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The quest for metabolic biomarkers of agrochemicals exposure *via in vitro* studies and suspect screening

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Abbreviations: ACN, acetonitrile; DBE, double bond equivalent; DMSO, dimethyl sulfoxide; EU, European Union; HLMs, human liver microsomes; HRMS, high resolution mass spectrometry; MeOH, methanol; NADPH, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate; QSAR, quantitative structure-activity relationship; REACH, Registration, Evaluation, Authorization and Restriction of Chemicals; TEST, toxicity estimator software; UHPLC-QTOF-MS, ultrahigh performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometer; VEGA, virtual models for property evaluation of chemicals within a global architecture.

Abstract

Numerous agrochemicals, including pesticides and herbicides, are applied in modern agriculture, resulting in concerns for the ecosystem and human safety as humans are easily exposed to these compounds. Many agrochemicals, and their transformation products or metabolites, have shown toxicity in *in vitro* and *in vivo* studies. However, given the rapid development of novel agrochemicals, for many there is no information about their effects nor about metabolic transformations when ingested by humans. Tracing biomarkers may be the best method for assessing the impacts of agrochemicals. A combination of *in vitro* metabolism study and suspect screening of human samples (e.g., urine, blood) can be utilized to efficiently find biomarkers for agrochemical exposure. In the work reported here, we determined the *in vitro* metabolic profiling of six prioritized pesticides and synergists, namely boscalid, carbendazim, piperonyl butoxide, spiroxamine, dimethomorph and fludioxonil, in human liver microsomes. 17 major metabolites were structurally elucidated by high resolution mass spectrometry (HRMS). Major metabolic transformation processes (e.g., hydroxylation, demethylation and oxidation) were proposed for each pesticide. Individual *in silico* toxicity assessments showed that some metabolites had the same or even enhanced toxicity compared to parent compounds. Information about these metabolites obtained from HRMS was used for suspect screening in human activities related samples. Carbendazim and a metabolite of fludioxonil were identified in wastewater and laboratory urine samples, respectively. Our findings provide concrete evidence for the use of *in vitro* metabolites as biomarkers in biomonitoring studies of potential exposure to toxic chemicals.

Keywords

Emerging agrochemicals; *in vitro* metabolism; metabolites identification; *in silico* toxicity; exposure biomarkers; suspect screening

1. Introduction

Agrochemicals, including pesticides, herbicides, insecticides, fungicides and synergists, are an integral part of modern agriculture; more than 4.1 million tons are used worldwide each year (Riedo et al., 2021). Although registration of new agrochemicals requires both human and environmental risk assessments to ensure their safety, such intensive and widespread use of agrochemicals still raises concern about their effects on non-targeted organisms and the environment. Humans may also be potential victims of agrochemicals through contamination of food and drinking water. For instance, pesticide residues have been detected not only in fruits and vegetables, but also in soft drinks and drinking water (Castilla-Fernández et al., 2021; EFSA et al., 2020; Syafrudin et al., 2021). Furthermore, and most seriously, chronic exposure to some pesticides has been conclusively demonstrated to have significant correlation with some diseases such as cancer, Parkinson's disease, Alzheimer's disease and reproductive disorders (Sabarwal et al., 2018). This is especially true for those occupationally exposed to pesticides.

Study of the effect of agrochemicals on humans is important. However, controlled studies in which human volunteers consume agrochemical-containing diets (i.e., *in vivo* studies) could be problematical if not prohibited. Today, human biomonitoring provides information vital to understanding the exposure of the general population to chemicals. Concentrations of agrochemicals and their metabolites are measured in specimens (e.g., blood, urine) and correlated with exposure levels; these concentrations can then be correlated with health-related outcomes (e.g., specific diseases). In these studies, the human metabolism adds a layer of complexity by converting contaminants into other forms with unknown toxicity. Thus,

63 monitoring only parent compounds may, firstly, underestimate the exposure level; and/or
64 secondly, miss the compound if it has been metabolized to trace levels, below the detection
65 limit of the method used. We need to establish the suitable exposure biomarkers for
66 biomonitoring. For this, we need metabolism study. However, for a metabolite to be used as a
67 biomarker generally requires verification in a small group of the general population (Bury et
68 al., 2018; Kolossa-Gehring et al., 2017), which needs analytical standards either from
69 commercial sources or synthesis. However, there are so many metabolites, it is hard to get all
70 their analytical standards/ relatively very few have been established, commercially. Nowadays,
71 utilizing suspect screening analysis by high resolution mass spectrometry (HRMS) provides
72 more feasibility to fill in this gap. Using *in vitro* human liver microsomes has two advantages.
73 First, it reduces the need for animal experiments. Second, it provides potential candidates for
74 suspect screening list of biomarkers that can be used for human biological samples and
75 wastewater samples – the former indicates the individual exposure while the latter represents
76 the community-wide exposure. Lopardo et al. (2017, 2018) firstly proposed the framework
77 combining human metabolism and wastewater fingerprinting assay to identify metabolic
78 biomarkers, and successfully applied on antimicrobial agents and personal care products.
79 Considering the ever increasing number and variety of chemicals we are exposed to daily,
80 Pellizzari et al. (2019) prioritized 155 emerging chemicals in different categories that may affect
81 children's health for biomonitoring and identified gaps needing additional research. Those
82 compounds were divided into four categories – two of the categories (B, C) are chemicals with
83 toxicity concerns but without exposure biomarkers. Category B chemicals include more

84 external exposure evidence (e.g., contaminations in food/water) compared to category C
85 chemicals. In this study, we included four Category B pesticides – boscalid, carbendazim,
86 dimethomorph and fludioxonil and two Category C pesticide and synergist – spiroxamine and
87 piperonyl butoxide.
88 Among these six agrochemicals, all four category B pesticides have been found in
89 environmental matrices to which humans are exposed. For example, carbendazim, boscalid and
90 dimethomorph were found in both the raw water and finished water in drinking water treatment
91 plants in Shanghai (Dong et al., 2021), while fludioxonil was detected in 26% of ambient air
92 samples in Spain (Coscollà et al., 2011). Further, these pesticides have been proven to induce
93 toxicological effects in both *in vitro* and *in vivo* models. Nearly all of them exhibit varying
94 degrees of developmental and/or reproductive toxicity in mammals such as rats, goats and dogs
95 (EU, 2017; Singh et al., 2016; WHO, 2007). Furthermore, fludioxonil and dimethomorph have
96 shown potential endocrine disrupting properties, apparently due to affinity with certain
97 androgen receptors, *in vitro* (Orton et al., 2011; Teng et al., 2013). For the two category C
98 pesticides/synergist additive – spiroxamine and piperonyl butoxide – the former is a relatively
99 new fungicide. Hence there is limited occurrence data and there have been few studies for it;
100 however, it has already been shown to have some developmental toxicity in rabbits (EFSA,
101 2010). Piperonyl butoxide, a pyrethroid synergist, has been measured in 75% of 48-hr prenatal
102 personal air samples in pregnant women (Williams et al., 2008), and this prenatal exposure has
103 been found to have associations with childhood cough in an urban cohort (Liu et al., 2012).
104 In this study, to facilitate the establishment of exposure biomarkers of these six

agrochemicals for biomonitoring studies, *in vitro* human phase I liver metabolites of each compound were identified using accurate mass spectrometry and built up the suspected list for suspect screening. Suspect screening was then conducted in two samples. To evaluate the toxicological hazard of each identified metabolite, several *in silico* models were used to predict their developmental, reproductive, and carcinogenic toxicity, bioaccumulation factors, and endocrine disruption potency.

2. Materials and methods

2.1 Chemicals and reagents

Analytical standards of pesticides dimethomorph (>98%), carbendazim (>98%), spiroxamine (>98%) and piperonyl butoxide (>95%) were purchased from Dr Ehrenstorfer (Ausberg, Germany) while fludioxonil (>99%) and boscalid (>98%) was from Chemservice Inc. (West Chester, PA). Pooled human liver microsomes (HLMs) (50 donors, mixed gender) were purchased from ThermoFisher (Waltham, MA), and β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH, >95%) was bought from Sigma-Aldrich (St. Louis, MO, USA). β-glucuronidase from *Helix pomatia* (Type HP-2, >100.000 U/mL) with sulfatase activity (≤7500 U/mL) was obtained from Sigma-Aldrich (USA). A 100 mM TRIS-buffer was prepared by dissolving 1.21 g Trizma base (Sigma-Aldrich) in 80 mL Milli-Q water, adjusting pH to 7.4 by hydrochloric acid (VWR International) and then making up to a volume of 100 mL. The buffer used in enzymatic deconjugation was prepared by adding 20 µL of β-glucuronidase in 10 mL 1 M ammonium acetate (pH to *ca.* 5). All chemicals used were at least

of AR grade unless specified. Dimethyl sulfoxide (DMSO, RCI Labscan), methanol (MeOH, Duksan Pure Chemicals), acetonitrile (ACN, Duksan Pure Chemicals), ethanol (EtOH, VWR Chemicals) and acetone (VWR Chemicals) used during sample preparation were AR or HPLC grade. For chromatographic analysis, LC-MS grade ACN (RCI Labscan) and LC-MS grade formic acid (99.0+%, Fisher Chemical) were utilized. Milli-Q water was obtained from an ultrapure water system (18.2 MΩ cm, Millipore, Billerica, MA, USA).

2.2 *In vitro* biotransformation assays

Phase I metabolites were generated using pooled HLMs and NADPH as activating agent. A reaction mixture containing 955 µL of TRIS-buffer (pH 7.4, 100 mM), 25 µL of HLMs (20 mg/mL) and 10 µL of target analytes (0.5 mM in DMSO or MeOH) was incubated in a 1.5 mL Eppendorf tube at 37 °C in a shaker bath. 10 µL of NADPH (0.1 M in TRIS-buffer) was added after 5, 60 and 120 min. Generation of Phase I metabolites was investigated at 1 h and 3 h. Reaction was stopped after 1 h or 3 h by adding 250 µL ice-cold ACN (containing 1% formic acid, v/v). Then the solution was vortexed for 30 s, centrifuged at 8000 rpm for 5 min and concentrated under stream of nitrogen gas to around 20 µL. After being reconstituted to 200 µL by ACN, the samples were filtered through a 0.22 µm PTFE syringe filter for UHPLC-QTOF-MS analysis. A positive control was also included by incubating 10 µL benzophenone-3 (0.5 mM in DMSO). The formation of benzophenone-1, as reported phase I metabolite, was monitored (Fig S1) (Watanabe et al., 2015). Besides, method blanks (without target analytes) and negative controls (either without HLMs or NADPH) were prepared in parallel.

147

148 **2.3 Pooled urine assay**

149 In April 2022, urine samples were collected from four laboratory volunteers who had not
150 been occupationally exposed to pesticides; samples were pooled. 40 mL urine samples (10 mL
151 from each volunteer) were enzymatically deconjugated with the addition of 10 mL prepared
152 buffer and incubated overnight at 37 °C. Solid phase extraction was performed using HLB Oasis
153 cartridges (Waters, UK) to reduce the matrix effect and concentrate samples. In detail, 40 mL
154 of deconjugated pooled urine samples were centrifuged, and supernatants were loaded onto
155 cartridges, which had been preconditioned with 6 mL of MeOH and Milli-Q water. After sample
156 loading, the cartridges were washed with 9 mL of Milli-Q water, dried for 20 min and eluted
157 with 3 mL ACN, EtOH and acetone, successively. Eluents were dried by a gentle nitrogen
158 stream and reconstituted in 500 µL of 1:1 v/v MeOH:H_2O. Procedural blank samples were
159 analyzed using Milli-Q water throughout the entire analytical procedure.

160

161

162 **2.4 Wastewater collection**

163 In December 2019, raw wastewater samples were collected from the wastewater tank of the
164 Science Tower of the Hong Kong Baptist University and filtered through a 0.45 µm nylon filter.
165 After that, 200 mL of filtered wastewater was enzymatically deconjugated to free any phase II
166 metabolites, incubated overnight, and then subjected to the solid phase extraction. All the
167 treated samples were filtered through 0.22 µm PTFE filters before instrumental analysis.

Procedural blank samples were analyzed using of Milli-Q water throughout the entire analytical procedure.

2.5 Analytical method

Metabolites were identified with UHPLC-QTOF-MS (Agilent 1290 UHPLC system coupled with Agilent 6540 Accurate Mass Quadrupole Time-of-Flight mass spectrometer, Agilent Technologies, USA). Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm, Waters, Ireland) was used to separate metabolites. The mobile phase was a mixture of Milli-Q water (A) and ACN (B), both containing 0.1% formic acid. Injection volume was set at 5 µL with 0.4 mL/min flow rate. An isocratic elution condition was used on boscalid (40%A; 60%B), carbendazim (20%A; 80%B) and dimethomorph (20%A; 80%B). For the remaining three, gradient separation was performed as: 0-1 min 98% A and 2% B; 1-8 min gradually changed to 0% A and 100% B; 8-12 min 0% A and 100% B; 12-12.5 min gradually changed to 98% A and 2% B and re-equilibration. Q-TOF mode was applied with ion source of Dual AJS ESI with mass correction at reference mass of *m/z* 112.9855 in negative mode and *m/z* 149.0233 in positive mode. High resolution mass spectra (*m/z* 100–1000) were obtained at a rate of 2 spectra per second, with metabolites screened in both positive and negative ESI modes. Conditions were as follows: dry nitrogen gas flow, 10 L/min at 325 °C; sheath nitrogen gas flow, 10 L/min at 400 °C; nebulizer, 20 psig; fragmentor voltage, 120 V; skimmer voltage, 65 V; octapole RF peak voltage, 750 V; Vcap, 4000 V; nozzle voltage, 500 V. The instrument was operated at 4 GHz (High Res Mode), with a low mass range setting of limit up to 1700 *m/z*. MassHunter

(Agilent Technologies) was applied for instrumental control, data acquisition and evaluation. The detected compounds were putatively characterized by candidate formulae. Thereafter, molecular ions were fragmented to obtain fragmentation patterns of metabolites. This information was used to define the confidence level of identification of these metabolites according to Schymanski et al. (2014).

Suspect screening of selected agrochemicals and their metabolites in human urine and municipal wastewater was conducted with the SCIEX TripleTOF 6600 hybrid mass spectrometer coupled with the ExionLC^{TM} AD Series UPLC system (SCIEX, Concord, Ontario, Canada). Chromatographic separation was performed using the same conditions as for metabolite identification, except with a change of injection volume to 10 µL. The TripleTOF mass spectrometer was operated in both positive and negative ESI modes, with the IonSpray Voltage Floating (ISVF) set to a value of ±4500 V and the declustering potential set to 80 V. The nebulizer gas (GS1), heater gas (GS2) and the curtain gas (CUR) were set at 35, 40 and 25 respectively, with heater temperature at 400 °C. Collision energies of 10 V and 30 V were used for TOF-MS survey scan and product ion scan, respectively, with dynamic background filtering. Instrument control and data acquisition were done with Analyst TF 1.8.1 (SCIEX), while data evaluation and analysis were conducted with SCIEX OS (Version 2.1.6.59781).

2.6 *In silico* evaluation of toxicity

The individual toxicity of each identified metabolites was evaluated comprehensively by several *in silico* models used in previous studies (Pellizzari et al., 2019). Virtual models for property Evaluation of chemicals within a Global Architecture (VEGA), a consortium of

models based on quantitative structure-activity relationship (QSAR) methodologies, was used to assess the developmental and developmental/reproductive effects and carcinogenicity of each pesticide and metabolites (Benfenati et al., 2013). Considering some pesticides have potential endocrine disrupting properties, VEGA was also used to predict the estrogen receptor-related binding affinity, estrogen receptor-mediated effect and androgen receptor-mediated effect of all pesticides and metabolites. Bioaccumulation factors related to persistence in tissues of organisms were determined by Toxicity Estimator Software (TEST, version 5.1), a model developed by the U.S. Environmental Protection Agency using QSAR methodologies (U.S. EPA, 2020).

3. Results and Discussion

3.1 Identification of metabolites of six agrochemicals

The characterization of the six agrochemicals and their metabolites was achieved using UHPLC-QTOF-MS. In total, 17 metabolites were identified for the six targeted agrochemicals. Details of the UHPLC-QTOF-MS analysis, including retention time, chemical formula, mass-to-charge ratio, and mass error, are provided in Table S1. In addition, due to their higher priority indicated by Pellizzari et al. (2019), metabolites of four category B pesticides (boscalid, carbendazim, fludioxonil and dimethomorph) were semi-quantified using relative signal intensity of parent compounds at 1 and 3 h (Fig S8). Although, with mass spectrometry, signal sensitivity is compound-dependent, semi-quantification with structurally similar compounds is still a common method to assess relative abundance, especially for compounds without

analytical standards (Tue et al., 2021; Vervliet et al., 2020).

3.1.1 Boscalid

Boscalid undergo metabolism primarily by hydroxylation (Fig 1a). Detailed structural elucidation of metabolites is presented in Fig S2. Boscalid was subjected to fragmentation *via* UHPLC-QTOF-MS as reference for its metabolites. The protonated molecule $[C_{18}H_{13}Cl_2N_2O]^+$ yielded a series of characteristic fragment ions at *m/z* 111.9949 and 139.9897 (Fig S2). TP-359 and TP-375, with the tentative formulae as $[C_{18}H_{13}Cl_2N_2O_2]^+$ and $[C_{18}H_{13}Cl_2N_2O_3]^{+\cdot}$ These ions were proposed to be mono-hydroxylated and di-hydroxylated metabolites with mass difference of 16 Da. Considering boscalid consists of a pyridine ring and two phenyl rings, hydroxylation could occur on several sites. TP-359 consists of the fragment at *m/z* 139.9897, representing the 2-chloropyridine-4-carboxy fragment of boscalid, but without the fragment at *m/z* 155, indicating that hydroxylation did not happen on the pyridine ring (Jabot et al., 2016). Therefore, the hydroxylation of TP-359 was proposed to occur in the chlorobenzene ring, as implied by the loss of HCl to form the fragment at *m/z* 323.0580 $[C_{18}H_{12}ClN_2O_2]^+$. While for TP-375, the distinctive fragment at *m/z* 155 $[C_6H_3ClNO_2]^+$ indicated that one of the hydroxylations occurred on the pyridine ring. Further hydroxylation of TP-359 on the pyridine ring leads to the formation of TP-375 $[C_{18}H_{13}Cl_2N_2O_3]^+$. According to the semi-quantitative result (Fig S8), the signal response ratio of TP-359 was well above TP-375 (0.28 vs. 0.01), indicating TP-359 has the highest potential to become the human biomarker of boscalid.

252 3.1.2 Carbendazim

253 Only one metabolite, TP-208, of carbendazim was formed after human phase I metabolism
254 through mono-hydroxylation (Fig 1b), with the tentative formulae as $[C_9H_{10}N_3O_3]^+$ at *m/z*
255 208.0713. Compared to the fragments of carbendazim at *m/z* 132.0548 and 117.0439, TP-208
256 represents fragment ions at *m/z* 148.0510 $[C_7H_6N_3O]^+$ and 133.0403 $[C_7H_5N_2O]^+$, with mass
257 differences of 16 Da compared to those from parent compound (i.e., *m/z* 132.0548 $[C_7H_6N_3]^+$
258 and 117.0439 $[C_7H_5N_2]^+$, respectively). This indicates that the hydroxylation occurred in the
259 aromatic ring of carbendazim (Fig S3). Yet, the exact position of the hydroxylation on the
260 aromatic ring could not be confirmed, even though previous study suggested that the
261 hydroxylation would lead to the formation of 5-hydroxy carbendazim (Douch, 1973).
262

263 3.1.3 Fludioxonil

264 As for fludioxonil, several hydroxylated metabolites were formed, with TP-263, TP-279 and
265 TP-295 as the mono-, di-, and tri-hydroxylated compounds, respectively (Fig 1c). For mono-
266 hydroxylated compound TP-263 (*m/z* 263.0280 $[C_{12}H_5F_2N_2O_3]^-$), considering it shared the
267 same fragment ions with fludioxonil at *m/z* 141, *m/z* 169 and *m/z* 180, hydroxylation was
268 proposed to happen on the aromatic ring (Fig S4). In contrast, TP-279 was believed to involve
269 hydroxylation of the pyrrole ring at the 2-position, as observed from the fragment ion of
270 $[C_{11}H_4F_2NO_4]^-$ at *m/z* 252.0119 after loss of -CN of TP-279, followed by the loss of -OH group
271 at the aromatic ring to form fragment $[C_{11}H_4F_2NO_3]^-$ at *m/z* 236.0169. Hydroxylation on the
272 pyrrole ring of fludioxonil has been previously reported (U.S. EPA, 2004).The addition of a

third hydroxyl group leads to the formation of TP-295, with two hydroxyl groups on the pyrrole ring at both the 2- and 5-positions as well as on the aromatic ring, as indicated by the loss of -CO, H_2O and -CN groups to form fragments of *m/z* 267.0215 $[C_{11}H_5F_2N_2O_4]^-$, 249.0125 $[C_{11}H_3F_2N_2O_3]^-$ and 224.0166 $[C_{10}H_4F_2NO_3]^-$, respectively. In addition, the signal response ratio of TP-279 was the highest (0.68), far above TP-263 (0.03) and TP-295 (0.004), indicating its potential to be used as a human biomarker (Fig S8).

3.1.4 Dimethomorph

For the three remaining pesticides, the metabolic pathway appears to be more complicated. Three metabolites of dimethomorph were identified after HLM-mediated *in vitro* metabolism (Fig S5). Fragmentation of dimethomorph $[C_{21}H_{23}ClNO_4]^+$ cleaves the morpholine ring, forming a fragment at *m/z* 301.0630 $[C_{17}H_{14}ClO_3]^+$. TP-374 is proposed to have the formula of $[C_{20}H_{21}ClNO_4]^+$, with double bond equivalent (DBE) of 10, which signifies in the same degree of unsaturation as dimethomorph. The fragment at *m/z* 301.0630 suggests that the transformation (loss of $-CH_2$) occurs in the ring of morpholine to form oxazolidine. With the same fragment at *m/z* 301, TP-404 and TP-420, with the tentative formulae of $[C_{21}H_{23}ClNO_5]^+$ and $[C_{21}H_{23}ClNO_6]^+$, respectively, are likely generated through mono- and di-hydroxylation, also respectively, of the morpholine ring. The proposed metabolic pathways of these three metabolites, as shown in Fig 2a, involve demethylation to form TP-374 and hydroxylation to form TP-404 and TP-420. According to the semi-quantitative result (Fig S8), although TP-404 had the highest response ratio of 0.17, TP-374 and TP-420 also followed behind with ratio as

294 0.10 and 0.06, respectively.

295

296 3.1.5 Piperonyl butoxide

297 As for piperonyl butoxide, three metabolites were identified. Piperonyl butoxide and its
298 metabolites formed NH_4^+ adduct in QTOF-MS. Fragmentation of piperonyl butoxide
299 ($[C_{19}H_{30}O_5+NH_4]^+$, *m/z* 356.2432) generates a characteristic fragment at *m/z* 177.0919
300 $[C_{11}H_{13}O_2]^+$ through cleavage of the 2-(2-butoxyethoxy)ethoxymethyl side-chain. TP-344, with
301 the formula $[C_{18}H_{30}O_5+NH_4]^+$ at *m/z* 344.2433, is proposed to be a catechol product of its parent
302 compound through opening of the methylenedioxy ring due to its mass difference of 12 Da.
303 Considering its key fragment at *m/z* 165.0914, $[C_{10}H_{13}O_2]^+$ appears to have the same mass
304 difference (12 Da) as the key fragment of piperonyl butoxide (*m/z* 177.0919, $C_{11}H_{13}O_2^+$). Ring
305 opening is believed to occur on the 1,3-dioxole side of piperonyl butoxide and to form the
306 corresponding catechol structure (Fig S6). TP-358 also showed the key fragment at *m/z*
307 165.0914, suggesting that it has the same catechol structure as TP-344. The fragments at *m/z*
308 177.1123 $[C_8H_{17}O_4]^+$ and *m/z* 159.1018 $[C_8H_{15}O_3]^+$ indicate that oxidation occurred on the 2-
309 (2-butoxyethoxy)ethoxymethyl side-chain of TP-344. TP-372, with an accurate mass at *m/z*
310 372.2371 and tentative structure of $[C_{19}H_{30}O_6+NH_4]^+$, is proposed to be a hydroxylation
311 product of piperonyl butoxide. With key fragment ions at *m/z* 177.0916 $[C_{11}H_{13}O_2]^+$ and
312 193.0860 $[C_{11}H_{13}O_3]^+$, hydroxylation is proposed to occur on the methylenedioxy ring of
313 piperonyl butoxide. In summary, the principal route of piperonyl butoxide metabolism proceeds
314 *via* an initial hydroxylation on the methylenedioxy ring to form TP-372 and then opening of the

315 methylenedioxy ring to form TP-344. Further oxidation of TP-344 on the 2-(2-
316 butoxyethoxy)ethoxymethyl side-chain then leads to the transformation to TP-358 (Fig 2b).
317
318 3.1.6 Spiroxamine
319 As for spiroxamine, it generated the most metabolites – a total of five. Sukul et al. (2010)
320 previously reported four biodegradation products of spiroxamine in soil by comparison with
321 the authentic standards in LC-MS. Fragmentation of TP-270 appeared to produce the same
322 fragments at *m/z* 116.1069 $[C_6H_{14}NO]^+$ as those reported for the standard compound desethyl-
323 spiroxamine, while TP-256 is proposed to be despropyl-spiroxamine (Fig S7). TP-314A and
324 TP-314B, although with the same tentative formula $[C_{18}H_{36}NO_3]^+$, appeared as separate,
325 distinctive peaks according to liquid chromatography and with different retention times and
326 fragmentation patterns according to mass spectrometry. Therefore, they are considered as two
327 different metabolites. TP-314B, with fragment ions at *m/z* 160.1332 and 130.1226 identical to
328 N-oxide-spiroxamine (Sukul et al., 2010), is proposed to undergo oxidation of the tertiary amine.
329 And for TP-314A, which was only reported in this study, considering its fragment at *m/z*
330 296.2589 $[C_{18}H_{34}NO_2]^+$ (loss of H_2O) and fragments at *m/z* 144, 126 and 100 (identical to the
331 parent compound), it is proposed to be a hydroxylated metabolite at the methylpropane position.
332 TP-244 (*m/z* 244.1905, $[C_{13}H_{26}NO_3]^+$) was also firstly reported. It is proposed to metabolize
333 through losing both the ethyl and propyl chains of TP-314A, with fragment at *m/z* 228.1967
334 $[C_{13}H_{26}NO_2]^+$. In conclusion, the primary metabolic pathway for spiroxamine in HLMs
335 involved desethylation, despropylation, hydroxylation and N-oxidation, producing TP-256, TP-

314A, TP-270 and TP-314B, respectively. Further transformation of the first three would lead to the formation of TP-244 (Fig 2c).

3.2 *In silico* toxicity assessment

As a variety of metabolites are produced during HLM-mediated metabolism, investigating their toxicity is essential for assessing the actual toxicity of HLM metabolites in the body/environment, especially knowing that some metabolites have demonstrated even greater adverse effects compared to their parent compounds (Su et al., 2014). Experimental toxicological approaches (i.e., *in vivo* and *in vitro* methods) are often limited by many pragmatic issues, such as cost, ethics and time, and – for this case – availability of chemical standards of the identified metabolites for toxicological assessment. Computational toxicology can overcome these challenges and commonly used by many regulatory agencies to preliminarily assess toxicity. For example, REACH – a legislation on chemicals used in EU – encourages the use of QSAR model predictions when the experimental data are not sufficient or as supplementary information for experimental data (Worth et al., 2005). Therefore, we used several QSAR *in silico* toxicological models (VEGA and TEST) to assess the potential health hazards of the selected pesticides and their individual human metabolites.

Boscalid is proven to have developmental toxicity in zebrafish embryos (Qian et al., 2018), which is in consistent with the developmental toxicant prediction using the VEGA test (Fig S9). Its metabolites, TP-359 and TP-375, probably retain similar toxicity with the parent compound. In addition, both boscalid and its two metabolites demonstrate certain probability of binding to

357 human estrogen receptor (Fig S9).

358 Carbendazim has shown developmental and reproductive toxicity in many *in vivo* studies and
359 is categorized in the priority list of endocrine disrupting chemicals by EU (Singh et al., 2016).
360 Its metabolite TP-208 demonstrated even higher potential as a developmental and reproductive
361 toxicant than its parent compound (Fig S10). Moreover, the position of the hydroxylated group
362 of TP-208 may affect not only its carcinogenicity, but also with the estrogen receptor relative
363 binding affinity.

364 As for fludioxonil, it caused an increase in incidence of dilated renal pelvis in fetuses and
365 litters observed in a rat developmental toxicity study (U.S. EPA 2012). According to the VEGA
366 prediction, all of its metabolites seem to retain that developmental toxicity, regardless the
367 position of the hydroxylated group (Fig S11), while the hydroxylated group in some specific
368 positions may elicit carcinogenicity.

369 Dimethomorph has been reported as an androgen receptor antagonist in some *in vitro* assays
370 (Kleinstreuer et al., 2017; Orton et al., 2011). VEGA predicts that both dimethomorph and its
371 metabolites (TP-404, TP-420 and TP-374) could possibly induce androgen receptor-mediated
372 effects (Fig S12).

373 Toxicological studies of piperonyl butoxide and spiroxamine are relatively limited.
374 According to the *in silico* assessment (Fig S13), piperonyl butoxide exhibited high reliability
375 of being a carcinogen, while its metabolite TP-358 and TP-344 also retain this feature. As for
376 spiroxamine, this fungicide showed developmental toxicity in rabbits (EFSA, 2010) and in
377 VEGA testing (Fig S14). For TP-314B, this concern is retained, and, unlike its parent compound,

TP-256, TP-270 and TP-314B showed some possibility of being carcinogens.

According to the *in silico* prediction, most of the metabolites, in general, would still retain the possibility of causing relative toxicity effects with their parent compounds, while some metabolites – such as TP-208 of carbendazim – have a higher toxicity potential. These results suggested that future toxicological or risk assessment studies need to take into account of not only the parent compounds, but also their metabolites/transformation products.

3.3 Suspect screening analysis

All the metabolites identified in the *in vitro* HLM by HRMS were added to the suspected metabolite list. A method was then adopted that screened the suspected metabolites for all the *in vitro* metabolites identified based on i) data-dependent acquisition using HRMS and ii) software-matching algorithms based on accurate mass and isotope patterns in the pooled urine and wastewater samples. Considering that phase II metabolism was not included to build up the database in suspect screening, enzymatic deconjugation was applied to both human urine and wastewater samples, aiming to convert phase II metabolites into phase I/parent compounds to increase analytical sensitivity.

Our suspect screening of pooled urine samples led to identification of one metabolite of fludioxonil, TP-263. Fig 3 shows a tentative formula of TP-263 $[C_{12}H_5F_2N_2O_3]^-$ with mass error of 0.38 ppm (*m/z* 263.0275). Characteristic fragments (*m/z* 169 and *m/z* 141) and the matched retention time (approx. 5.1 min) confirmed that mono-hydroxylated metabolite of fludioxonil appeared in the *in vivo* pooled urine sample.

We also screened the wastewater sample for potential biomarkers of community-wide exposure. Neither fludioxonil nor TP-263 was detected in the wastewater samples; however, another pesticide, carbendazim was found with exact mass of *m/z* 192.0764 (mass error: -2.08 ppm, R.T. = 0.6 min) and unambiguously confirmed by an analytical standard (Fig 4).

Both carbendazim and fludioxonil are permitted pesticides in Hong Kong with maximum residue limits ranging from 0.01 to 500 mg/kg in different foods (Hong Kong e-Legislation, 2022). Therefore, it is not surprising to detect them in human activities related samples. Furthermore, the presence of TP-263 but not its parent compound – fludioxonil – indicated the importance of investigating the biotransformation products for assessing exposure to pesticides. Also, according to the semi-quantification results, TP-279, not TP-263, was the most abundant metabolite in the HLM assay, indicating the variation between *in vitro* and *in vivo/*wastewater samples. Still, the result of this study constitute evidence that HLM metabolites can be used as exposure biomarkers.

At least two factors can be used to explain the differences in detection pattern of selected pesticides and their metabolites: 1) variation in collection period, resulting in different exposure signatures; and 2) the complexity of the wastewater/urine matrices, restricting the discovery of contaminants and metabolites at trace levels. Nevertheless, combining *in vitro* HLM with non-target screening in human urine and wastewater samples has several advantages. First, *in vitro* studies reduce the need for *in vivo* animal/human experiments, after the applicability of the metabolites has been verified in human urine and wastewater samples. On the other hand, traditional *in vitro/in vivo* metabolism studies may lead to identification of multiple metabolites,

while commercial standards or organic synthesis of these metabolites are necessary for further human exposure verification (Huang et al., 2019; Bury et al., 2018). Utilizing HRMS with *in vitro* HLM metabolites to build up the user-defined suspect list for non-target screening could help to narrow down the potential biomarkers among all these metabolites and suggest the most likely ones for further exposure assessment. In other words, using HLM metabolites as biomarkers candidates and further verifying by suspect screening can save time, money and labor.

4. Conclusion

With the aim of identifying biomarkers of exposure to agrochemicals, this study presents a comprehensive investigation of the phase I *in vitro* metabolism of six prioritized agrochemicals. A total of 17 metabolites were systematically identified in HLM by UHPLC-QTOF-MS and used to build up the user-defined database for suspect screening. Carbendazim and TP-263, a metabolite of fludioxonil, were detected in wastewater and pooled urine samples, respectively. Besides, *in silico* predictions showed that metabolites of piperonyl butoxide (i.e., TP-344 and TP-358) and metabolites of spiroxamine (i.e., TP-256, TP-270 and TP-314B) should raise concerns for their potential developmental toxicity and/or carcinogenicity. Further comprehensive human biomonitoring studies are suggested to better assess these metabolites.

Declaration of Competing Interest

The authors declare they have no conflict of interest.

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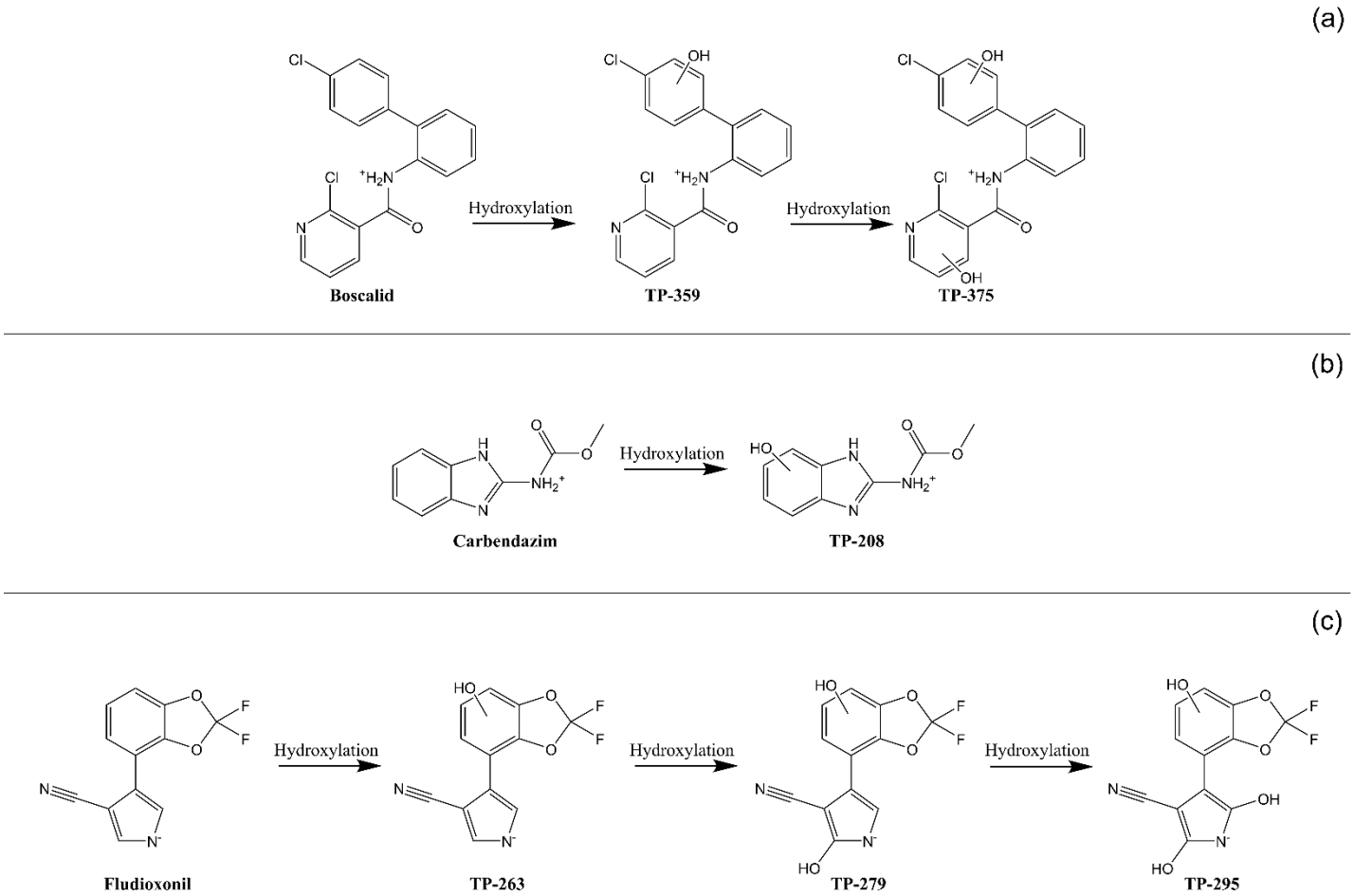
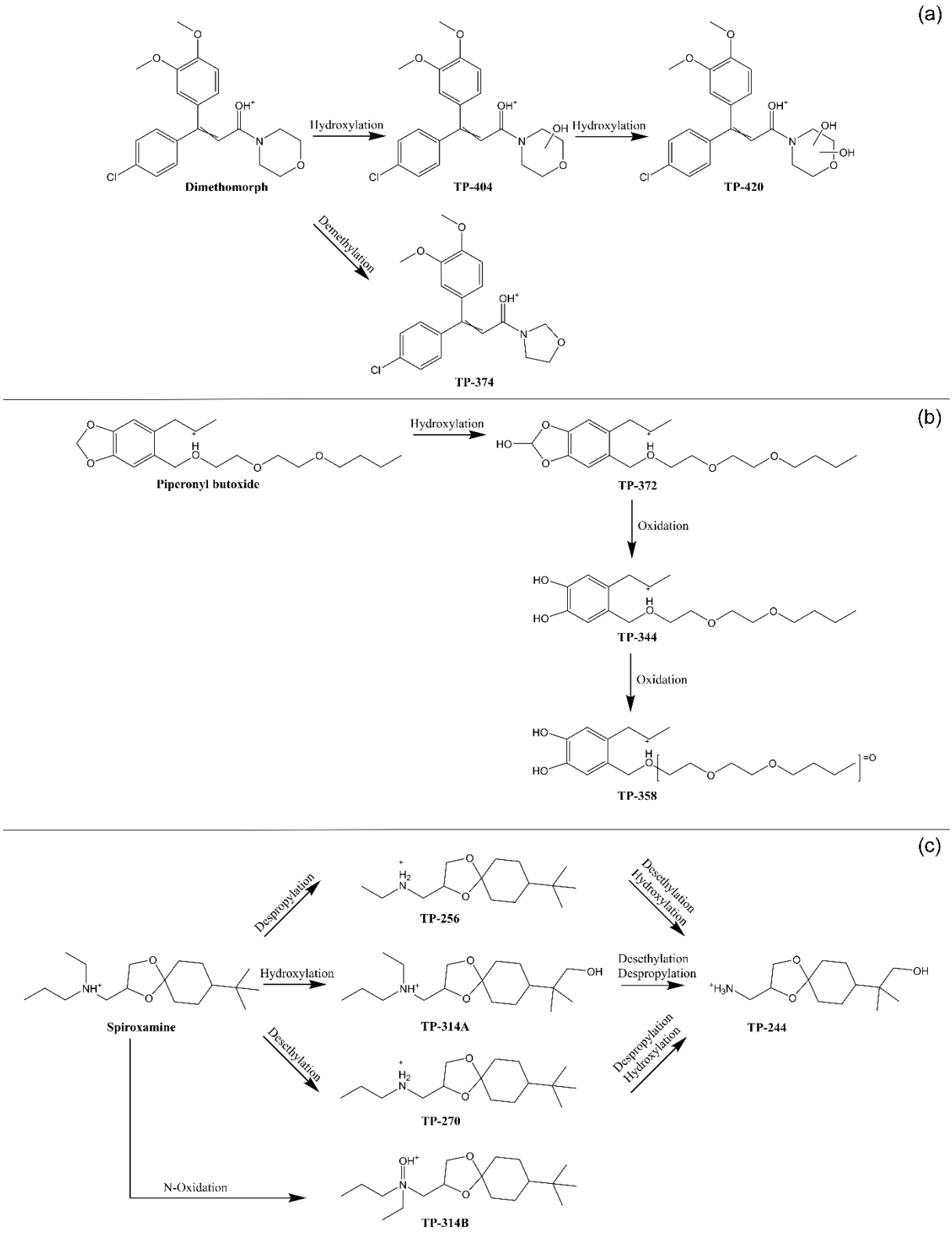


575

576 Fig 1. Proposed in vitro phase I metabolic pathway of targeted agrochemicals. (a) Boscalid; (b) Carbendazim; (c) Fludioxonil.



577

578 Fig 2. Proposed in vitro phase I metabolic pathway of targeted agrochemicals. (a)
579 Dimethomorph; (b) Piperonyl butoxide; (c) Spiroxamine.

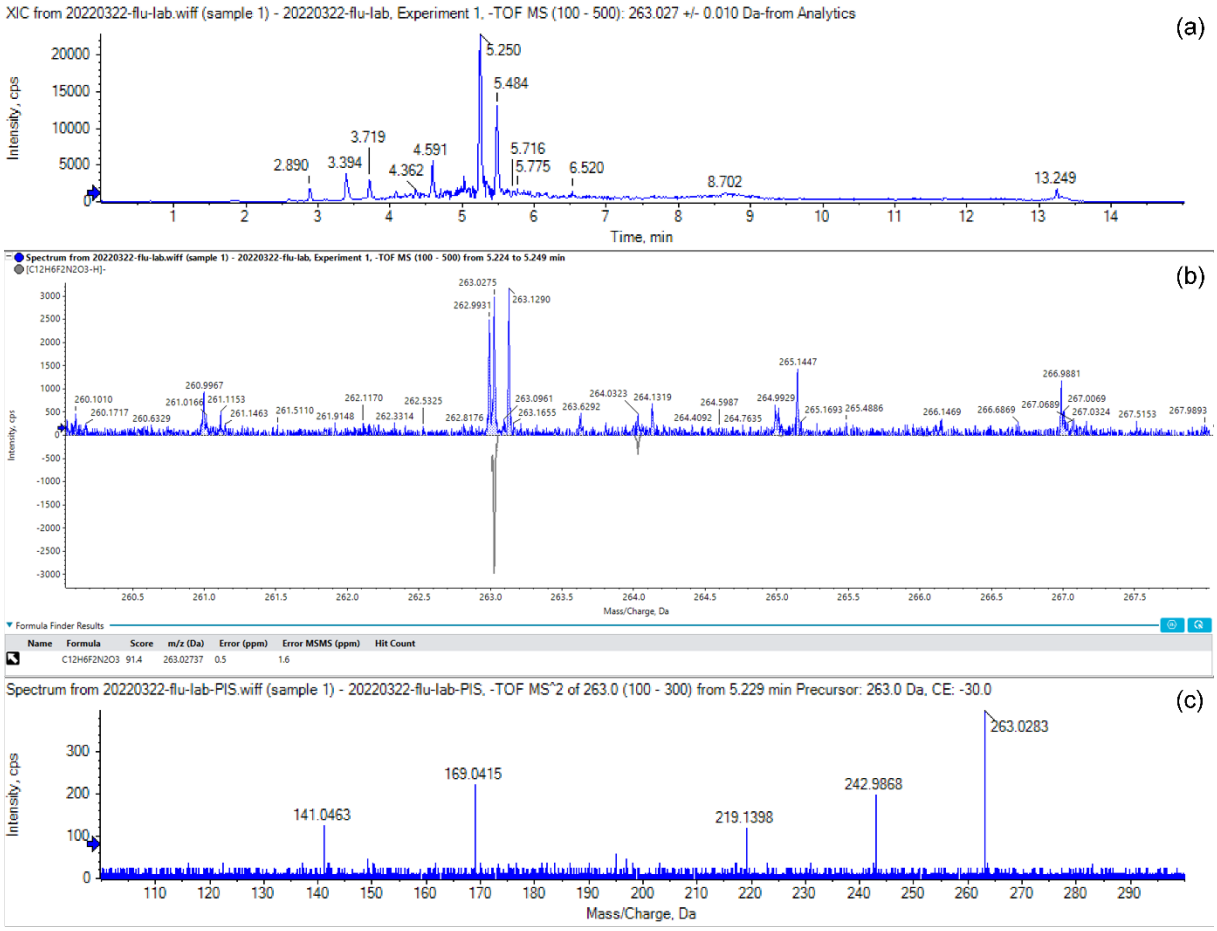


Fig 3. Detection and identification of FLU-TP-263 by UHPLC-QTOF-MS in urine sample. (a) Extracted Ion Chromatogram of *m/z* 263.0270 ± 0.010 in urine sample; (b) TOF-MS spectrum for peak at R.T. = 5.250 min in (a), showing the potential presence of FLU-TP-263 (with mirror spectrum of proposed formula $C_{12}H_6F_2N_2O_3$); (c) Fragmentation spectrum of *m/z* 263.0 for peak at R.T. = 5.250 min in (a), showing the characteristic fragments (*m/z* 141 and 169) to confirm the identity as FLU-TP-263.

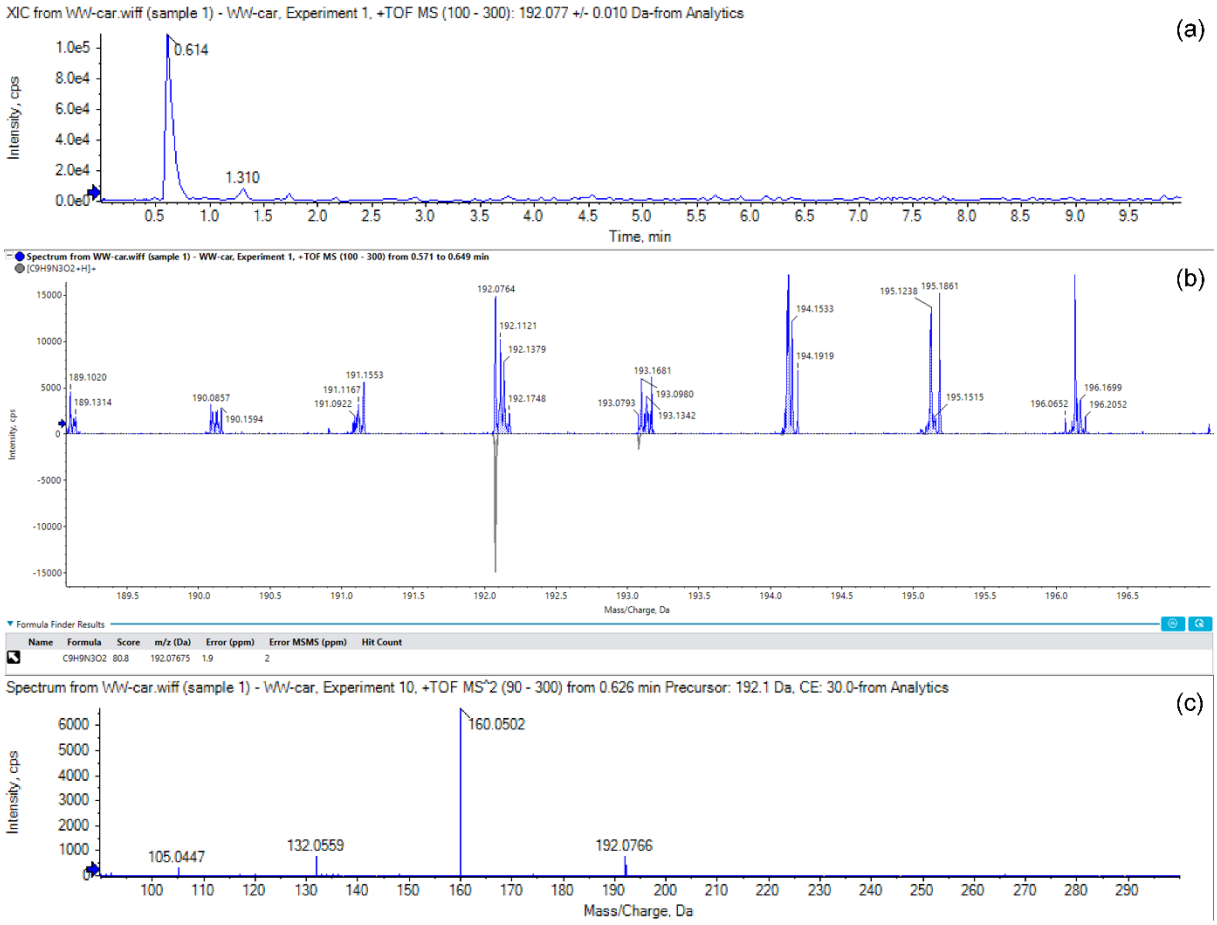


Fig 4. Detection and identification of carbendazim by UHPLC-QTOF-MS in wastewater sample. (a) Extracted Ion Chromatogram of *m/z* 192.0770 ± 0.010 in wastewater sample; (b) TOF-MS spectrum for peak at R.T. = 0.614 min in (a), showing the potential presence of carbendazim (with mirror spectrum of proposed formula $C_9H_9N_3O_2$); (c) Fragmentation spectrum of *m/z* 192.1 for peak at R.T. = 0.626 min in (a), showing the characteristic fragments (*m/z* 132 and 160) to confirm the identity as carbendazim.