Analytical Methods

Increasing accuracy of field-scale studies to investigate plant uptake and soil dissipation of pharmaceuticals

Journal:	*Analytical Methods*
Manuscript ID	AY-ART-04-2021-000623.R1
Article Type:	Paper
Date Submitted by the Author:	29-May-2021
Complete List of Authors:	Dickman, Rebecca; University at Buffalo, Chemistry Brunelle, Laura; University at Buffalo, Chemistry Kennedy, Bradley; Rich Earth Institute Noe-Hays, Abe; Rich Earth Institute Love, Nancy; University of Michigan, Civil & Environmental Engineering Aga, Diana; University at Buffalo, Chemistry

Increasing accuracy of field-scale studies to investigate plant uptake and soil dissipation of pharmaceuticals

Authors: Rebecca A. Dickman[1], Laura D. Brunelle[1], Bradley Kennedy[2], Abe Noe-Hays[2], Nancy G. Love[3], Diana S. Aga[1*]

Affiliations:

1. *Department of Chemistry, University at Buffalo, The State University of New York, Buffalo, NY 14260, USA*
2. *Rich Earth Institute, Brattleboro, VT 05301, USA*
3. *Department of Civil and Environmental Engineering, University of Michigan, Ann Arbor, MI 48109, USA*

* *Corresponding Author*

Abstract:

Pharmaceuticals and personal care products (PPCPs) can enter agricultural fields through wastewater irrigation, biosolid amendments, or urine fertilization. Numerous studies have assessed the risk of PPCP contamination, however there are no standardized methodologies for sample treatment, making the interpretation of results challenging. Various time periods between sampling and analysis have been reported (shipping, storage, drying, etc.), but literature is lacking in the evaluation of PPCP degradation amidst this process. This study assessed the stability of 20 pharmaceuticals (200 μg/L) in soil and crops stored at -40ºC for 7, 30, and 310 days. After 310 days, caffeine, meprobamate, trimethoprim, primidone, carbamazepine, anhydro-erythromycin and dilantin were found to be stable (≥75% recovered) in all matrices. On the other hand, acetaminophen, amitriptyline, bupropion, lamotrigine, sulfamethoxazole, naproxen, ibuprofen, paroxetine, were unstable after 30 days in at least one of the matrices investigated. Due to variations in analyte stability, fortification with isotopically-labelled surrogates at the point of sample collection was evaluated in comparison to fortification after shipment and storage, immediately prior to extraction. Chromatographic peak areas of stable analytes were found to be reproducible (±15%) in field-fortified samples, indicating that no additional errors occurred during sample handling under field conditions despite having a less controlled environment. Unstable analytes revealed notable differences in peak areas between fortification times, suggesting that fortification immediately after sample collection is

crucial to account for analyte losses during shipping and storage, resulting in accurate quantification of PPCPs.

1. Introduction:

Greenhouse and field-scale studies are commonly used to assess fate and transport of organic contaminants, such as pharmaceuticals and personal care products (PPCPs), in soil and crops resulting from wastewater irrigation,[1, 2] biosolids amendments,[3, 4] application of urine derived fertilizers (UDF),[5, 6, 7] and other resource reuse systems. Evaluating the residues of PPCPs in agricultural systems is an important step in assessing potential risks to consumers.[8] However, standard procedures for sample preparation, shipping, storage, and analysis do not yet exist. Due to their large-scale and environmental variability, field-scale trials introduce daunting challenges to quantitative chemical analysis, and without standardized methodologies many of these challenges are not properly addressed. A major challenge that is often overlooked is the analyte stability during sample collection, storage, and transportation.[9] An overview of different methodologies utilized in field and greenhouse studies that assess PPCP-uptake into common crops is shown in Table 1, exemplifying the variability of the analytical protocols used. Most studies fail to report details regarding sample storage conditions and duration, even though extended storage times are often unavoidable in large field studies. It is also notable that the temperature of storage ranges significantly from -18 to -70°C. The effects of sample storage on the integrity of PPCPs needs to be evaluated because studies have revealed the occurrence of catalyzed abiotic transformations at sub-zero temperature in the presence of nitrites.[10-12] This exemplifies why the stability of each analyte must be assessed and accounted for under the intended storage conditions to obtain accurate reporting of residual PPCPs in plants and soil samples. Furthermore, many PPCPs are unstable in the presence of water; hence, lyophilization prior to storage is desired for accurate reporting.[13] Notably, this step is time-consuming and may not always be possible to perform immediately prior to storage. In this regard, potential analyte losses between storage and sample extraction must be evaluated for each analyte under relevant sample handling conditions.

A common practice in trace chemical analysis is the use of surrogate standards for quantification, often a stable isotope-labelled analogue of each analyte, to correct for the extraction efficiencies and any

instrument variability during analysis.[14, 15] Stable isotope standards have the same chemical and physical properties as their analogous native analytes, and therefore will undergo the same rate of degradation and transformation within the samples. In this regard, isotopically-labelled standards can be used for quantification to correct for degradation of analytes over time, providing more accurately quantified results. Surprisingly, only five of the studies listed in Table 1 included stable isotope surrogates to correct for analyte losses during the whole analytical procedure, suggesting that many of these studies could be underreporting contaminant concentrations. Other techniques, such as matrix-matched external calibration and the method of standard addition, have also been utilized for quantification in field studies. These methods can account for signal variabilities arising from matrix interferences, but they fail to correct for analyte losses that occur throughout the whole analytical process.

In environmental water analyses, fortification of isotopically-labelled standards at the time of sample collection can be utilized with ease because aqueous samples require minimal sample preparation, with only volume measurements being involved at the time of collection.[14, 15] For solid samples, such as plant tissues and soil, additional sample preparation is needed (e.g. grinding, homogenizing, weighing, and sieving), hence field fortification of surrogate standards must be done carefully at the proper step of the procedure. Of the five studies listed in Table 1 that used isotopically-labelled surrogates, some fortified the samples prior to extraction (after a period of sample storage) while others fortified the sample extract just prior to injection into the instrument. None of these studies fortified samples at the time of collection. This lack of agreement in methodologies highlights the need to develop in-depth protocols for field surrogate fortification to accurately account for PPCP uptake in crops and determine their persistence in soil.

In this regard, this study aims to evaluate the stability of pharmaceutical analytes in various periods of storage, as well as to demonstrate the importance of field fortification of surrogate standards to obtain accurate results. This research promotes the use of standardized protocols and techniques to improve quantitative analysis of contaminants of emerging concern in agricultural and environmental field trials and support comparison of results between studies. To achieve these goals, we evaluated samples from a larger project that assessed the effectiveness of urine-derived fertilizer in crop production[16] and the potential risk

of plant uptake of pharmaceutical residues found in urine.[7] The present study consists of: (1) a laboratory-scale stability assessment of PPCPs to gain an understanding of the analyte recoveries in relevant matrices during long-term storage, and (2) a field-scale study that evaluates spiking samples with isotopically-labelled PPCPs using the technique of field fortification in comparison to lab fortification. In this paper we use the term "field fortification" to mean spiking the samples as close to the sampling collection time as possible. This does not mean literally in the field, but rather in a local or mobile lab where samples are processed immediately after field collection. We use the term "lab fortification" to describe the more common practice of fortifying in the analytical laboratory, prior to extraction and analysis. Results from this study provide much needed information that will improve our ability to accurately determine the risks associated with PPCP contamination of food crops.

2. Materials and Methods:

2.1 Chemicals and Reagents:

Acetaminophen, d4-acetaminophen, acetyl-sulfamethoxazole, d4-acetyl-sulfamethoxazole, amitriptyline, bupropion, caffeine, $^{13}C_3$-caffeine, ciprofloxacin, d8-ciprofloxacin, citalopram, d6-citalopram, desvenlafaxine, d6-desvenlafaxine, d3-diphenhydramine, erythromycin, ^{13}C-d3-erythromycin, ibuprofen, d3-ibuprofen, meprobamate, d7-meprobamate, naproxen, d3-naproxen, paroxetine, d6-paroxetine, sulfamethoxazole, trimethoprim, and d9-trimethoprim, were obtained from Sigma Aldrich (Saint Louis, MO). Carbamazepine, d10-carbamazepine, dilantin, d10-dilantin, and d4-sulfamethoxazole were obtained from Cambridge Isotopes Inc. (Andover, MA). D-10 bupropion and lamotrigine were obtained from Cayman Chemicals (Ann Arbor, MI).

Acetonitrile and methanol of liquid chromatography- mass spectrometry (LCMS) grade for instrumental analysis and high-performance liquid chromatography (HPLC) grade for extraction solvent

were obtained from Omnisolv® through Millipore Sigma (Saint Louis, MO) and Fisher Chemical (Pittsburg, PA) respectively. American Chemical Society (ACS) grade nitric acid, glacial acetic acid, formic acid, and phosphoric acid were obtained from J. T. Baker (Philipsburg, NJ). Waters™ Cortecs™ C18+ (2.7 μm particle size, 2.1 mm internal diameter, 150 mm length) analytical column and Waters™ Oasis® hydrophilic–lipophilic balance™ (HLB) solid phase extraction (SPE) cartridges (6 cc, 500 mg) were obtained from Waters™ (Milford, MA). Reference soil was collected by collaborators from control field plots at Rich Earth Institute's field study site (Westminster, VT). Organically grown carrot and lettuce were purchased from Wegmans™ grocery store (Buffalo, NY).

Study conditions/ Sample type	Collection/ preparation before storage	Storage conditions	Length of storage	Fortification	Quantification (Recovery correction?)	Reference
Field – carrot, lettuce, spinach, cabbage, celery, cucumber, bell pepper, tomato, soil	Divided (root, fruit, stem, leaves) washed, lyophilized, ground	-20°C	"until time of analysis"	Deuterated PPCPs; prior to extraction, after storage	Isotope dilution (Y)	Wu *et al*. **2014**[17]
Field – barley, eggplant, zucchini, chili pepper, cabbage, green beans, soil	Composite samples	conditions not reported	not reported	Deuterated PPCPs; prior to extraction, after storage	Matrix matched calibration curve (Y)	Pico *et al*. **2019**[1]
Field - tomato, eggplant, zucchini, pepper, cabbage, lettuce, parsley, arugula, potato, carrot	Divided (root, fruit, shoot, leaves), washed, lyophilized, ground	conditions not reported	not reported	No fortification	Standard addition (N)	Riemenschneider *et al.* **2016**[18]
Field – tomato, soil	Composite samples, washed, air dried (soil)/ tissue dried (tomato)	-18°C in plastic bags	"until analysis"	No fortification	External calibration (N)	Christou *et al.* **2017**[19]
Field – corn, soil	Homogenized, lyophilized (ground after storage)	conditions not reported	not reported	Deuterated PPCPs; prior to extraction (in fume hood overnight)	Isotope dilution (Y)	de Santiago-Martín *et al.* **2020**[20]
Field – tomato, carrot, corn, potato	Washed	-70°C	"until analysis at a commercial lab"	Labelled internal standards; prior to analysis, after extraction and storage	Isotope dilution (N)	Sabourin *et al.* **2012**[4]
Lysimeter field plot – carrot, sweet potato	Composite samples, washed, air dried	-20°C	"until processing"	Deuterated PPCPs; after storage prior to extraction	Isotope dilution (N)	Malchi *et al.* **2014**[21]
Greenhouse – tomato, cucumber, soil	Washed (lyophilized/ ground after storage)	-20°C	"until time of analysis"	Deuterated PPCPs; after extraction & storage, prior to analysis	Isotope dilution (N)	Goldstein *et al.* **2014**[22]

Table 1. Summary of field, lysimeter, and greenhouse studies assessing plant uptake and soil persistence of pharmaceuticals and personal care products in agriculture, demonstrating different sample preparation and storage conditions, as well as surrogate fortification and quantification techniques

Greenhouse – lettuce, carrot, tomato, soil	fruit, leaves), washed, lyophilized	conditions not reported	not reported	lyophilization, before extraction	Isotope dilution (Y)	Pan *et al.* 2017[23]
Greenhouse – lettuce, carrot, soybean, radish, wheat, corn	Divided (root, stem, fruit, leaves)	conditions not reported	not reported	Deuterated PPCPs; time of spiking not discussed	Isotope dilution (Unknown)	Prosser *et al.* 2014[3]
Greenhouse - tomato	Composite samples homogenized	-20°C	"until their analysis"	^{13}C-caffeine, d10-carbamazepine; prior to extraction	Matrix matched calibration curve (Y)	Martínez-Piernas *et al.* 2019[2]

2.2 Storage Stability Assessment:

Soil and crop samples were homogenized, frozen, and pre-weighed (wet-weight) into polypropylene centrifuge tubes (15 mL). For each sample matrix, 9 replicates were measured and fortified with 100 µL (1 mg/L) of 20 pharmaceutical standards mixture (Table 2) to a concentration of 200 ppb (µg/L, ng/g). The pharmaceutical standards mix was prepared from individual pharmaceutical stocks (100 mg/L) measured with a glass syringe and diluted into LCMS grade methanol. For this study, a high concentration of 200 ppb was used to ensure that analyte degradation could be assessed over 310 days. A spiking volume of 100 µL ensures that the spike will be dispersed across the entire sample, ensuring that the spike is homogenous within the samples. Replicate samples (n=3) were stored for each time point of 7, 30, and 310 days at -40°C, representing ideal storage conditions. The 310-day time point far exceeds the typical sample storage, but this represents a worst-case scenario for long term storage. Triplicate "day 0" samples for each matrix were fortified and extracted at each time point to represent control samples without loss due to storage.

Samples were measured to 1000.0 (± 5%) mg, 250.0 (± 5%) mg, and 1.00 mL for soil, crops, and urine, respectively. Samples were then fortified with 100 µL (1 mg/L) isotopically labeled pharmaceutical mix to achieve a final concentration of 200 ppb (µg/L, ng/g) for each labeled PPCP to be used as surrogate standards to account for analyte losses during extraction. Table 2 lists the 17 isotopically labelled standards added, and the analytes for which they represent as surrogates. Next, 1% acetic acid in 50:50 H_2O: MeOH

(v/v) (10 mL) was added to soil and crop samples, vortexed, sonicated on ice (20 min), and centrifuged (20 min, 4°C, 1968 g). Extracts were then collected into 500-mL amber glass jars. This process was done twice for crops and three times for soil, pooling extracts. Each sample extract was then diluted with Nanopure™ water to decrease the organic fraction to less than 5 % to retain analytes in the SPE sorbent. Salts and proteins were precipitated out of urine samples with the addition of methanol at -4°C (10 mL), which was then vortex, sonicated and centrifuged as above. The solution was collected and diluted with Nanopure™ water in amber glass jars so that the final sample will contain <5% organic solvent. Diluted extracts (220, 330 and 400 mL for crops, soil, and urine respectively) were loaded (6 mL/min) onto Waters™ Oasis® HLB™ SPE cartridges (6 cc, 500 mg). The SPE cartridges were dried by leaving them under vacuum for 30 min, then stored in freezer (-40°C) overnight. The SPE cartridges were eluted with two aliquots of acetonitrile (4 mL), pooling the eluents into acid washed glass centrifuge tubes. Each eluent was fully dried under nitrogen and resuspended with 500 µL of starting LC mobile phase that contains 100 µg/L d3-diphenhydramine, which served as an instrument internal standard to account for any drift in LC retention times or variations in MS ionization efficiencies.[24] The LCMS/MS method used a Waters™ Cortecs™ C18+ analytical column (2.7 µm particle size, 2.1 mm internal diameter, 150 mm length) with a gradient program of 0.3% formic acid in water and acetonitrile as mobile phases A and B, respectively. The details of the method are defined in a previous publication. [24]

Analyte	Corresponding Surrogate
Acetaminophen	D4-Acetaminophen
Acetyl SMX	**D4-Acetyl Sulfamethoxazole**
Amitriptyline	**D7-Meprobamate**
Bupropion	**D9-Bupropion**
Caffeine	**13C3-Caffeine**
Carbamazepine	**D10-Carbamazepine**
Ciprofloxacin	D8-Ciprofloxacin
Citalopram	**D6-Citalopram**
Desvenlafaxine	**D6-Desvenlafaxine**
Dilantin	**D10-Dilantin**
Erythormyocin-H2O	13C,D3-Erythromyocin
Ibuprofen	**D3-Ibuprofen**
Lamotrigine	**D7-Meprobamate**
Meprobamate	**D7-Meprobamate**
Naproxen	**D3-Naproxen**
Paroxetine	**D6-Paroxetine**
Primidone	D6-Paroxetine
Sertraline	13C6-Norsertraline
Sulfamethoxazole	**D4-Sulfamethoxazole**
Trimethoprim	**D9-Trimethoprim**
Venlafaxine	**D6-Venlfaxine**

Table 2. Target analytes and their corresponding surrogates. Surrogates are the isotopically labelled standards of the native analytes, with the acceptation of lamotrigine, amitriptyline, and primidone. These analytes were assigned surrogates based on the isotope standard which is structurally related to the native compound. Bolded surrogates were those included in the field fortification training session at the Rich Earth Institute.

2.3 Field Fortification Collaboration

This study proposes modifying fortification procedures, from a fortification prior to extraction and analysis to field fortification at the time of sample collection. Field fortification has a less controlled

environment compared to spiking in the analytical lab and therefore thorough trainings and protocols must be set in place to avoid the introduction of error. This study aims to provide more accuracy in quantification, but if the field technicians are not trained sufficiently, this technique may introduce more error to the quantification process. Therefore, thorough protocols were written including pre-weighing samples (in terms of wet weight), equipment preparation and cleaning, fortification techniques, as well as standard and sample handling and storage. These were shared with scientists at Rich Earth Institute (Westminster, VT) for quality assurance during the trial (supplemental information Table 1). Protocols are valuable tools for minimizing confusion and errors that could be easily introduced with field fortification, as well as lack of experience. Typically, fortification occurs within a laboratory setting by a trained chemist who has experience in handling very small volumes with syringes and micropipettes. Often, the training include blinded sample analysis to ensure that fortification and quantification are performed accurately. Field scientists often have a unique set of expertise, different than that of a lab chemist, and so easing the transition to field fortification of standards for quantification through protocols and trainings is critical.

As such, a training session on sampling modifications and fortification was done at Rich Earth Institute's Research Center in Vermont that focuses on resource recycling through urine diversion. Two resident Rich Earth Institute scientists participated, practicing weighing and fortifying samples with a glass syringe prior to the soil dissipation study. Soil that had no fertilization with urine in previous studies was collected in bulk prior to the training session. This soil was weighed (1.21 ± 0.05 g) (n=3) into 15 mL

polypropylene centrifuge tubes by each participant (n=3). Using a glass syringe, 100 μL of a 500 μg/L pharmaceutical standard mix (Table 2) was fortified into each soil sample by the trainer and gently shaken to homogenize. The pharmaceutical standard mix was prepared in LCMS grade methanol from individual pharmaceutical stocks (100 mg/L) each measured with a glass syringe. For this study, a spiking concentration of 100 ppb was used to assess the accuracy of each participant's fortification ability at PPCP concentrations that are relevant in urine. The volume of 100 μL was used to ensure that the sample was fully covered by the fortification solution and allow sufficient mixing within the sample. Following this step, each sample was fortified with a mixture of 14 surrogates (Table 2, bold) by each Rich Earth Institute participant. Samples were gently shaken to homogenize, and then placed on ice for transport to the University at Buffalo (UB) overnight. Soil samples were briefly frozen (-40°C) for 1 hour, lyophilized, and extracted as described above.

2.4 Soil Dissipation Study:

Soil was homogenized and sifted through a 2-mm sieve to remove pebbles and plant materials. Soil was then measured into acid-washed glass jars (50.00 ± 0.05 g, dry weight). Each jar was adjusted to 21% soil moisture (50% water holding capacity) and covered with a lid containing a small hole (1 cm) for gas exchange. Soil moisture was adjusted weekly to maintain a 21% soil moisture through the 2-week pre-incubation and 8-week trial period. Jars were incubated prior to fertilization (2 weeks), allowing the soil microbiome to equilibrate. Pharmaceutical-free urine was fortified to predetermined concentrations and

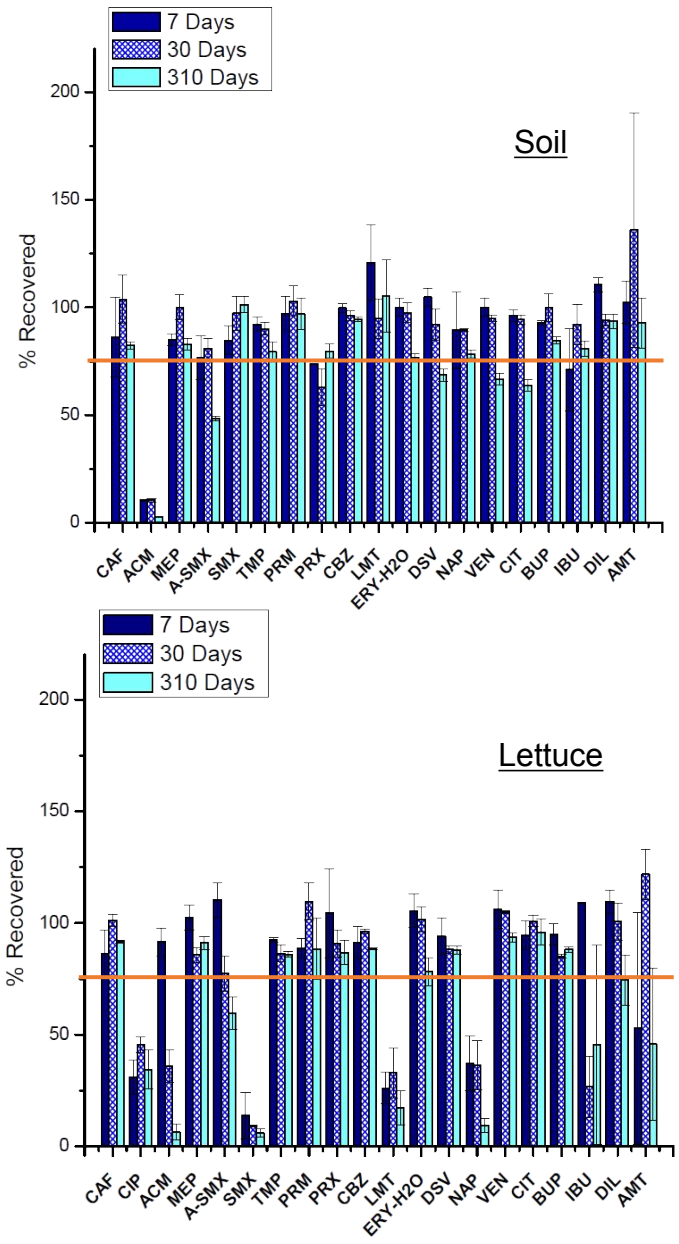
thoroughly mixed into each jar, with four replicates for each treatment (SI Figure 1). The study was held for 0, 2, 4, and 8 weeks in a dark enclosure at 23 ± 1°C. Prior to sampling, an isotope standard solution (250 µg/L) was prepared from individual pharmaceutical stocks (100 mg/L), each measured with a glass syringe, in LCMS grade methanol. The solution was divided into halves; one half was shipped overnight on ice to Rich Earth Institute then stored in a -20°C freezer. The other half was kept at UB and stored in a -40°C freezer until analysis.

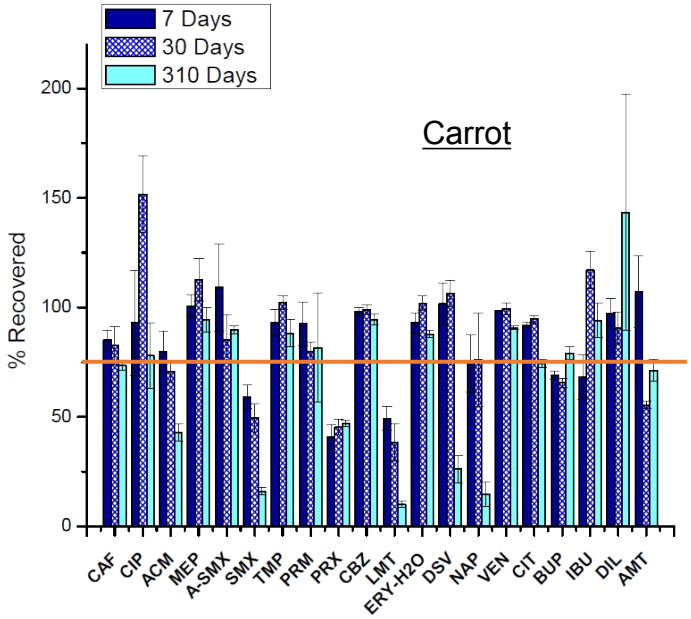
During sampling, water was added to all jars to maintain the soil moisture at 21%. Jars were homogenized and samples (n=2) were measured (1.21 ± 0.05 g) into polypropylene tubes (15 mL). One replicate from each jar was designated "FF" (field fortified) and spiked with 200 µL of isotope standard mix (250 µg/L) at the time of collection, yielding a final surrogate concentration of 100 ng/g. This volume was used to create a slurry of soil, which ensures that the standards are dispersed homogenously within the samples and produce surrogate concentrations typical of a urine-derived fertilizer. Additionally, the use of a larger spiking volume decreases the relative error during spiking. The second replicate was designated "LF" (lab fortified) and was not fortified in the field. Instead, the LF samples were frozen and shipped on ice overnight to UB. Upon arrival, the LF samples were frozen (1 h), lyophilized, then fortified as described above. Samples were extracted and analyzed as described previously.[24] The shipping and lyophilization lasted 2 days, and therefore FF samples were spiked 2 days prior to LF samples.

3. Results and Discussion:

3.1 Assessment of Stability during storage at -40 C:

The stability of various PPCPs evaluated across three time points (7, 30, and 310 days) is summarized in Figure 1. The percent recovery was calculated in comparison to "day 0" samples as defined by Equation 1. Error bars represent the standard deviation for each set of replicates (n=3). A 75% recovery after a 30-day storage period was classified as stable. The USEPA Residue Chemistry Test Guidelines defines a "30% rule of thumb" regarding analyte losses during storage. Analytes with sample loss of 30% or less during storage can be reported if a correction factor is applied. This "rule of thumb" was used as a benchmark for analytes with 75% recovery, classifying them as "stable" during long-term storage.





$$\textbf{\textit{Equation 1}}. \quad \%\ Recovered = \frac{Area\ "Day\ x"}{Area\ "Day\ 0"} \times 100$$

Figure 1. Stability of pharmaceuticals in: (a) soil, (b) carrot, and (c) lettuce after 7, 30, and 310 days of storage at -40ºC (n=3). Error bars represent the standard deviation for each set of replicates. The stability of pharmaceuticals varied significantly based on both analyte characteristics and matrix. The orange line indicates 75% recovered. Analytes recovered above this threshold are considered to be stable. Acetaminophen (ACM), Acetyl-Sulfamethoxazole (A-SMX), Amitriptyline (AMT), Bupropion (BUP), Caffeine (CAF), Carbamazepine (CBZ), Ciprofloxacin (CIP), Citalopram (CIT), Desvenlafaxine (DSV), Dilantin (DIL), Anhydro-erythromycin (A-ERY), Ibuprofen (IBU), Lamotrigine (LMT), Meprobamate (MEP), Naproxen (NAP), Primidone (PRM), Paroxetine (PRX), Sulfamethoxazole (SMX), Trimethoprim (TMP)

3.1.1 Stability in soil

As shown in Figure 1a, most analytes were recovered at or above 75% in soil after 310 days. Table 3 lists analytes, in increasing order of K_{ow}, with their recovered values at each time point and matrix. Bolded analytes are those that are classified as stable based on the ≥75% recovered criteria in all matrices. No trend was observed between analyte stability and K_{ow}. Caffeine, meprobamate, sulfamethoxazole, trimethoprim, primidone, paroxetine, carbamazepine, lamotrigine, anhydro-erythromycin, naproxen, bupropion, ibuprofen, dilantin and amitriptyline were all stable in soil at the 310-day time point. Paroxetine has a low extraction efficiency (17%), and therefore only two replicates were recovered at the 7-day trial. It is important to analyze this compound because it is a selective serotonin reuptake inhibitor (SSRI), which are known to be persistent in the environment.[25] Regardless, paroxetine was relatively stable even up to 310 days in storage. At 30 days, acetyl sulfamethoxazole, desvenlafaxine, venlafaxine, and citalopram were observed to be stable in soil, although they were unstable at 310 days. In contrast, acetaminophen was very unstable in soil, with a recovery of only 10% after 7 days and 3% after 310 days. This suggests that acetaminophen can degrade during storage of soil samples at -40ºC and therefore may be significantly underestimated in soil studies where samples have been stored longer than 7 days. This observation is consistent with the results found in a sorption study performed in both sterilized and non-sterilized soils, which showed a loss of acetaminophen over time. In that study, non-sterilized soil showed a half-life for acetaminophen of 2.1 days while an analyte loss of 30% was observed in sterilized soil after 15 days.[26] This is important to note, as acetaminophen is an over-the-counter pharmaceutical, widely used across the United States and world-wide. The study also reported that sorption and degradation of caffeine are significant mechanisms of loss in soil.[26] This trend, however, was not observed in these stability trials, as caffeine was stable for the duration of the experiment. It is also valuable to note that amitriptyline was recovered at 135 ± 54% after 30 days of storage. This variability suggests that minor variation in sample composition can drastically impact its stability. Amitriptyline does not have an analogous isotopically-labelled standard in this method. Therefore, variation in extraction efficiency between samples cannot be corrected for this

analyte, creating increased variation relative to other target analytes that were normalized to their isotope analogue.

3.1.2 Stability in carrot

Figure 1b displays the stability of pharmaceuticals in carrot matrix. In this matrix, analytes including caffeine, ciprofloxacin, meprobamate, acetyl sulfamethoxazole, trimethoprim, carbamazepine, primidone, anhydro-erythromycin, venlafaxine, citalopram, ibuprofen, and dilantin were stable (≥75%) after 310 days. Acetaminophen showed a greater stability in carrot than in soil, and 70% was recovered after 30 days, dropping to 43% after 310 days. This suggests that degradation or sorption can occur, but the mechanism of loss for acetaminophen is not as rapid as was observed in soil. Lamotrigine, sulfamethoxazole, and paroxetine were observed to be unstable over 7 days of storage, with recoveries under 60% and only 10-16% recovered after 310 days. A study on analyte stability in HLB™ SPE cartridges stored at -4ºC over 8, 15, and 28 days observed similar results, with significant losses of sulfonamide antibiotics.[14] Sulfamethoxazole was classified as unstable after 15 days (30% recovered), and with only 70% recovered after 8 days. Additionally, it was found that SSRIs, including paroxetine, had significant loss and variability after 8 days.[14] These results support that paroxetine is not stable after 1 week of storage in this matrix. Naproxen and desvenlafaxine were stable at 30 days, with 76% and 106% recovered, respectively. However, these two analytes had significant losses after extended storage, with only 15% and 26% recovered after 310 days, respectively, suggesting that analysis after 30 days is not reliable for these compounds. Dilantin was stable to 30 days (90% recovered), after 310 days it was recovered at 143%, but was inconsistent, with an RSD of 38%.

3.1.3. Stability in lettuce

Figure 1c reveals that caffeine, meprobamate, trimethoprim, paroxetine, carbamazepine, anhydro-erythromycin, venlafaxine, citalopram, dilantin, and bupropion were stable (>75%) in lettuce after 310 days. Naproxen, ciprofloxacin, acetyl-sulfamethoxazole, sulfamethoxazole, lamotrigine, and amitriptyline showed recovery of 14 – 59% after one week, suggesting they are not stable in this matrix. Similar to soil, acetaminophen exhibited a low stability with only 35% recovered after 30 days. In lettuce, coextracted

matrix can impact the extraction efficiency and detection of ibuprofen significantly, which can account for the high variability in the calculated percent recoveries. Ibuprofen was not recovered in one replicate from the "day 7" sample set and therefore the standard deviation could not be calculated for stability in lettuce.

Table 3. Percent analyte stability at 7, 30 and 310 days in soil, lettuce, and carrot matrices. Bold numbers suggest analytes that are stable (>75%) up to 30 days in all matrices. NR denotes no recovery.

	Soil (%)			Lettuce (%)			Carrot (%)		
Analyte	7 d	30 d	310 d	7 d	30 d	310 d	7 d	30 d	310 d
CAF	**86**	**103**	**82**	**86**	**101**	**92**	**85**	**82**	**73**
CIP	NR	NR	NR	31	46	34	93	152	78
ACM	10	11	3	92	36	6	80	71	43
MEP	**85**	**100**	**83**	**102**	**86**	**91**	**101**	**113**	**94**
A-SMX	**77**	**81**	**48**	**110**	**77**	**60**	**109**	**85**	**90**
SMX	85	97	101	14	9	6	59	50	16
TMP	**92**	**90**	**79**	**92**	**86**	**86**	**93**	**102**	**88**
PRM	**97**	**103**	**97**	**89**	**110**	**89**	**93**	**80**	**82**
PRX	74	63	80	104	91	87	40	45	47
CBZ	**100**	**96**	**95**	**91**	**96**	**88**	**98**	**99**	**94**
LMT	121	95	105	26	33	17	49	38	10
ERY-H2O	**100**	**98**	**77**	**106**	**102**	**78**	**93**	**102**	**88**
DSV	**105**	**92**	**69**	**94**	**88**	**88**	**102**	**106**	**27**
NAP	89	90	78	37	36	9	75	76	15
VEN	**100**	**95**	**67**	**106**	**105**	**94**	**99**	**99**	**91**
CIT	**96**	**94**	**64**	**95**	**101**	**96**	**92**	**95**	**74**
BUP	93	100	85	95	85	88	69	65	79
IBU	71	92	81	109	27	45	68	117	94
DIL	**111**	**94**	**93**	**109**	**101**	**74**	**97**	**91**	**143**
AMT	102	136	93	53	122	46	107	56	71

3.2 Field fortification training

The fortification accuracy of each scientist was assessed following the training session. Figure 2 shows the quantified results using isotope dilution for three scientists, identities blinded, in soil samples. For the stable and well recovered analytes, such as bupropion, venlafaxine, citalopram, meprobamate, acetyl sulfamethoxazole, carbamazepine, and dilantin were quantified within 5% of the fortified value (100 ng/g) with 5% residual standard deviation (RSD) within replicates, as well as when compared to the other scientists. Trimethoprim, desvenlafaxine, caffeine, sulfamethoxazole, and ibuprofen were over quantified by all scientists, suggesting that there were background signals interfering with the quantification of these

analytes. Paroxetine and naproxen had large RSD between each set of replicates as well as between sample sets. These variations can be attributed to the poor extraction efficiency and low ionization efficiency of these two analytes, respectively. With the consistent results for stable analytes, it was concluded that each scientist was properly trained and was proficient in field fortification of samples.

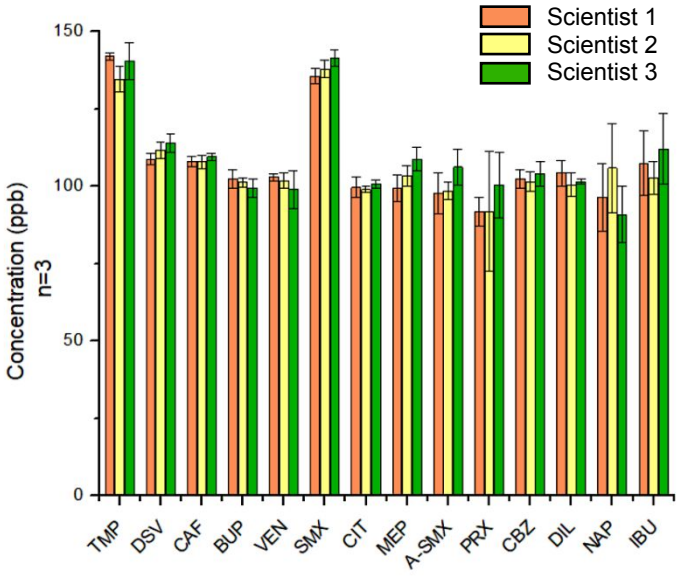


Figure 2. Quantified concentrations of 14 pharmaceuticals in soil (n=3) as a result of fortification field training for three scientists (blinded). Results indicate that each participant was proficient in field fortification of isotopically labelled standards.

3.3 Evaluation of field vs lab fortification

As observed, analyte stabilities are widely variable over time and are dependent on the matrix type. Therefore, field fortification with isotopically-labelled standards is proposed to achieve more accurate quantification. Samples for a soil dissipation trial were used as proof-of-concept to demonstrate reliability of the field fortification technique. In this trial, urine was fortified with 20 pharmaceuticals to pre-determined concentrations. For each soil sample, the areas of each isotopically-labeled analogue was normalized to the area of internal standard, d3-diphenhydramine. Figure 3 shows the variation in the spiking of selected isotope analogues at the zero- and two-week time points. At each time point, FF (n=8) replicates and LF (n=8) replicates were compared and plotted as the average of the normalized areas for each

analogue. For analytes with stabilities beyond 7 days in soil (d10-carbamazepine, d6-desvenlafaxine, d10-dilantin, d7-meprobamate, d4-sulfamethoxazole, d10-bupropion, $^{13}C_3$-caffeine, ^{13}C,d3-anhydro erythromycin, and d3-ibuprofen) the variations between field and lab fortification were minimal (<15%). These results suggest that no additional errors occurred during the field fortification of isotopically-labelled surrogates as compared to fortification in the lab setting. Alternatively, those analytes with low stabilities after 7 days showed notable variations in percent recoveries between the field and lab fortification. For example, d4-acetaminophen and d6-paroxetine revealed large variation between field-fortified and lab-fortified samples. Acetaminophen and paroxetine have low stabilities in soil, such that even a 2-day delay between collection and extraction can lead to significant analyte losses. Studies have observed low concentrations and highly variable detection frequencies of acetaminophen in crop samples.[2] The variability of acetaminophen observed in the training session could be attributed to the instability of the analyte within soils and storage. Wu et al. found acetaminophen in irrigation water but not in crops, attributing the lack of uptake to the rapid degradation of acetaminophen in soil. It is possible for this loss to cause analyte concentrations to fall below the method's limit of detection.[17] Loss of acetaminophen was observed when quantifying in soil at the 0-day time point. In this case, FF samples were quantified just above the methods limit of quantification, while the LF samples were quantified below this limit. It should be emphasized here that when isotope analogues are spiked at the time of extraction rather than at the time of collection, these spiked surrogates do not correct for any analyte loss throughout the sampling process. The observed results in this current study strongly support the proposed hypothesis that field-fortification of isotopically-labelled analogues is a particularly beneficial technique for real-world field analyses. Additionally, in field-scale studies, a two-day delay in analysis, due to shipping and lyophilization, is very short. Although this study required no extended storage due to a minimal number of samples spread across a two-month period, crop trials often require extended storage due to the number of samples and laboratory limitations. After the second sampling week, it was shown that field fortification was reproducible and introduced very minimal error, if any, to the quantification of samples. Therefore, in the subsequent sampling campaigns,

fortification was only performed in the field and lab spiking was discontinued for the 4- and 8-week sample periods.

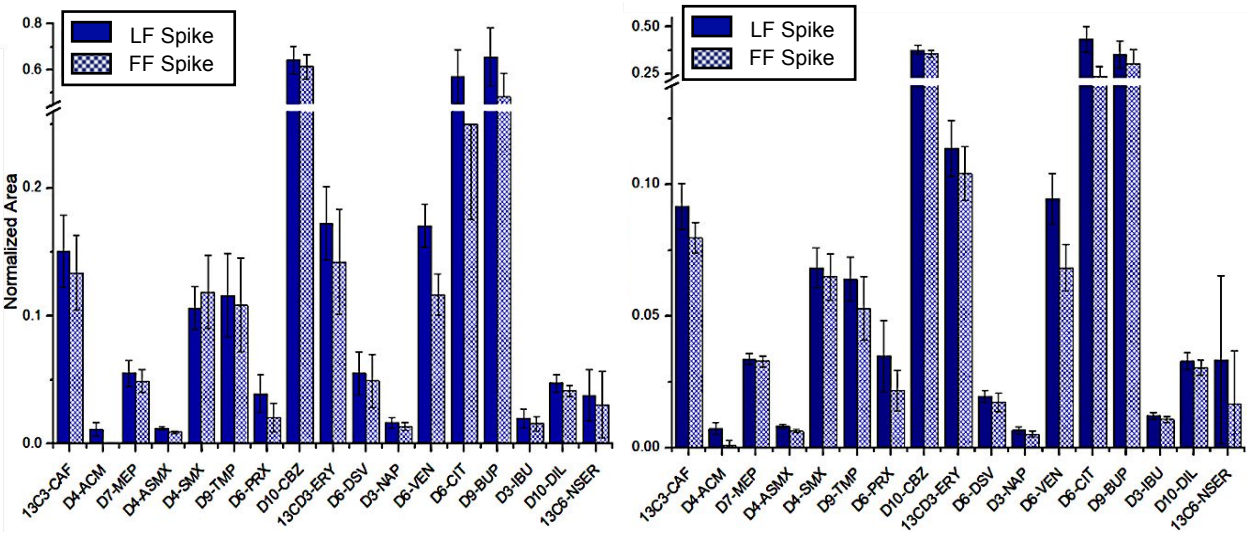


Figure 3. Normalized areas of isotopically labelled analogues from 0-week (left) and 2-week (right) time points. Field fortification compared to lab fortification showed consistent areas with stable analytes. Unstable analytes, including acetaminophen and paroxetine, show significant variation in area, with over 40% loss after 2 days.

4. Conclusion:

Wide variations in analyte stabilities were observed for pharmaceutical analytes in three agricultural matrices across the 7-, 30-, and 310-day sampling time points, even when stored under ideal conditions (-40ºC, dark). Many analytes including caffeine, meprobamate, trimethoprim, primidone, carbamazepine, anhydro-erythromycin and dilantin were stable (≥75% recovered) in all matrices after 310 days. Other analytes including acetaminophen, sulfamethoxazole, paroxetine, ciprofloxacin, lamotrigine, amitriptyline, naproxen, and ibuprofen showed significant losses (<75% recoveries) of analyte concentrations in at least one of the matrices. This study supports the conclusion that accurate quantification in risk-based assessments requires the use of isotopically-labelled standards at the time of sample collection. Furthermore, ensuring that the technicians and scientists who conduct field fortification have the proper materials and training makes field fortification achievable without significant error. It was observed that brief delays in analysis can cause significant underestimation in quantities of unstable analytes, such as acetaminophen and paroxetine in soil. The implementation of standard practices for fortification of

surrogate standards at the time of collection will create notable benefits to the fields of environmental and agricultural chemistry by improving the accuracy of quantification.

Statement of human consent

For these trials, urine was collected through a urine diverting toilet with the stipulation that those using pharmaceuticals are discouraged from donation. The urine samples were aggregated and is not traceable to specific people – therefore protecting the identities of the donors. In this regard formal consent from the people that used the urine diverting toilet is not considered necessary.

Author Contributions

Rebecca Dickman: Conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing – original draft, writing – review and editing. Laura Brunelle: Investigation. Bradley Kennedy: Conceptualization, investigation, writing – review and editing. Abe Noe-Hays: Conceptualization, supervision. Nancy Love: Conceptualization, funding acquisition, supervision. Diana S. Aga: Conceptualization, funding acquisition, supervision, methodology, writing – review and editing.

Acknowledgements

The authors acknowledge National Science Foundation (Award # 1639244) for support of this research. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the NSF. We would also like to acknowledge Kate Kusiak Galvin, who coordinated meetings between the teams in Vermont, Michigan, and New York, making the sharing of information seemless.

5. Corresponding author information:

* Phone: (716) 645-4220;; email: dianaaga@buffalo.edu

6. References:

1. Picó, Y.; Alvarez-Ruiz, R.; Alfarhan, A. H.; El-Sheikh, M. A.; Alobaid, S. M.; Barceló, D., Uptake and accumulation of emerging contaminants in soil and plant treated with

wastewater under real-world environmental conditions in the Al Hayer area (Saudi Arabia). *Science of The Total Environment* **2019,** *652*, 562-572.

2. Martínez-Piernas, A. B.; Plaza-Bolaños, P.; García-Gómez, E.; Fernández-Ibáñez, P.; Agüera, A., Determination of organic microcontaminants in agricultural soils irrigated with reclaimed wastewater: Target and suspect approaches. *Analytica Chimica Acta* **2018,** *1030*, 115-124.

3. Prosser, R. S.; Lissemore, L.; Topp, E.; Sibley, P. K., Bioaccumulation of triclosan and triclocarban in plants grown in soils amended with municipal dewatered biosolids. *Environmental Toxicology and Chemistry* **2014,** *33* (5), 975-984.

4. Sabourin, L.; Duenk, P.; Bonte-Gelok, S.; Payne, M.; Lapen, D. R.; Topp, E., Uptake of pharmaceuticals, hormones and parabens into vegetables grown in soil fertilized with municipal biosolids. *Science of The Total Environment* **2012,** *431*, 233-236.

5. de Boer, M. A.; Hammerton, M.; Slootweg, J. C., Uptake of pharmaceuticals by sorbent-amended struvite fertilisers recovered from human urine and their bioaccumulation in tomato fruit. *Water Research* **2018,** *133*, 19-26.

6. Winker, M.; Clemens, J.; Reich, M.; Gulyas, H.; Otterpohl, R., Ryegrass uptake of carbamazepine and ibuprofen applied by urine fertilization. *Science of The Total Environment* **2010,** *408* (8), 1902-1908.

7. Wigginton, K.; Love, N.; Lahr, R.; Goetsch, H.; Aga, D.; Mullen, R.; Noe-Hays, A.; Nace, K.; Bott, C.; Gagnon, A.; District, H. R. S.; Jimenez, J.; Caldwell, B. Nutrient recovery through urine Separation; *Water Environment Research Foundation* **2017**.

8. Keerthanan, S.; Jayasinghe, C.; Biswas, J. K.; Vithanage, M., Pharmaceutical and personal care products (PPCPs) in the environment: Plant uptake, translocation, bioaccumulation, and human health risks. *Critical Review Environmental Science & Technology* **2020**, 1-38.

9. Angeles, L. F.; Aga, D. S., Establishing analytical performance criteria for the global reconnaissance of antibiotics and other pharmaceutical residues in the aquatic environment using liquid chromatography-tandem mass spectrometry. *Journal of Analytical Methods in Chemistry* **2018**, 9

10. Min, D. W.; Kim, K.; Lui, K. H.; Kim, B.; Kim, S.; Cho, J.; Choi, W., Abiotic formation of humic-like substances through freezing-accelerated reaction of phenolic compounds and nitrite. *Environmental Science & Technology* **2019,** *53* (13), 7410-7418.

11. Kim, K.; Ju, J.; Kim, B.; Chung, H. Y.; Vetráková, L. u.; Heger, D.; Saiz-Lopez, A.; Choi, W.; Kim, J., Nitrite-induced activation of iodate into molecular iodine in frozen solution. *Environmental Science & Technology* **2019,** *53* (9), 4892-4900.

12. Kim, K.; Chung, H. Y.; Kim, B.; Wong, G.; Nguyen, A. Q. K.; Kim, S.; Kim, J., Freezing-induced simultaneous reduction of chromate and production of molecular iodine: mechanism, kinetics, and practical implications. *Environmental Science & Technology* **2020,** *54* (24), 16204-16211.

13. USFDA, Lyophilization of parenterals. Dept of Health, and Welfare Public Health Service, Ed. 1986; Vol. 43.

14. Singh, R. R.; Angeles, L. F.; Butryn, D. M.; Metch, J. W.; Garner, E.; Vikesland, P. J.; Aga, D. S., Towards a harmonized method for the global reconnaissance of multi-class antimicrobials and other pharmaceuticals in wastewater and receiving surface waters. *Environment International* **2019,** *124*, 361-369.

15. Angeles, L. F.; Mullen, R. A.; Huang, I. J.; Wilson, C.; Khunjar, W.; Sirotkin, H. I.; McElroy, A. E.; Aga, D. S., Assessing pharmaceutical removal and reduction in toxicity provided by advanced wastewater treatment systems. *Environmental Science: Water Research & Technology* **2020,** *6* (1), 62-77.

16. Goetsch, H. E.; Zhao, L.; Gnegy, M.; Imperiale, M. J.; Love, N. G.; Wigginton, K. R., Fate of the urinary tract virus bk human polyomavirus in source-separated urine. *Applied and Environmental Microbiology* **2018,** *84* (7), e02374-17.

17. Wu, X.; Dodgen, L. K.; Conkle, J. L.; Gan, J., Plant uptake of pharmaceutical and personal care products from recycled water and biosolids: A review. *Science of The Total Environment* **2015,** *536*, 655-666.

18. 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. *Journal of Agricultural and Food Chemistry* **2016,** *64* (29), 5784-5792.

19. Christou, A.; Karaolia, P.; Hapeshi, E.; Michael, C.; Fatta-Kassinos, D., Long-term wastewater irrigation of vegetables in real agricultural systems: Concentration of pharmaceuticals in soil, uptake and bioaccumulation in tomato fruits and human health risk assessment. *Water Res* **2017,** *109*, 24-34.

20. de Santiago-Martín, A.; Meffe, R.; Teijón, G.; Martínez Hernández, V.; López-Heras, I.; Alonso Alonso, C.; Arenas Romasanta, M.; de Bustamante, I., Pharmaceuticals and trace metals in the surface water used for crop irrigation: Risk to health or natural attenuation? *Science of The Total Environment* **2020,** *705*, 135825.

21. 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. *Environmental Science & Technology* **2014,** *48* (16), 9325-9333.

22. Goldstein, M.; Shenker, M.; Chefetz, B., Insights into the uptake processes of wastewater-borne pharmaceuticals by vegetables. *Environmental Science & Technology* **2014,** *48* (10), 5593-5600.

23. Pan, M.; Chu, L. M., Fate of antibiotics in soil and their uptake by edible crops. *Science of The Total Environment* **2017,** *599-600*, 500-512.

24. Mullen, R. A.; Wigginton, K. R.; Noe-Hays, A.; Nace, K.; Love, N. G.; Bott, C. B.; Aga, D. S., Optimizing extraction and analysis of pharmaceuticals in human urine, struvite, food crops, soil, and lysimeter water by liquid chromatography-tandem mass spectrometry. *Analytical Methods* **2017,** *9* (41), 5952-5962.

25. Arnnok, P.; Singh, R. R.; Burakham, R.; Perez-Fuentetaja, A.; Aga, D. S., Selective uptake and bioaccumulation of antidepressants in fish from effluent-impacted niagara river. *Environmental Science & Technology* **2017,** *51* (18), 10652-10662.

26. Lin, A. Y.-C.; Lin, C.-A.; Tung, H.-H.; Chary, N. S., Potential for biodegradation and sorption of acetaminophen, caffeine, propranolol and acebutolol in lab-scale aqueous environments. *J Hazard Mater* **2010,** *183* (1), 242-250.