, R.; Schroder, P. Metabolism of acetaminophen (paracetamol) in plants-two independent pathways result in the formation of a glutathione and a glucose conjugate. *Environ. Sci. Pollut. Res.* **2009**, *16*, 206−213.
(19) Marsik, P.; Sisa, M.; Lacina, O.; Motkova, K.; Langhansova, L.; Rezek, J.; Vanek, T. Metabolism of ibuprofen in higher plants: A model Arabidopsis thaliana cell suspension culture system. *Environ. Pollut.* **2017**, *220*, 383−392.
(20) Fu, Q. G.; Zhang, J. B.; Borchardt, D.; Schlenk, D.; Gan, J. Direct conjugation of emerging contaminants in Arabidopsis: Indication for an overlooked risk in plants? *Environ. Sci. Technol.* **2017**, *51*, 6071−6081.
(21) Choi, E. J.; Bae, S. H.; Park, J. B.; Kwon, M. J.; Jang, S. M.; Zheng, Y. F.; Lee, Y. S.; Lee, S. J.; Bae, S. K. Simultaneous quantification of caffeine and its three primary metabolites in rat plasma by liquid chromatography-tandem mass spectrometry. *Food Chem.* **2013**, *141*, 2735−2742.
(22) Bispo, M. S.; Veloso, M. C. C.; Pinheiro, H. L. C.; De Oliveira, R. F. S.; Reis, J. O. N.; De Andrade, J. B. Simultaneous determination of caffeine, theobromine, and theophylline by high-performance liquid chromatography. *J. Chromatogr. Sci.* **2002**, *40*, 45−48.
(23) Dalmazio, I.; Santos, L. S.; Lopes, R. P.; Eberlin, M. N.; Augusti, R. Advanced oxidation of caffeine in water: On-line and real-time monitoring by electrospray ionization mass spectrometry. *Environ. Sci. Technol.* **2005**, *39*, 5982−5988.
(24) Silverman, K.; Evans, S. M.; Strain, E. C.; Griffiths, R. R. Withdrawal syndrome after the double-blind cessation of caffeine consumption. *N. Engl. J. Med.* **1992**, *327*, 1109−1114.
(25) White, B.; Lincoln, C.; Pearce, N.; Reeb, R.; Vaida, C. Anxiety and muscle tension as consequences of caffeine withdrawal. *Science* **1980**, *209* (4464), 1547−1548.
(26) Tong, A. Y. C.; Peake, B. M.; Braund, R. Disposal practices for unused medications around the world. *Environ. Int.* **2011**, *37*, 292−298.
(27) Thach, A. V.; Brown, C. M.; Pope, N. Consumer perceptions about a community pharmacy-based medication take back program. *J. Environ. Manage.* **2013**, *127*, 23−27.
(28) Loos, R.; Carvalho, R.; Antonio, D. C.; Comero, S.; Locoro, G.; Tavazzi, S.; Paracchini, B.; Ghiani, M.; Lettieri, T.; Blaha, L.; Jarosova, B.; Voorspoels, S.; Servaes, K.; Haglund, P.; Fick, J.; Lindberg, R. H.; Schwesig, D.; Gawlik, B. M. EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents. *Water Res.* **2013**, *47*, 6475−6487.
(29) Fram, M. S.; Belitz, K. Occurrence and concentrations of pharmaceutical compounds in groundwater used for public drinking-water supply in California. *Sci. Total Environ.* **2011**, *409*, 3409−3417.
(30) Gardinali, P. R.; Zhao, X. Trace determination of caffeine in surface water samples by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS). *Environ. Int.* **2002**, *28*, 521−528.
(31) Pierattini, E. C.; Francini, A.; Raffaelli, A.; Sebastiani, L. Degradation of exogenous caffeine by Populus alba and its effects on endogenous caffeine metabolism. *Environ. Sci. Pollut. Res.* **2016**, *23*, 7298−7307.
(32) Ashihara, H.; Sano, H.; Crozier, A. Caffeine and related purine alkaloids: Biosynthesis, catabolism, function and genetic engineering. *Phytochemistry* **2008**, *69*, 841−856.
(33) Yoneyama, N.; Morimoto, H.; Ye, C. X.; Ashihara, H.; Mizuno, K.; Kato, M. Substrate specificity of N-methyltransferase involved in purine alkaloids synthesis is dependent upon one amino acid residue of the enzyme. *Mol. Genet. Genomics* **2006**, *275*, 125−135.
(34) McLean, C.; Graham, T. E. Effects of exercise and thermal stress on caffeine pharmacokinetics in men and eumenorrheic women. *J. Appl. Physiol.* **2002**, *93*, 1471−1478.
(35) U.S. EPA. *Exposure Factors Handbook 2011 Edition (Final Report)*; U.S. Environmental Protection Agency, Washington, DC, EPA/600/R-09/052F, 2011; https://cfpub.epa.gov/ncea/risk/recordisplay.cfm?deid=236252 (accessed June 01, 2017).
(36) Temple, J. L.; Bernard, C.; Lipshultz, S. E.; Czachor, J. D.; Westphal, J. A.; Mestre, M. A. The Safety of ingested Caffeine: A Comprehensive Review. *Front. Psychiatry* **2017**, *8*, 1−19.
(37) Rousu, T.; Herttuainen, J.; Tolonen, A. Comparison of triple quadrupole, hybrid linear ion trap triple quadrupole, time-of-flight and LTQ-Orbitrap mass spectrometers in drug discovery phase metabolite screening and identification in vitro - amitriptyline and verapamil as model compounds. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 939−957.
(38) Macherius, A.; Seiwert, B.; Schroder, P.; Huber, C.; Lorenz, W.; Reemtsma, T. Identification of plant metabolites of environmental contaminants by UPLC-QToF-MS: the in vitro metabolism of triclosan in horseradish. *J. Agric. Food Chem.* **2014**, *62*, 1001−1009.
(39) Li, Y. Y.; Wang, H. X.; Zhao, C. Y.; Huang, Y. Q.; Tang, X. W.; Cheung, H. Y. Identification and characterization of kukoamine metabolites by multiple ion monitoring triggered enhanced product ion scan method with a triple-quadruple linear ion trap mass spectrometer. *J. Agric. Food Chem.* **2015**, *63*, 10785−10790.
(40) Wu, J. L.; Liu, J.; Cai, Z. W. Determination of triclosan metabolites by using in-source fragmentation from high-performance liquid chromatography/negative atmospheric pressure chemical ionization ion trap mass spectrometry. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 1828−1834.

DOI: 10.1021/acs.jafc.8b02235
J. Agric. Food Chem. 2018, 66, 7907−7915

(41) Schymanski, E. L.; Singer, H. P.; Slobodnik, J.; Ipolyi, I. M.; Oswald, P.; Krauss, M.; Schulze, T.; Haglund, P.; Letzel, T.; Grosse, S.; Thomaidis, N. S.; Bletsou, A.; Zwiener, C.; Ibanez, M.; Portoles, T.; de Boer, R.; Reid, M. J.; Onghena, M.; Kunkel, U.; Schulz, W.; Guillon, A.; Noyon, N.; Leroy, G.; Bados, P.; Bogialli, S.; Stipanicev, D.; Rostkowski, P.; Hollender, J. Non-target screening with high-resolution mass spectrometry: critical review using a collaborative trial on water analysis. *Anal. Bioanal. Chem.* **2015**, *407*, 6237−6255.
(42) Sumner, L. W.; Amberg, A.; Barrett, D.; Beale, M. H.; Beger, R.; Daykin, C. A.; Fan, T. W. M.; Fiehn, O.; Goodacre, R.; Griffin, J. L.; Hankemeier, T.; Hardy, N.; Harnly, J.; Higashi, R.; Kopka, J.; Lane, A. N.; Lindon, J. C.; Marriott, P.; Nicholls, A. W.; Reily, M. D.; Thaden, J. J.; Viant, M. R. Proposed minimum reporting standards for chemical analysis. *Metabolomics* **2007**, *3*, 211−221.
(43) Li, J.; Chen, Y.; Diao, Y. Y.; Su, Y. Q.; Wang, Q. L.; Yao, Z. C.; Yi, T. X.; Jin, W. T.; Zhao, D.; Wang, C. H.; Liu, M. R.; Liu, H. M. Identification of metabolites of the novel anti-tumor drug candidate MDH-7 in rat urine by liquid chromatography coupled with triple quadrupole linear ion trap mass spectrometry. *Rapid Commun. Mass Spectrom.* **2016**, *30*, 1001−1010.
(44) European Comission. Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Document No. 2002/657/EC*, Legis. 221/8, 17.8.2002; Off. J. Eur. Communities: 2002.
(45) Summers, R. M.; Louie, T. M.; Yu, C. L.; Gakhar, L.; Louie, K. C.; Subramanian, M. Novel, highly specific N-demethylases enable bacteria to live on caffeine and related purine alkaloids. *J. Bacteriol.* **2012**, *194*, 2041−2049.
(46) Daniel, W. A.; Syrek, M.; Rylko, Z.; Kot, M. Effects of phenothiazine neuroleptics on the rate of caffeine demethylation and hydroxylation in the rat liver. *Polish Journal of Pharmacology* **2001**, *53*, 615−621.
(47) Gu, L.; Gonzalez, F. J.; Kalow, W.; Tang, B. K. Biotransformation of caffeine, paraxanthine, theobromine and theophylline by cDNA-expressed human CYP1A2 and CYP2E1. *Pharmacogenetics* **1992**, *2*, 73−77.
(48) Kim, J.; Buell, C. R. A revolution in plant metabolism: Genome-enabled pathway discovery. *Plant Physiol.* **2015**, *169*, 1532−1539.
(49) Koshiishi, C.; Kato, A.; Yama, S.; Crozier, A.; Ashihara, H. A new caffeine biosynthetic pathway in tea leaves: utilisation of adenosine released from the S-adenosyl-L-methionine cycle. *FEBS Lett.* **2001**, *499*, 50−54.
(50) Mazzafera, P. Catabolism of caffeine in plants and microorganisms. *Front. Biosci., Landmark Ed.* **2004**, *9*, 1348−1359.
(51) Mazzafera, P. Growth and biochemical alterations in coffee due to selenite toxicity. *Plant Soil* **1998**, *201*, 189−196.
(52) Uehara, S.; Uno, Y.; Inoue, T.; Suzuki, T.; Utoh, M.; Sasaki, E.; Yamazaki, H. Caffeine 7-N-demethylation and C-8-oxidation mediated by liver microsomal cytochrome P450 enzymes in common marmosets. *Xenobiotica* **2016**, *46*, 573−578.
(53) Peri-Okonny, U. L.; Wang, S. X.; Stubbs, R. J.; Guzman, N. A. Determination of caffeine and its metabolites in urine by capillary electrophoresis-mass spectrometry. *Electrophoresis* **2005**, *26*, 2652−2663.
(54) Chen, Z. Q.; Kang, Y.; Zhang, C. H.; Tao, J.; Xue, Y. Metabolic mechanisms of caffeine catalyzed by cytochrome P450 isoenzyme 1A2: A theoretical study. *Theor. Chem. Acc.* **2015**, *134*, 110.
(55) Petermann, J. B.; Baumann, T. W. Metabolic relations between methylxanthines and methyluric acids in Coffea L. *Plant Physiol.* **1983**, *73*, 961−964.
(56) Tanoue, R.; Sato, Y.; Motoyama, M.; Nakagawa, S.; Shinohara, R.; Nomiyama, K. Plant uptake of pharmaceutical chemicals detected in recycled organic manure and reclaimed wastewater. *J. Agric. Food Chem.* **2012**, *60*, 10203−10211.

DOI: 10.1021/acs.jafc.8b02235
J. Agric. Food Chem. 2018, 66, 7907−7915