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Potential metabolism of pharmaceuticals in radish: Comparison of *in vivo* and *in vitro* exposure

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ABSTRACT

Metabolism of pharmaceuticals in plants is important to evaluate their fate and accumulation in vegetables, and subsequently the risks to human health. However, limited knowledge is available to evaluate the metabolism of pharmaceuticals in plants due to the lack of appropriate research approaches. In this study, radish was selected as a model plant to investigate the metabolism of pharmaceuticals in intact plants (*in vivo*) growing in hydroponic solution and in plant tissue enzyme extracts (*in vitro*). For caffeine, six phase-I demethylation metabolites identified in the intact radish plant were also found in the plant enzyme extracts. After 7 days of *in vivo* exposure, the amount of the identified metabolites was about 5.4 times greater than the parent caffeine in radish roots. Furthermore, the metabolism potential of fifteen pharmaceuticals in radish was evaluated on the basis of mass balance. After 7 days of hydroponic exposure, oxytetracycline, trimethoprim, carbamazepine, lincomycin, monensin and tylosin manifested relatively less extent of metabolism with the mass recoveries ranging from 52.3 to 78.2%. In contrast, 17 β-estradiol, sulfamethoxazole, sulfadiazine, estrone, triclosan, acetaminophen, caffeine, carbadox and lamotrigine underwent extensive metabolism with only 3.0 to 32.1% of the parent compound recovered. In the *in vitro* system, 17 β-estradiol, estrone, triclosan, oxytetracycline, acetaminophen, sulfadiazine and sulfamethoxazole were readily metabolized in radish root

25 enzyme extracts with 1.8 to 34.0% remaining after 96-h exposure. While in the leaf enzyme

26 extracts, only triclosan was rapidly metabolized with 49.2% remaining, and others

27 pharmaceuticals were $\geq 60\%$, indicating that the varying extents of metabolism occurred in

28 different plant parts. This study highlights the importance of pharmaceutical metabolism in

29 plants, and suggests that plant tissue enzyme extracts could serve as an alternative tool to assess

30 pharmaceutical metabolism in plants.

31

32 Capsule: Similar metabolism patterns were observed for rapidly metabolized pharmaceuticals in

33 both *in vivo* (radish tissue enzyme extracts) and *in vivo* (the intact plant) exposure.

34

35 *Keywords:* Plant metabolism; Plant uptake; Pharmaceuticals; Plant tissue enzyme extracts.

36

37 **1. Introduction**

38   Reclaimed water reuse in agricultural irrigation has been increasingly practiced to

39 alleviate the burden of water scarcity, especially in arid and semiarid regions (Bischel et al., 2011;

40 Sato et al., 2013). Biosolids and animal manures are also commonly applied to agricultural lands

41 for their fertility values, and as a convenient disposal approach as well (Clarke and Smith, 2011;

42 Cogger et al., 2013; Kumar et al., 2005). These practices also release pharmaceuticals to the

43 environment, resulting in their ubiquitous presence in soils with concentrations at ng kg^{-1} to μg

44 kg^{-1} levels (Chen et al., 2014; Durán-Alvarez et al., 2009; Kinney et al., 2006; Vazquez-Roig et

45 al., 2010). These pharmaceuticals could enter vegetables from soils via root uptake, which serves

46 as the starting point in the food chain of human and animal dietary consumption. It has been well

47 documented that vegetables could accumulate a range of pharmaceuticals from soils (Malchi et

2

48 al., 2014; Prosser and Sibley, 2015; Tanoue et al., 2012; Wu et al., 2013). However, only a few

49 attempts have been made to elucidate the metabolism of pharmaceuticals in plants. In addition to

50 uptake and translocation, metabolic transformation of pharmaceuticals is also an important

51 process influencing their residue levels in plants and their potential risk of food safety.

52   Pharmaceuticals could be metabolized in plants by multiple pathways and form a variety

53 of transformation products (Macherius et al., 2012; Miller et al., 2016; Riemenschneider et al.,

54 2017). In general, the metabolic processes involve three phases of reactions. Pharmaceuticals

55 could be mediated by plant enzymes and form more polar and water-soluble products via phase I

56 reactions (oxidation, reduction and hydrolysis). In phase II reactions, pharmaceuticals and their

57 phase-I metabolites could be conjugated to endogenous plant biomolecules such as amino acids,

58 sugars and glutathione, and form relatively larger-sized molecules. Compared to their parent

59 compounds these conjugates generally demonstrate an enhanced water solubility and higher

60 mobility in plants. These conjugates formed by phase II reactions could be sequestered in plant

61 vacuoles or cell walls by phase III reactions (He et al., 2017; Macherius et al., 2012; Sandermann,

62 1992). The metabolic transformations of some pharmaceuticals in plants have been studied such

63 as triclosan (Macherius et al., 2012), benzotriazole (LeFevre et al., 2015), ibuprofen (He et al.,

64 2017), diclofenac (Huber et al., 2012), iopromide (Cui et al., 2017) and carbamazepine

65 (Riemenschneider et al., 2017). Carbamazepine was moderately metabolized in tomato after 35

66 days of exposure, 21 phase I and II transformation products were identified, and their total

67 amount was equivalent to ~ 45% of carbamazepine accumulated in plants (Riemenschneider et

68 al., 2017). Among these metabolites, the 10,11-epoxycarbamazepine manifested even greater

69 toxic potency than carbamazepine (Tomson et al., 1990). In many cases, the amount of formed

70 metabolites exceeded that of the parent compounds remaining in plants. For instance, the sum of

3

71 eight phase II triclosan conjugates was 5 times greater than triclosan in carrots during two-month

72 growth in greenhouse (Macherius et al., 2012). In another study, three major metabolites of

73 benzotriazole were found to be >1.5 times more than benzotriazole after 8-day exposure in

74 hydroponic *Arabidopsis* (LeFevre et al., 2015). Besides the intact plants, the metabolism of

75 pharmaceuticals was also studied using plant cell cultures (Fu et al., 2017; Huber et al., 2009;

76 Macherius et al., 2012; Marsik et al., 2017; Sauvêtre et al., 2018; Wu et al., 2016). Triclosan,

77 naproxen, diclofenac, ibuprofen, gemfibrozil, sulfamethoxazole and atorvastatin were found to

78 rapidly disappeared in carrot cell cultures with 0.4 to 47.3% remaining after 90 h of exposure

79 (Wu et al., 2016). The lack of assessment of metabolism could underestimate the total

80 accumulation of pharmaceuticals and metabolites in plants and the potential risk related to the

81 consumption of contaminated agricultural products. Paltiel et al. (2016) recently reported that

82 human consumption of fresh produce irrigated with reclaimed water could lead to the appearance

83 of bioactive metabolites of carbamazepine in their urine.

84   *In vivo* exposure (intact plants) is an effective way to examine the fate and metabolism of

85 pharmaceuticals in plant-water (or -soil) systems. However, the intact plant experiments

86 conducted in hydroponic solution or soil are usually labor-intensive and time-consuming. The

87 presence of microorganisms and root exudates also influence pharmaceutical metabolism, which

88 cannot be excluded from the *in vivo* studies (Yu et al., 2013; Zhang et al., 2016). The uptake and

89 transport processes could further complicate the metabolism of pharmaceuticals in plants by

90 governing the input and output of parent compounds/metabolites in a specific plant part. In

91 plants, enzyme-mediated transformation has been considered as a major process to the

92 metabolism of xenobiotics in plants (Huang et al., 2013; Van Eerd et al., 2003). Plant enzyme

93 extracts have been used in the *in vitro* system to investigate the enzyme-mediated metabolism of

94 polybrominated diphenyl ethers (Huang et al., 2013), estrogens (Card et al., 2013) and metformin

95 (Cui and Schröder, 2016). These studies showed that the metabolites from the reactions with

96 plant enzyme extracts were similar to the products formed in the intact plants. Therefore, plant

97 enzyme extracts may serve as an alternative system to provide a simple and quick approach to

98 evaluate the metabolism of pharmaceuticals in plants, which is minimally affected by plant

99 microbial, uptake and transport processes.

100 The objective of this study was to compare the pharmaceutical metabolism in radish

101 using both *in vitro* and *in vivo* experiments. Radish was selected because this root vegetable is

102 usually consumed in raw, and might represent the worst case of exposure scenarios. Caffeine was

103 selected as one representative pharmaceutical for further evaluation of the formed metabolites in

104 both intact radish and radish tissue enzyme extracts because it is one of the most frequently

105 detected pharmaceuticals in vegetables (Goldstein et al., 2014; Malchi et al., 2014; Wu et al.,

106 2014; Wu et al., 2013). In addition, we expanded the experiments to fifteen commonly-used

107 pharmaceuticals with a wide range of physicochemical properties to assess their metabolism

108 potential in radish. This study provides new knowledge on the metabolic transformation of

109 pharmaceuticals in plants. The *in vitro* exposure method could serve as an alternative to quickly

110 screen the metabolism potential of pharmaceuticals in plants.

111

112 **2. Materials and methods**

113

114 *2.1. Chemicals and regents*

115 Fifteen pharmaceuticals were purchased from Sigma-Aldrich (St. Louis, MO, USA)

116 including acetaminophen, caffeine, carbamazepine, sulfadiazine, sulfamethoxazole, lamotrigine,

117 carbadox, estrone, 17 β-estradiol, triclosan, trimethoprim, lincomycin, oxytetracycline, monensin

118 and tylosin. These pharmaceuticals were selected because they are frequently detected in the

119 environment, and demonstrate a wide range of physicochemical properties (Table S1). Caffeine

120 metabolites xanthine, 3-methylxanthine, 7-methylxanthine, theophylline, paraxanthine and

121 theobromine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ceramic homogenizers,

122 octadecylsilane (C18), primary secondary amine (PSA), and graphitized carbon black (GCB)

123 were purchased from Agilent Technologies (Santa Clara, CA, USA). Disodium

124 ethylenediaminetetraacetate (Na_2EDTA), formic acid, glacial acetic acid, and sodium chloride

125 (NaCl) were purchased from J.T. Baker (Phillipsburg, NJ, USA). All organic solvents were of

126 HPLC grade from Fisher (Fair Lawn, NJ, USA). Water used in this study was produced from a

127 Milli-Q water purification system (Billerica, MA, USA).

128

129 *2.2. In vivo hydroponic experiment*

130 Radish seeds (*Raphanus sativus,* Burpee & Co., Warminster, PA) were germinated on

131 moistened filter paper for 4 days. The seedlings were then transferred to a plastic container with

132 10 L of nutrient solution (pH 6.5) (Hydrodynamics International, Lansing, MI, USA). After 14

133 days of growth, the radish plants (~10 cm in height) were used in the exposure experiments.

134 Each radish plant was carefully transferred to a 250-mL glass jar containing 230 mL of nutrient

135 solution with spiked pharmaceuticals. This exposure experiment was conducted with two

136 treatments (1) the radish was exposed to 200 ng mL^{-1} of caffeine in nutrient solution; (2) the

137 radish was exposed to a mixture of fifteen pharmaceuticals with the initial concentration of 100

138 ng mL^{-1} for each compound. The solution was continuously aerated via Teflon tubing connected

139 to a fusion air pump. The jars were wrapped with aluminum foil to prevent the potential

140 photolysis of pharmaceuticals and to minimize algae growth. Pharmaceutical-free controls

141 (radish only) and pharmaceutical solution controls (without radish) were also conducted in the

142 experiments. All controls and exposure experiments were performed in triplicate. During the

143 experiment, nutrient solution (without pharmaceuticals) was added daily to each jar to

144 compensate the water loss from plant transpiration. The glass jars were placed in a controlled

145 growth chamber at 20 ± 2 °C. The light cycle was set up as 10 h of fluorescent light every day

146 with the intensity of 150 mmol/m^2/s followed by 14 h of darkness. For the treatment with only

147 caffeine, radish roots, leaves and solution were sampled at day 1, 3 and 7. For the exposure of the

148 mixture of pharmaceuticals, aqueous solution, radish roots and leaves were collected after 7 days

149 of growth. The radish samples were thoroughly rinsed with deionized water, wiped with tissue

150 paper, weighed, chopped into small pieces, freeze-dried and ground to fine powders. All samples

151 were stored in freezer at −20 °C prior to the extraction of pharmaceuticals.

152

153 *2.3. In vitro reactions with radish enzyme extracts*

154 Pharmaceutical metabolism was also examined *in vitro* using the crude enzyme extracts

155 from radish tissues. Radish enzyme extracts were prepared using the method reported by Card *et*

156 *al.* (Card et al., 2013). In brief, fresh radish tissues were separated into roots and leaves, chopped

157 into small pieces, quickly frozen, ground into powders in liquid nitrogen, and then extracted with

158 50 mM potassium phosphate buffer (pH 7.0) at the mass to solution ratio of 1: 2 (g: mL) in ice

159 for 30 min. The homogenate was centrifuged at 8000 g for 40 min at 4 °C. The supernatant of

160 tissue enzyme extracts was collected, passed through a 0.22-µm cellulose ester membrane

161 (Millipore, Cork, Ireland), and immediately stored in an ice bath prior to use.

7

162   The *in vitro* exposure experiment was performed by mixing 200 ng mL^{-1} of caffeine or a

163   mixture of fifteen pharmaceuticals at 100 ng mL^{-1} for each chemical with the radish enzyme

164   extracts. The reactant mixtures were incubated in 1.5-mL centrifuge tubes in a water bath (25 °C),

165   and the reaction was quenched by adding 50 µL of glacial acetic acid. The experiments were

166   conducted in triplicate, along with enzyme extracts-free controls. The resultant solution was

167   sampled at the time intervals of 0, 4, 10, 24, 48, 72 and 96 h. An aliquot of the solution (0.5 mL)

168   was diluted to 1.0 mL with methanol and subject to pharmaceutical analysis. The proteins

169   concentration in radish enzyme extracts was used to estimate the activity of the enzyme mixture,

170   which was measured during the experiment using Bradford assay (Bradford, 1976). The proteins

171   concentration in the radish enzyme extracts demonstrated relatively stable during 96 h of

172   reaction (Figure S1).

173

174   *2.4. Analysis of pharmaceuticals*

175   Pharmaceuticals in radish root and leaf samples were extracted using a modified

176   QuEChERS method (Chuang et al., 2015). The detailed extraction procedure is given in the

177   supporting information (SI). Hydroponic solution was filtered through a 0.22-µm cellulose ester

178   membrane, and an aliquot of the filtrate (0.5 mL) was diluted to 1.0 mL with methanol and

179   injected directly into a LC-MS/MS system. The LC-MS/MS system consisted of a Shimadzu

180   prominence high-performance liquid chromatography (Columbia, MD, USA) coupled to a Sciex

181   4500 triple quadrupole mass spectrometer (Foster City, CA, USA), and a 50 mm × 2.1 mm

182   Agilent C18 column (Torrance, CA, USA). The optimized conditions and quality assurance of

183   the LC-MS/MS is detailed in SI. Multiple reaction monitoring (MRM) parameters are listed in

184 Tables S2 and S3. The corresponding matrix recoveries and method detection limits (MDLs) for

185 the studied pharmaceuticals are summarized in Tables S4-S7.

186   The metabolism of pharmaceuticals in radish enzyme extracts was fit to the first-order

187 kinetic model $C_i = C_0 e^{-kt}$, where C_i and C_0 is pharmaceutical concentration at sampling time t (h)

188 and the beginning time, respectively, and k is the rate constant (h^{-1}). The dissipation half-life ($T_{1/2}$)

189 was calculated as $T_{1/2} = \ln 2/k$.

190

191 **3. Results and discussion**

192 *3.1. Biotransformation of caffeine in hydroponic system*

193   The mass distribution of caffeine in radish leaves, roots and hydroponic solution is shown

194 in Figure S2. On the basis of the initial amount of caffeine added to the solution, 11.8, 48.9 and

195 82.6% disappeared in the system after 1, 3 and 7 days of exposure, respectively. The quick

196 depletion of caffeine in the solution, compared to the unplanted controls (< 5% of loss after 7

197 days), suggests that caffeine could be taken up by radish and undergoes relatively rapid

198 transformation in the hydroponic system. Six demethylation metabolites from phase I reactions

199 were identified and quantified using their authentic standards including xanthine, 3-

200 methylxanthine, 7-methylxanthine, theophylline, paraxanthine and theobromine. All six

201 metabolites were detected in both radish roots and leaves. None of the metabolites was found in

202 the radish-free controls indicating that the metabolism is plant-associated. Only a small amount

203 of xanthine was found in the hydroponic solution with radishes at day 3 and 7, and no other

204 metabolites were found in the solution (Table S8). The presence of xanthine in the solution could

205 be attributed to the excretion from plants and/or caffeine transformation by root exudates.

206   The molar fractions of the six metabolites increased with exposure time in both roots and

207   leaves (Figure 1). In the radish roots, the parent compound caffeine gradually decreased from

208   67.1% at day 1 to 49.8% at day 3, and to 15.6% at day 7, indicating that caffeine was

209   substantially metabolized. The product xanthine (loss of three –CH_3 groups from caffeine) was

210   the most abundant metabolite with the molar fraction increasing from 16.7% at day 1 to 58.5% at

211   day 7. Theobromine (loss of one –CH_3 group) was the major intermediate with the fraction

212   increasing from 9.0% at day 1 to 13.2% at day 7. This time-dependent transformation pattern

213   was also observed for 3- and 7-methylxanthine with their molar fractions up to 5.3% at the end

214   of experiment. Theophylline and paraxanthine (loss of one –CH_3 group) were the least abundant

215   metabolites with the molar fractions < 3.8% (day 3); their fractions increased between day 1 and

216   3 and then decreased from day 3 to 7. Compared with the metabolism occurring in radish roots,

217   caffeine experienced less transformation in the leaves (Figure 1). The parent compound caffeine

218   was predominant with the molar fraction of 94.6% at day 1, 77.5% at day 3, and 68.1% at day 7.

219   Xanthine was the most abundant metabolite at day 1 (2.2%) and day 3 (9.6%), and theobromine

220   was most abundant at day 7 (9.9%). Given that these phase I reaction products all contained the

221   moiety of xanthine that maintains the bioactivity of the parent compound (Goth and Cleaver,

222   1976), the extensive biotransformation of caffeine in radish, particularly in the edible root

223   fraction, could lead to an underestimate of the risk associated with food safety if only

224   considering the presence of the parent compound.

225

226   *3.2. Transformation of caffeine in radish enzyme extracts*

227   The results of the *in vivo* hydroponic experiment provided the information on the

228   metabolism of caffeine in the intact radish. However, the uptake and translocation of caffeine in

229   radish, as well as radish growth, could add more confounding impacts to elucidating the

230   metabolism process. The *in vitro* exposure experiments with radish enzyme extracts offer an

231   alternative to investigate the metabolism of pharmaceuticals in plants. The amount of caffeine

232   and its metabolites formed in the radish tissue enzyme extracts as a function of time is shown in

233   Figure 2. During 96-h exposure, the loss of caffeine in root enzyme extracts was 29.9%, which

234   was approximately 2 times that in leaf enzyme extracts (15.4%) (Figure 2). Meanwhile, the total

235   amount of metabolites formed by demethylation reactions in root enzyme extracts was

236   approximately 2 times that in leaf enzyme extracts, suggesting that a larger extent of

237   biotransformation occurred in radish roots. This is consistent with the results of the *in vivo*

238   hydroponic experiment in which the larger fraction of metabolites was formed in radish roots

239   than in leaves. The amount of the six demethylation metabolites continued to increase over time

240   in both root and leaf enzyme extracts, except for xanthine in the leaf enzyme extracts which

241   decreased slightly from 72 h to 96 h. The amount of metabolites formed in both tissue enzyme

242   extracts at 96 h of exposure ranked in the order of xanthine > theobromine > 3-methylxanthine

243   ≈ 7-methylxanthine > theophylline ≈ paraxanthine (Figure 2), which is also consistent with the

244   ranking of demethylation metabolites found in the *in vivo* intact radish. In the *in vivo* hydroponic

245   experiment, caffeine accumulation in roots and leaves varied with time due to the continuous

246   water movement into radish; therefore, it is not feasible to compare the absolute amount of the

247   formed metabolites between the *in vitro* and *in vivo* experiments. However, the consistence in the

248   types of metabolites and their rankings in magnitude in both radish roots and leaves suggests that

249   the *in vitro* exposure to plant tissue enzyme extracts could provide a convenient alternative to

250   evaluate the metabolism of pharmaceuticals in a specific plant compartment (Card et al., 2013;

251   Cui and Schröder, 2016).

11

252   In both *in vivo* and *in vitro* studies, phase I demethylation reaction is identified as a

253   metabolism pathway for caffeine in radish (Figure 3). The possible transformation route follows

254   stepwise N-demethylation reactions via the loss of one –CH_3 group to form paraxanthine,

255   theobromine and theophylline, loss of the second –CH_3 group to form 7- and 3-methylxanthine,

256   and loss of all three –CH_3 groups to form the end product xanthine. The initial N-demethylation

257   reaction (loss of one –CH_3 group) is common in mammalians and insects, which is believed to be

258   mediated by cytochrome P450 enzyme systems (i.e. CYP1A2 isoenzyme) (Berthou et al., 1991;

259   Berthou et al., 1992; Coelho et al., 2015; Kot and Daniel, 2008). The N-demethylation reaction

260   of antidiabetic agent metformin was also observed in *Typha latifolia* (Cui and Schröder, 2016).

261   P450 enzymes are also commonly present in plants, and catalyze many metabolic reactions of

262   xenobiotics including some pharmaceuticals and herbicides (Fonne-Pfister et al., 1988; Huber et

263   al., 2009; Siminszky, 2006; Thies et al., 1996). Caffeine is believed to undergo similar enzyme-

264   mediated transformation in radish. In mammalians paraxanthine represents the large fraction of

265   the primary metabolites (70-80%) during the first step of demethylation (Berthou et al., 1991;

266   Kalow and Tang, 1993; Kot and Daniel, 2008). However, in this study theobromine was the most

267   abundant intermediate in radish.

268

269   *3.3. Mass distribution of pharmaceuticals in hydroponic system*

270   Pharmaceuticals have been frequently detected in vegetables grown in fields amended

271   with biosolids or irrigated with reclaimed water (Christou et al., 2017; Malchi et al., 2014; Wu et

272   al., 2014); however, their metabolism in plants still remains largely unknown. To obtain more

273   basic information on the metabolism of pharmaceuticals in vegetables, we further assessed

274   metabolism potential of fifteen pharmaceuticals in radish on the basis of mass balance using the

275 hydroponic experiment. The mass recoveries of pharmaceuticals in the radish-free controls

276 ranged from 92.0 to 108.2% indicating that pharmaceuticals in nutrient solution were relatively

277 stable, with the only exception of oxytetracycline (14.5%) (Table S9). The substantial loss of

278 oxytetracycline could be due to the rapid hydrolysis in the nutrient solution at pH 6.5 (near

279 neutral), which is the most favorable condition for the hydrolysis of oxytetracycline (Xuan et al.,

280 2009).

281 The mass distribution of the fifteen pharmaceuticals in solution, roots and leaves after 7-d

282 exposure is presented in Figure 4. The mass recoveries varied between 3.0 and 78.2% of the

283 initial pharmaceutical input, with the relatively high recoveries for tylosin (78.2%), monensin

284 (76.4%), lincomycin (74.3%), carbamazepine (68.4%), trimethoprim (58.6%) and

285 oxytetracycline (52.3%). Among these six pharmaceuticals, the majority of trimethoprim

286 (36.7%), lincomycin (69.4%), monensin (54.5%), and tylosin (43.8%) remained in the nutrient

287 solution, whereas large fractions of oxytetracycline (27.1%) and carbamazepine (40.8%) were

288 accumulated in radish plants. The metabolism of carbamazepine in vegetables such as sweet

289 potato, carrot, tomato, and cucumber could produce 10,11-epoxycarbamazepine and 10,11-

290 dihydroxycarbamazepine (Goldstein et al., 2014; Malchi et al., 2014). Riemenschneider *et al.*

291 (2017) found 21 phase-I and II transformation products derived from carbamazepine in tomato

292 after 35 days of exposure, and accounted for ~45% of carbamazepine uptake into plant. The

293 recoveries were relatively low for other nine pharmaceuticals ranging from 3.0 to 32.1%, only

294 3.0-4.0% for 17 β-estradiol, sulfamethoxazole, sulfadiazine and estrone, 11.7-18.4% for triclosan,

295 acetaminophen and caffeine, 20.7 and 32.1% for carbadox and lamotrigine. Similar metabolism

296 potential of triclosan was also observed in carrot by Macherius et al. (2012), who identified 8

297 triclosan conjugation products and their total amounts was about 5 times more than the parent

13

298 compound triclosan. The total recovered mass was < 80% for all tested pharmaceuticals after 7

299 days of exposure, and the metabolism in radish is expected to be responsible for such

300 discrepancies between the initial input and the remaining amount. The potential risk originating

301 from the metabolites are still unclear, particularly for those quickly and intensively metabolized

302 pharmaceuticals such as estrogens and sulfonamide antibiotics.

303

304 *3.4. Metabolism of pharmaceuticals in radish enzyme extracts*

305   The metabolism potential of the fifteen pharmaceuticals in radish root and leaf enzyme

306 extracts was examined by measuring their dissipation during 96 h of exposure (Figure 5). In root

307 enzyme extracts the tested pharmaceuticals demonstrated a wide range of variation in mass

308 recoveries from 1.8 to 98.1% of the initial dosage (Figure 5). Lincomycin, monensin, tylosin,

309 carbadox, carbamazepine, lamotrigine, trimethoprim and caffeine showed relatively low to

310 minimal transformation with the mass recoveries ranging between 72.3 and 98.1%.

311 Carbamazepine and trimethoprim demonstrated high stability in an *in vitro* study, where their

312 concentration remained nearly unchanged in carrot cell cultures during the 90 h of exposure, and

313 < 5% of carbamazepine was transformed after 22 days of incubation (Wu et al., 2016). In another

314 lately *in vitro* study, about 5% of carbamazepine was transformed in horseradish hairy root cell

315 cultures after 6 days of exposure (Sauvêtre et al., 2018). Sulfamethoxazole, sulfadiazine,

316 acetaminophen, oxytetracycline, triclosan, estrone and 17 β-estradiol manifested a rapid

317 metabolism with the recovered amount of < 34.0% of the initially spiked dosage. The fast

318 metabolism of estrogens, acetaminophen, sulfamethoxazole and triclosan is consistent with

319 previous *in vitro* studies (Card et al., 2013; Huber et al., 2009; Macherius et al., 2012; Wu et al.,

320 2016). Three conjugates (acetaminophen–glucoside, acetaminophen-glutathione, and cysteine

14

321 conjugate) of acetaminophen in the cell cultures of *A. rusticana* were identified after 6 h of

322 exposure, and their total amounts was about 4.6 times that of acetaminophen (Huber et al., 2009).

323 Wu et al. (2016) found that about 55.5 and 91.7% of triclosan and sulfamethoxazole disappeared

324 in carrot cell cultures within 90 h. Macherius et al. (2012) reported that ~95% of triclosan was

325 quickly metabolized within 24 h in carrot cell cultures. In this study, the rapidly depleted

326 pharmaceuticals were found for sulfamethoxazole, sulfadiazine, acetaminophen, oxytetracycline,

327 triclosan, estrone and 17 β-estradiol in both intact radish and root enzyme extracts. However, the

328 highly metabolized carbadox and caffeine in intact radish showed less depletion in the enzyme

329 extracts.

330   The extent of metabolism of pharmaceuticals in leaf enzyme extracts was less than that in

331 root extracts with the exception of carbadox and lamotrigine (Figure 5). Among the

332 pharmaceuticals studied, triclosan demonstrated the highest transformation in leaf enzyme

333 extracts (50.8%); however, the remaining fraction was still ~5 times greater than that in root

334 enzyme extracts (9.5%). Sulfadiazine, carbadox and sulfamethoxazole were moderately

335 metabolized in leaf enzyme extracts with the remaining fractions of 59.4%, 61.4% and 72.1%,

336 respectively. For the remaining ten pharmaceuticals, the majority of the initially spiked

337 pharmaceuticals still existed in the leaf enzyme extracts (79.2-98.8%) (Figure 5). The different

338 metabolism rates of pharmaceuticals between root vs leaf enzyme extracts could be related to the

339 types and amount of extracted enzymes, and their metabolic activities in different radish tissues.

340 The changes in metabolism magnitude could alter the distribution and accumulation of

341 pharmaceuticals in different plant parts, especially in the scenario that pharmaceuticals enter

342 plants by root uptake. For instance, only 1.8% of 17 β-estradiol remained in root enzyme extracts

343 after 96-h exposure, while > 90% of 17 β-estradiol was still present in leaf enzyme extracts

344 (Figure 5). In the *in vivo* experiment, 17 β-estradiol concentration in the leaves was 16 ng g^{-1}

345 which was >10 times greater than that in roots (1.4 ng g^{-1}). The results from the *in vitro*

346 experiments clearly support the rapid metabolism of 17 β-estradiol in radish roots, which is

347 believed to lead to the corresponding lower concentration in roots in the *in vivo* experiments.

348 Pharmaceuticals in different plant parts could have varying metabolism rates such as

349 carbamazepine in sweet potato, carrot, and tomato (Malchi et al., 2014; Riemenschneider et al.,

350 2017) and iopromide in cattail (Cui et al., 2017).

351   The rapid metabolism of sulfamethoxazole, sulfadiazine, acetaminophen, oxytetracycline,

352 triclosan, estrone and 17 β-estradiol in radish root enzyme extracts was fit to the first-order

353 kinetic model, and the fittings are shown as curves in Figure 6 with R^2 = 0.94-0.97 (Table S10).

354 The corresponding half-life was estimated as 17 β-estradiol (15.4 h) < estrone (17.3 h) <

355 triclosan (30.1 h) < acetaminophen (33.0 h) < oxytetracycline (36.5 h) < sulfadiazine (49.5 h) <

356 sulfamethoxazole (63.0 h). The rapid dissipation of triclosan and sulfamethoxazole were also

357 found in the carrot cell cultures with half-life of ~0.2 h and ~7.7 h, respectively (Wu et al., 2016).

358 Compared with the results of carrot cell cultures, triclosan and sulfamethoxazole both exhibited a

359 much slower dissipation rate in the radish enzyme extracts. Triclosan demonstrated a faster

360 dissipation rate than sulfamethoxazole in both *in vitro* systems. The high removal of these

361 pharmaceuticals in root enzyme extracts is consistent with their low mass recoveries in the *in*

362 *vivo* study (Figure 4), suggesting that these biotransformation reactions in root enzyme extracts

363 could also take place in radish roots in the *in vivo* experiments. Application of plant tissue

364 enzyme extracts to react with pharmaceuticals could serve as an appropriate approach for rapid

365 evaluation of their metabolism potential in plants. This particularly simplifies the evaluation of

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366 plant compartment-dependent metabolism, and could also eliminate the impact of uptake and

367 translocation to metabolism process in the intact plant experiments.

368

369 **4. Conclusion**

370 The deficient knowledge on metabolism of pharmaceuticals in plants limits the accurate

371 risk assessment of pharmaceuticals accumulated in vegetables because of the lack of appropriate

372 experimental or technique protocols for identification and quantification of metabolites. In this

373 study, the comparison in caffeine metabolism in radish between *in vivo* and *in vitro* exposure

374 experiments demonstrates that the demethylation metabolites identified in radish tissue enzyme

375 extracts (*in vitro* study) were similar to those formed in the intact radish roots and leaves in the *in*

376 *vivo* experiment. Similar metabolism patterns (dissipation of parent compounds) were observed

377 for rapidly metabolized pharmaceuticals including 17 β-estradiol, estrone, acetaminophen,

378 triclosan, oxytetracycline and sulfonamides in both radish tissue enzyme extracts and in the

379 intact plant. These results suggest that the metabolic reactions with plant tissue enzyme extracts

380 could be used as an appropriate approach for rapid examination of metabolism potential of

381 pharmaceuticals in a specific plant tissue, with the minimal impact from uptake and translocation.

382 For those extensively metabolized pharmaceuticals such as 17 β-estradiol, acetaminophen,

383 triclosan and sulfonamides, the measurement of the parent compounds in plants is not sufficient

384 to assess their accumulation and potential risks since the formed metabolites might still carry the

385 bioactive moieties and pose similar adverse impacts to plant and human health. Further

386 investigation of the metabolism of these pharmaceuticals in plants and their related

387 bioactivity/toxicity are needed to warrant the safe use of reclaimed water for irrigation and land

388 application of biosolids and animal manures.

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Acknowledgements

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Appendix A. Supporting Information

Additional details are provide in the Supporting Information including physicochemical properties of pharmaceuticals, analytical methods, mass recovery of pharmaceuticals in the hydroponic controls, protein concentration in enzyme extracts, mass distribution of caffeine in hydroponic-radish system, dissipation curves of pharmaceuticals in radish enzyme extracts, and representative chromatograms of pharmaceuticals.

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