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Author(s)	Nimako, Collins; Ikenaka, Yoshinori; Akoto, Osei; Fujioka, Kazutoshi; Taira, Kumiko; Arizono, Koji; Kato, Keisuke; Takahashi, Keisuke; Nakayama, Shouta M. M.; Ichise, Takahiro; Ishizuka, Mayumi
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1 **Simultaneous Quantification of Imidacloprid and its Metabolites in Tissues of Mice**
2 **upon Chronic Low-dose Administration of Imidacloprid**

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4 Collins NIMAKO¹, Yoshinori IKENAKA^{1,2,8,9}, Osei Akoto³, Kazutoshi FUJIOKA⁴,
5 Kumiko TAIRA⁵, Koji ARIZONO⁶, Keisuke KATO⁷, Keisuke TAKAHASHI⁷, Shouta
6 M.M. NAKAYAMA¹, Takahiro ICHISE¹ and Mayumi ISHIZUKA¹

7 ¹Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Faculty of
8 Veterinary Medicine, Hokkaido University, Japan.

9 ²Water Research Group, Unit for Environmental Sciences and Management, North-West U
10 niversity, Potchefstroom, South Africa

11 ³Chemistry Department, Kwame Nkrumah University of Science and Technology, Ghana

12 ⁴Pharmaceutical Research Institute, Albany College of Pharmacy and Health Sciences

13 ⁵Department of Anesthesiology, Tokyo Women's Medical University Center east, Tokyo, Ja
14 pan

15 ⁶Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto,
16 Kumamoto, Japan

17 ⁷Faculty of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba,
18 274-8510, Japan

19 ⁸Translational Research Unit, Veterinary Teaching Hospital, Faculty of Veterinary
20 Medicine, Hokkaido University, Japan

21 ⁹One Health Research Center, Hokkaido University, Japan

22
23
24
25 *Corresponding Author: Yoshinori IKENAKA

26 Faculty of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo,

27 Hokkaido 606-0818, Japan

28 Tel : +81-11-706-6949

29 Fax : +81-11-706-5150

30 E-mail: y_ikenaka@vetmed.hokudai.ac.jp

31 **Abstract**

32 This study aimed to (i) develop a sensitive method for simultaneous detection and
33 quantification of imidacloprid (IMI) and seven of its metabolites in tissue specimens, and to
34 (ii) determine the biodistribution of the IMI compounds in tissues of C57BL/6J male mice;
35 after exposure to 0.6 mg/kg bw/day of IMI (10% of no observable adverse effect level of
36 IMI) through a powdered diet for 24 weeks. We successfully developed a method which was
37 accurate (recoveries were $\geq 70\%$ for most compounds), sensitive (LODs ≤ 0.47 ng/mL and
38 LOQs ≤ 1.43 ng/mL were recorded for all detected compounds, $R^2 \geq 0.99$) and precise (RSDs
39 $\leq 20\%$) for routine analysis of IMI and seven of its metabolites in blood and various tissue
40 matrices. After bio-distributional analysis, IMI and five of its metabolites were detected in
41 mice. Brain, testis, lung, kidney, inguinal white adipose tissue and gonadal white adipose
42 tissue mainly accumulated IMI, blood and mesenteric white adipose tissue mainly
43 accumulated IMI-olefin; liver mainly accumulated desnitro-IMI; pancreas predominately
44 accumulated 4-hydroxy-IMI. The desnitro-dehydro-IMI and the desnitro-IMI metabolites
45 recorded tissue-blood concentration ratios ≥ 1.0 for testis, brain, lung and kidney. The
46 cumulative levels of the six detected IMI compounds ($\Sigma 6$ IMI compounds) were found in the
47 decreasing order: blood > testis > brain > kidney > lung > iWAT > gWAT > mWAT > liver
48 > pancreas. Altogether, this study provided essential data needed for effective mechanistic
49 elucidation of compound-specific adverse outcomes associated with chronic exposures to
50 IMI in mammalian species.

51 **Keywords:** *Imidacloprid, IMI-olefin, 4-Hydroxy-IMI, N-Desnitro-IMI, 5-Hydroxy-IMI, N-*
52 *Desnitro-4,5-dehydro-IMI.*

53 **1. Introduction**

54 Neonicotinoid insecticides (NNs) stand out as the world's most favorite class of insecticides,
55 used extensively for pest extermination in households, veterinary health care and in
56 agriculture [1]. The global affection for NNs is largely ascribed to their competitive
57 insecticidal potencies, low mammalian toxicity, broad insecticidal spectra, enhanced
58 systemic properties and their high plant compatibilities [2,3,4]. Being neurotoxicants, NNs
59 agonize the nicotinic acetylcholine receptors (nAChRs), expressed within the central nervous
60 systems of both vertebrates and invertebrates [5]. They are known to be effective against
61 different species of biting, chewing and sucking insects.

62 Imidacloprid (IMI; *1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine*;
63 *Fig.1*) is one of the most popular NNs across the world; accounting for about 41.5% of the
64 total neonicotinoid use in the world [2,6]. It is also labeled as the second most widely used
65 insecticide worldwide; with an annual production estimate of 20000 tonnes [7]. As a result
66 of the high global use of IMI, its residues are now pervasive in many environmental
67 compartments. Several studies have detected high residual levels of IMI in environmental
68 matrices such as soil, water, sediments and in human food [8,9,10,11]. Reports from
69 biomonitoring studies have also indicated steady increases in IMI exposure trends among
70 human populations worldwide [12,13,14,15,16,17].

71 Like other NNs, IMI was previously considered to be less toxic to mammalian species, due
72 to its limited binding affinity for the mammalian nAChR. In recent times however, the advent
73 of *in vivo* and *in vitro* studies has disputed this assumption, by revealing significant
74 toxicological potencies of IMI on physiological functioning of key mammalian organs such

75 as brain [18,19], liver [20], adipose tissues [6], ovaries [21], heart and kidney [22]. From
76 pharmacokinetic studies however, IMI is known to have a quick excretion rate in mammalian
77 models (more than 90% of radiolabeled IMI was recovered in rats within 24 h after
78 administration [23]). It is, therefore, perplexing whether the recent IMI-related toxicological
79 outcomes observed in mammalian species are specifically induced by IMI itself or by any of
80 its metabolites.

81 Following enteric absorption, IMI is transformed by phase I and phase II enzymes into
82 various metabolite forms; and each of the metabolites tend to elicit different effects, affinities
83 or potencies towards the nAChRs [2,3,24,25]. For instance, the olefin metabolite of IMI (4,5-
84 dehydro-imidacloprid, IMI-olefin or IMI-ole) is known to be selectively toxic to insects than
85 the parent IMI [25,26]. Also, the desnitro metabolite of IMI (*N*-desnitro-imidacloprid, dn-
86 IMI) agonizes the mammalian nAChRs than the parent IMI compound [27,28]. Conceivably,
87 an *in vivo* evaluation of tissue-specific accumulation trends of all the IMI-related compounds
88 in mammalian species, may provide essential data needed for effective mechanistic
89 elucidation of compound-specific adverse outcomes associated with chronic exposures to
90 IMI in mammalian species.

91 Currently, information on the tissue-specific accumulation of IMI and its associated
92 metabolites within the mammalian system is highly limited. So far, the only study cited in
93 literature, used a single exposure assay to elucidate the tissue-specific distribution of some
94 IMI-compounds in a lizard model [3]. However, the use of such a one-time exposure assay
95 may not reveal the exact tissue-specific accumulation trends associated with chronic low-

96 dose exposures to IMI, as expected in real-life situations. Besides, a reptile model may not
97 be suitable for efficient prediction of IMI distribution, accumulation and excretion in human
98 systems; largely because of existing differences in the xenobiotic excretion pathways
99 between humans (excretion occurs largely through urine and faeces) and reptiles (excretion
100 occurs largely through molting). To this end, the use of a rodent model may offer a better
101 alternative for extrapolating the biodistribution data of IMI into human situations. So far,
102 only a handful of studies have reported on IMI metabolism and its tissue-dependent
103 distribution in rodent models. However, almost all those studies focused on the excretion
104 kinetics and transient tissue distribution patterns of IMI, rather than its long-term
105 accumulation in tissues [26]. The lack of a long-term exposure-related tissue accumulation
106 data on IMI throws a big challenge to scientific advances towards uncovering the mechanistic
107 pathways that underlie the recent IMI-mediated adverse outcomes observed in mammalian
108 models.

109 Determination and quantification of xenobiotics in biological specimens require a precise,
110 reliable and accurate analytical method. Already, some few studies have succeeded in
111 developing methods for imidacloprid analysis in biological specimens; however, those
112 studies focused on urine specimen only [4,13,29]. Besides, all the published IMI-detection
113 methods published were efficient for the detection of only few IMI-related compounds. So
114 far, no method has been established for the simultaneous analysis of multiple IMI-related
115 compounds in different kinds of tissue specimens.

116 Previously, Wang *et al.* [3] and Yang *et al.* [30] optimized an analytical protocol for
117 quantification of IMI and its metabolites in tissues of lizards. However, the protocol was
118 valid for only four IMI compounds. Also, Ford and Casida [31] published a detection
119 technique for quantification of IMI and its metabolites in tissues of mice, however, their
120 method was applicable to only brain and liver. Most of the published detection techniques of
121 IMI compounds [3, 30, 31] lacked robust purification systems. In those methods, tissue
122 extracts were injected directly into HPLC systems, after passing through membrane filters.
123 Due to the complexity of tissue matrices, the lack of appropriated clean-up procedures in
124 such analytical methods may present a considerable amount of matrix interference in tissue
125 analysis of IMI compounds. The incorporation of an SPE clean up procedure in the current
126 method will offer an optimum accuracy for routine analysis of multiple IMI compounds in
127 various tissue specimens.

128 This study aimed to (i) develop rugged, robust and sensitive SPE (solid phase extraction) and
129 LC-MS/MS-based technique for simultaneous detection and quantification of IMI and its
130 metabolites (IMI compounds) in tissues, and to (ii) determine the tissue-specific
131 accumulation trends of IMI and its related metabolites in tissues of C57BL/6J male mice,
132 after a long-term low dose exposure of IMI to the mice. A triple quadrupole LC-MS/MS
133 system was used for this study because of its high sensitivity, good accuracy, low limit of
134 detection (LOD) and low limit of quantification (LOQ).

135 **2. Materials and Methods**

136 **2.1. Materials**

137 Eight (8) IMI compounds; IMI, IMI-olefin, dn-IMI, 4-hydroxy-imidacloprid (4OH-IMI), 5-
138 hydroxy-imidacloprid (5OH-IMI), *N*-desnitro-4,5-dehydro-imidacloprid (dn-dh-IMI), 6-
139 chloronichotinic acid (6-CNA) and 6-chloronichotinic acid-glycine (6-CNA-glycine)
140 (*Fig.1*); and two isotope-labelled internal standards; Imidacloprid-d4 (IMI-d4) and 6-
141 Chloronicotinoic acid-13C6 (6-CNA-13C6), were analyzed in the current study. The IUPAC
142 nomenclature, molar masses and CAS numbers of the target IMI compounds are presented
143 in *Table S2*. IMI was purchased from Kanto Chemical Co., Inc. (Chuo-Ku, Tokyo, Japan);
144 standards of 5OH-IMI, 4OH-IMI, 6-CNA-glycine, dn-IMI and dn-dh-IMI were synthesized
145 in Toho university (Chiba, Japan) [32, 33, 34]; 6-CNA standard was purchased from Wako
146 Pure Chemical Industries (Osaka, Japan); IMI-ole standard was obtained from Supelco
147 (USA); IMI-d4 and 6CNA-13C6 internal standards were purchased from Cambridge Isotope
148 Laboratories, Inc. (Tewksbury, MA, United States). Male C57BL/6J mice, aged 3 weeks
149 were obtained from Sakyo Labo Service Corporation, Inc. (Edogawa-ku, Tokyo, Japan).
150 Powdered diet (D12451M, with red dye) was purchased from Research Diets Inc. (New
151 Brunswick, NJ 08901, USA). All reagents and solvents used in the current study were of
152 HPLC grade; and they were purchased from Kanto Chemical Co., Inc. (Chuo-Ku, Tokyo,
153 Japan).

154 **2.2. *In-vivo* protocol**

155 This animal study was conducted in accordance with the Institutional Animal Care and Use
156 Committee of the Faculty of Veterinary Medicine, Hokkaido University, Japan. The animal
157 experiments were performed in accordance with the Guide for the Care and Use of

158 Laboratory Animals, in conformity with the Association for the Assessment and
159 Accreditation of Laboratory Animal Care International (AAALAC; approval number: 18-
160 0061; validity period: 04/2018 - 03/2023). The animal facility used for the present study was
161 controlled for temperature (20-25 °C), humidity (40-60 °C) and 12-hour light/dark cycle.
162 After 2 weeks of acclimatization, mice were divided into two groups (control-group and IMI-
163 exposed group; 4 mice per group). Mice in the IMI-exposed group were treated with 0.6
164 mg/kg bw/day of IMI (one-tenth of the NOAEL dose of IMI) through a powdered diet for 24
165 weeks. In the control-group, mice were provided with only powdered diet for the 24-week
166 period. In our previous study [20], a chronic (24 week) exposure to 1/10th of the NOEAL of
167 IMI drastically altered the lipid homeostasis of mice. Also, Sun *et al.* [6] found out that an
168 exposure to 1/10th of the NOAEL of IMI increases adiposity and alters glucose metabolism
169 in mice. Hence, 1/10th of the NOAEL of IMI was selected for the current study due to its
170 toxicological significance to mammalian studies. In the present study, diet and water were
171 given to mice ad libitum throughout the experiment; and were provided fresh three times in
172 a week. At the end of the entire study, mice were sacrificed by CO₂ asphyxiation. At necropsy,
173 blood, lung, liver, testis, brain, kidney, pancreas inguinal white adipose tissue (iWAT),
174 mesenteric white adipose tissue (mWAT), and gonadal white adipose tissue (gWAT), were
175 collected and processed accordingly, for chemical analysis.

176 **2.3. Optimization of sample preparation method**

177 **2.3.1. Tissue extraction**

178 The tissue extraction method adopted in the current study was a modification of the protocol
179 published previously by Ohno *et al.* [35] and Nimako *et al.* [20]. The method optimization
180 process of the current study was carried out using mice brain that had undetected levels of
181 all the target chemicals (brain samples from mice in the control group). Specifically, a 10 -
182 15 mg blank brain tissue sample was spiked with 100 μ L of IMI-internal standard master mix
183 containing 5 ng each of IMI-d4 and 6-CNA-13C6. The spiked tissue sample was
184 homogenized in 0.4 mL of 1% formic acid in acetonitrile, using Tissue Lyser (Qiagen GmbH,
185 Hilden, Germany). The homogenized tissue sample was mixed thoroughly by vortex; and
186 centrifuged at 10,000 G for 10 min. Following centrifugation, supernatant from the sample
187 (about 0.5 mL) was carefully separated into fresh 1.5 mL Eppendorf tube and labeled as SP1
188 (supernatant 1). The sample residue was subsequently reconstituted in 0.5 mL of methanol,
189 mixed thoroughly by vortex (2 min) and centrifuged again, at 10,000 G for 10 min.
190 Supernatant from the second extraction was carefully separated as before; and labeled as SP2
191 (supernatant 2). Both SP1 and SP2 were combined, mixed thoroughly to represent the final
192 tissue extract.

193 **2.3.2. Cleanup (Solid-Phase Extraction)**

194 A 0.2 mL of the tissue extract was diluted in 0.6 mL of 3% (v/v) methanol in distilled water
195 (HPLC grade). The diluted extract was purified with the InertSep CBA SPE cartridge (100
196 mg / 1 mL, GL Sciences, Tokyo, Japan), which contained a silica-based sorbent modified
197 with carboxyl ethyl functional groups. The cartridge was connected to the SPE manifold
198 (equipped with a vacuum pump), preconditioned with 2 mL methanol and dried under

199 vacuum for 10 min. The diluted tissue extract was loaded unto the pre-conditioned cartridge
200 and allowed to elute at a rate of 1 mL/min. Eluate was collected into a pre-cleaned translucent
201 test tube (15 mL) and labeled as F1 (fraction-one). The analytes were eluted from the
202 cartridge into F1 with 1 mL of 20% (v/v) acetonitrile in distilled water (HPLC grade), at the
203 same speed as stated previously. The eluate (F1) was evaporated to dryness under gentle
204 stream of nitrogen gas at 60 °C. Finally, the dried sample was reconstituted in 0.2 mL of 20%
205 (v/v) methanol in distilled water (HPLC grade), vortexed for 2 min and transferred into an
206 LC vial for LC–MS/MS analysis.

207 The clean-up process of the brain tissue extracts was optimized by testing (I) three (3)
208 additional SPE sorbents; thus, InertSep PSA cartridge (100 mg / 1 mL, GL Sciences, Tokyo,
209 Japan), InertSep Pharma cartridge (60 mg / 3 mL, GL Sciences, Tokyo, Japan) and InertSep
210 Phospholipid Remover (100 mg / 3 mL, GL Sciences, Tokyo, Japan); and (II) four different
211 SPE elution solvents; thus, 20% (v/v) acetonitrile in distilled water, 50% (v/v) acetonitrile in
212 distilled water, 20% (v/v) methanol in distilled water and 50% (v/v) methanol in distilled
213 water, with the current method for optimal purification and elution efficiencies (n=3)

214 **2.4. Instrumental analysis**

215 The sample analysis was carried out using a triple quadrupole LC-/MS/MS system (Agilent
216 6495B, Agilent Co., CA, USA). The LC system (1290 Infinity II, Agilent) was equipped with
217 a binary LC pump, a vacuum degasser, an autosampler and an oven. A gradient system of
218 0.1% formic acid + 10 mM ammonium acetate in; water (solvent A) and methanol (solvent
219 B) were chosen for routines as follows: t = 0–1 min. 5% B (isocratic), t = 6 min: 95% B

220 (gradient), $t = 6\text{--}8$ min (gradient): 95% B (isocratic). The column oven temperature was set
221 at 60 °C and the total mobile phase was pumped at a flow rate of 0.35 mL/min through a
222 Kinetex Biphenyl column (2.1 mm ID \times 150 mm, ϕ 1.7 μm ; Phenomenex, Inc., CA, USA).
223 The volume of each sample injected into the mobile phase flow was 10 μL .

224 The triple quadrupole mass spectrophotometer was equipped with an electrospray ionization
225 (ESI) interface; the IMI compounds in the column eluents were quantified with a triple
226 quadrupole mass filter. The sheath gas temperature and flow rate were 350 °C and 12 L/min
227 respectively. The drying gas temperature, drying gas flow rate and nebulizer pressure were
228 set at 210 °C, 17 L/min and 25 psi respectively. The capillary voltage was set at 3500 V for
229 positive detection mode and 3000 V for the negative detection mode. The ion signals were
230 acquired with multiple-reaction monitoring (MRM) in positive ionization mode; the selected
231 m/z ions for the all the IMI compounds considered in the current study have been shown in
232 *Table S1*. The MS/MS operating conditions were set in accordance with the manufacturer`s
233 instructions and the data acquisition and processing were carried out using the MassHunter
234 Workstation software (Agilent Technologies).

235 **2.5. Method Validation**

236 The current method was validated by evaluating method accuracy, matrix effects, linearity,
237 inter-day and intra-day precisions, freeze-thaw stability, limit of detection (LOD) and the
238 limit of quantification (LOQ). These quality assurance and quality control measures were
239 adopted based on guidelines for method performance recommended by the SANTE standard
240 (SANTE/11945/2019 [36])

241 Method accuracy was assessed by estimating the recoveries of matrix-matched standards of
242 the target chemicals. Blank brain tissues (obtained from the in-vivo experiment) were spiked
243 with working standard solutions containing all the target compounds and IMI internal
244 standards at 3 concentration levels; a low concentration (2.5 ng/mL; n=3), a medium
245 concentration (5.0 ng/mL; n=3) and a high concentration (10.0 ng/mL, n=3). In order to
246 normalize the influence of ME on method accuracy, recoveries of target chemicals that had
247 isotopically labelled standards (IMI and 6-CNA) were estimated by comparing analyte/IS
248 peak area ratios (37). Recoveries of target chemicals without internal standards were
249 estimated by comparing peak areas of matrix-matched standards to peak areas of post matrix-
250 matched standards.

251 Matrix effects were assessed by post-extraction matrix-spikes (tissue extracts were spiked
252 with 2.5 ng of the standard of each target compound, just before instrumental analysis); and
253 by comparing response of the target chemicals against response of their respective standards
254 prepared in 20% (v/v) MeOH in distilled water (n=3).

255 Linearity of the method was determined using matrix-matched calibration curves plotted
256 from nine different concentrations (0.03 to 21.87 ng/mL). The calibration plots were
257 subjected to linear regression analysis to obtain regression coefficients (r^2).

258 The LODs and LOQs were estimated from analysis of matrix matched standards as values at
259 which the signal-to-noise ratios were 3 and 10, respectively. The ratios of the analyte signal
260 to the noise in chromatogram were determined with the MassHunter Workstation software
261 (Agilent Technologies).

262 For Intra-day precision, blank brain samples were fortified with the target chemicals at 2.5,
263 5 and 10 ng/mL; and this was analyzed in five different batches (n=3). The inter-day precision
264 was also examined at 5 ng/mL for 4 successive days (n=3, a single measurement per day).
265 Both intra-day and inter-day precisions were estimated as percent standard deviations (%
266 RSD; [4]).

267 The freeze-thaw stabilities of the target IMI compounds were evaluated according to the
268 method described previously by Ueyama et al. [37].

269 **2.6. Method variability**

270 To ascertain conditions that may affect variability of the current method, we evaluated the
271 relative instrumental response of the target IMI compounds in each of the following steps (I)
272 three different tissue weights (10 mg, 20 mg and 50 mg) were tested to validate the tissue
273 weight-dependency of the method accuracy, (II) eight different biological matrices (blood,
274 brain, white adipose tissue, liver, lung, testis, pancreas and kidney) were tested with the
275 method to evaluate the effects of matrix variability on the method accuracy (n=3).

276 **2.7. Application of method for tissue distribution analysis of imidacloprid** 277 **compounds**

278 Following the 24-week IMI exposure, blood, liver, testis, brain, kidney, pancreas and adipose
279 tissues (including inguinal, mesenteric and gonadal white adipose tissues) were harvested
280 from mice and stored at -20 °C until analysis. Tissue concentrations of IMI and its
281 metabolites were determined by the method developed and optimized in the present study.
282 Four (4) types of matrix-matched calibration curves were employed to quantify the target

283 chemicals in various tissue specimens. (I) Brain matrix-matched samples were used to
284 quantify the IMI compounds in brain, adipose tissue, and liver (these matrices showed similar
285 matrix effect tendencies the target chemicals), (II) Pancreas matrix-matched samples were
286 used to quantify all compounds in pancreas, lung, and kidney (these matrices showed similar
287 matrix effect tendencies the target chemicals), (III) Testis matrix-matched samples were used
288 to quantify all compounds in testis, and (IV) Blood matrix-matched samples were used to
289 quantify all compounds in blood.

290 **2.8.Statistical Analysis**

291 Data from the present study were statistically analyzed using JMP Pro13 (SAS institute,
292 USA). The data from all the experimental groups were tested for normality using the Shapiro-
293 Wilk test; and for homogeneity of variance using the Levene's test. Figures were plotted
294 using JMP Pro13 or Microsoft excel 2016 (version: 16.0.12527.21378) 64-bit.

295

296 **3. Results and Discussion**

297 **3.1.Optimization of SPE sorbents**

298 Efficiencies of 5 SPE cartridges; cation exchange (InertSep SCX and InertSep CBA), anion
299 exchange (InertSep PSA) and neutral (InertSep Pharma and InertSep Phospholipid Remover),
300 were evaluated for purification of the target IMI compounds in tissue extracts; and the results
301 have been shown in *Fig.2*. Purification efficiencies of the cartridges were evaluated based on
302 recoveries and matrix effects (ME) obtained from analysis of matrix-matched standard
303 solutions containing the target chemicals.

304 Of the 5 SPE cartridges tested, the InertSep CBA and InertSep Pharma recorded best
305 purification efficiencies for all the compounds (both cartridges had recoveries of more than
306 70% for all the target compounds). However, elution with InertSep Pharma cartridge
307 produced higher matrix effects for most target compounds (ME range; from -11% of dn-dh-
308 IMI to -96% of IMI), compared to that of InertSep CBA (ME ranged; -15% of dn-dh-IMI to
309 -51% of IMI-olefin). The PSA recorded good recoveries of more than 70% for six out of the
310 eight (6/8) compounds (ME ranged; -18% of dn-IMI to -66% of IMI-olefin), InertSep
311 Phospholipid Remover recorded recoveries above 70% for five out of the eight (5/8) target
312 compounds (ME ranged; -15% of dn-IMI to -75% of IMI), SCX recorded acceptable
313 recoveries (> 70%) for four out of eight (4/8) of the target compounds (ME ranged; 11% of
314 4OH-IMI to -39% of IMI)

315 Most NN parent compounds are hydrophilic and electrically neutral [4]. However, NN
316 metabolites may differ from parent compounds in terms of charge densities, largely because
317 of structural and functional differences. For instance, the 6-CNA metabolite of IMI is acidic
318 and hence, may assume anionic charges when ionized, but the parent IMI is neutral in
319 solutions. Due to possible charge differences between IMI and its metabolites, designing a
320 simultaneous purification technique for all the metabolites of IMI could be challenging.

321 In the present study, we employ an ion exchange SPE with a trapping strategy (reverse phase
322 SPE). We selected ion exchange SPE cartridges which had the potential to trap impurities,
323 while allowing the target chemicals to elute. Prior to sample elution however, the tissue

324 extracts were diluted in DW to reduce viscosity and to facilitate elution of analyte through
325 the sorbents.

326 The InertSep CBA, a weak cation exchanger was highly effective in trapping most impurities;
327 and this was evident in the relatively low matrix effects obtained in relation to the CBA
328 elution. At the same time, CBA (weak cation exchanger) exhibited low binding affinity for
329 the target IMI compounds; and this resulted in high accuracies for all the target compounds.
330 In contrast, the strong cation exchanger, InertSep SCX, trapped four of the target compounds
331 (dn-IMI, dn-dh-IMI, 4OH-IMI and 5OH-IMI) along with impurities. Perhaps, dn-IMI, dn-
332 dh-IMI, 4OH-IMI and 5OH-IMI had high binding affinities for strong cation exchangers. As
333 such, SCX was considered unsuitable for the trapping SPE strategy employed in the current
334 method.

335 The anion exchanger (InertSep PSA) permitted the elution of majority of the compounds
336 (6/8); however, 6-CNA and 6-CNA-glycine were trapped in PSA. The 6-CNA and its glycine
337 conjugates have acidic functionalities; and hence may exhibit anionic tendencies in solutions.
338 The PSA cartridge probably trapped these metabolites through anion exchange interactions.

339 Although the neutral cartridge, InertSep Pharma, had good elution for all the target
340 compounds, the matrix effects obtained from its associated clean-ups were mostly higher
341 than those of other cartridges. This suggests that the neutral cartridge, Pharma, had lower
342 efficiency for trapping most impurities in tissue extracts; compared to the anion and cation
343 exchange cartridges. InertSep Phospholipid Remover is highly selective for removal of
344 phospholipids from tissue extracts; and this is helpful for minimizing ion suppression in

345 LC/MS/MS analysis. In the present study however, InertSep Phospholipid Remover was
346 found to trap 4OH-IMI, 5OH-IMI and 6-CNA along with tissue phospholipids; and this
347 probably resulted into low recoveries for these compounds. As a result of the selective
348 binding affinities of Phospholipid Remover for the target IMI compounds, it was considered
349 unsuitable for the current purification strategy.

350 **3.2. Optimization of SPE elution solvents**

351 Four aqueous organic solvents, thus; 20% (v/v) MeOH, 50% (v/v) MeOH, 20% (v/v) ACN
352 and 50% (v/v) were tested to determine their elution efficiencies for the target IMI
353 compounds, using the InertSep CBA and InertSep SCX cartridges (*Fig. S1*). Out of all the
354 solvents tested, 20% (v/v) ACN and 50% (v/v) MeOH showed the highest elution efficiencies
355 for the target compounds. In the current method however, 20% (v/v) ACN was considered
356 most suitable for elution of the target compounds, due to its likelihood to present lesser matrix
357 interferences in LC-MS/MS analysis, as compared to methanol. Phospholipids in tissue
358 extracts are known to be less soluble in acetonitrile, compared to methanol; as a result,
359 acetonitrile has been found to be more effective in reducing MS signals than methanol [38].

360 **3.3. Method validation**

361 **3.3.1. Accuracy**

362 Accuracy estimation at three concentration levels (2.5, 5 and 10 ng/mL) yielded recoveries
363 of more than or equal to 70% for all the target compounds (*Fig.S2*). Moreover, all the target
364 IMI compounds recorded recoveries within the range of 70 - 120% in all the mice tissues
365 considered in the current study, except for lung (*Fig 3A*). According to the

366 SANTE/11945/2019 guidelines, method performance is deemed acceptable if mean
367 recoveries fall within the range of 70-120%, with an associated repeatability $RSD \leq 20\%$. By
368 inference, recoveries obtained in the current study are suggestive that, the current method
369 had appropriate accuracy for detection and quantification of the target IMI-compounds in
370 most tissues considered in the present study. In lung, dn-dh-IMI, IMI-olefin, 5OH-IMI and
371 6-CAN-glycine had recoveries of 49%, 49%, 65% and 66% respectively (Fig 3A), however
372 these recoveries were recorded with good precisions ($RSD \leq 20\%$). In exceptional cases, the
373 SANTE/11945/2019 guideline gives room for acceptance of recovery rates that are within
374 the range of 30-140% (thus; recovery rates outside the range of 70-120%), provided they are
375 consistent ($RSD \leq 20\%$). This suggests that the current method had acceptable accuracy for
376 quantification of dn-dh-IMI, IMI-olefin, 5OH-IMI and 6-CAN-glycine in lungs as well.

377 When different tissue weights (10, 20 and 50 mg) were tested with the current method,
378 recoveries of all the target compounds were consistently found within acceptable range (70-
379 120%), regardless of the weight considered (Fig. S3). However, ion suppressions of the target
380 compounds were found to increase with increasing tissue weights (*Fig. S3*). These suggests
381 that the current method may produce optimum accuracies with minimum interferences, when
382 tested with low amounts of tissue specimens.

383 **3.3.2. Precision**

384 The intra-day precision estimated from the fortified tissue samples at 2.5, 5 and 10 ng/mL
385 yielded $RSDs \leq 20\%$ for all the target IMI compounds (*Table 1*). Moreover, inter-day
386 precision analysis yielded $RSDs < 20\%$ for all the target chemicals (*Table 1*). These results

387 suggest that the current method had high consistency for detection and quantification of
388 majority of the IMI compounds considered in the present study.

389 Meanwhile, the freeze and thaw stability evaluation yielded RSDs $\leq 20\%$ for IMI, IMI-ole,
390 5OH-IMI and 6-CNA-glycine; and RSDs $\geq 20\%$ for dn-dh-IMI, dn-IMI, 4OH-IMI and 6-
391 CNA (*Table 1*). These suggest that, beside IMI, IMI-ole, 5OH-IMI and 6-CNA-glycine, other
392 IMI compounds may not exude adequate stabilities when subjected to continuous freezing
393 and thawing cycles. A chromatogram of tissue-fortified samples has been shown in *Figs.4*.
394 The peaks of all the target IMI compounds were resolved; and they were without
395 interferences.

396 **3.3.3. Matrix Effect**

397 In the current study, matrix effects (MEs) of the target compounds were evaluated in all
398 tissues *Fig 3B*. From the results, dn-IMI and dn-dh-IMI were found with the least MEs in all
399 the tissues (median MEs were -6.1% and -10.8% respectively). According to
400 SANTE/11945/2019 guidelines, analytes with MEs (%) within the range of $\pm 20\%$ are less
401 susceptible to interferences during LC-MS/MS analysis. This suggest that dn-IMI and dn-
402 dh-IMI suffered from minimal matrix suppression; and hence can be quantified by solvent
403 calibrations, via the current method. Other IMI compounds targeted in the current study
404 recorded MEs beyond the range of $\pm 20\%$. Median MEs of IMI, 6-CAN, IMI-olefine, 5OH-
405 IMI, 4OH-IMI and 6-CNA-glycine recorded in all the target tissues were -27.0%, -30.0%, -
406 33.5% -44.8%, -64.3% and -63% respectively. This suggests that, apart from dn-IMI and dn-
407 dh-IMI, all the target IMI compounds suffered from matrix inhibition during the instrumental

408 analysis. Matrix effects in analytical measurements are mainly caused by ion suppression and
409 ion enhancement [39]. According to Ly *et al.*, [39], matrix effects in LC-MS/MS are more
410 dependent on co-eluting components rather than the properties of an analyte. Hence this may
411 be minimized by employing either isotopically labeled standards, matrix matched calibration
412 curves or sample dilution in analytical measurements [39, 40, 41]. In the current study, both
413 isotopically labeled standards and matrix matched calibrations curves were employed to
414 normalize MEs during instrumental analysis.

415 **3.3.4. Linearity**

416 Matrix-matched calibration curves were plotted for various IMI compounds within the
417 calibration range of 0.03 to 21.87 ng/mL. Linear responses and correlation coefficients of the
418 target IMI compounds have been shown in *Table 1*. Linearity was considered acceptable, if
419 a response yielded correlation coefficient greater than or equal to 0.99 [40]. Results from the
420 present study revealed regression coefficients that were greater than 0.99 for all the IMI
421 compounds considered in this study. This means that the current method produced excellent
422 linear relationships for all the IMI compounds considered in the present study.

423 **3.3.5. LOD and LOQ determination**

424 Based on the signal-to-noise ratios of the matrix-matched calibration standards, we evaluated
425 the method LODs and LOQs for all the target IMI compounds; and the results have been
426 shown in *Table 1*. The results indicate LODs ranging from 0.06 ng/mL to 0.47 ng/mL and
427 LOQs ranging from 0.17 ng/mL to 1.43 ng/mL for the target IMI compounds. These results

428 give strong indication that the current method had high sensitivities towards the detection
429 and quantification of all the IMI compounds considered in this study.

430

431 **3.4. Tissue distribution/accumulation of IMI and its metabolites**

432 We applied the method developed in the current study to determine the
433 distribution/accumulation trends of IMI and its metabolites in blood and tissues of C57BL/6J
434 male mice, following a 24-week exposure to a low dose (less than the NOAEL dose) of IMI
435 (*Figs. 5 and 6A; Table S3*). The tissues, considered in the present study include lung, liver,
436 kidney, brain, pancreas, testis, mesenteric white adipose tissue, inguinal white adipose tissue
437 and gonadal white adipose tissue.

438 **3.4.1. Biodistribution of IMI**

439 The parent IMI compound was detected in blood and all the target tissues, except for the liver
440 (*Fig. 6A*). The blood and tissue accumulation trends of IMI was found in the decreasing order;
441 Testis > blood > brain > lung > kidney > iWAT > gWAT > mWAT > pancreas > liver (*Fig.*
442 *5*). From pharmacokinetic studies, IMI is known to undergo rapid oral absorption in
443 mammalian systems; with a consequential distribution of its residues into various organs and
444 tissues [42]. Perhaps, IMI elicited a quick enteric absorption into blood; and this might have
445 facilitated its distribution into the organs and tissues considered in the current study model.
446 The testis-blood concentration ratio of IMI was 1.0 (Table 2), suggesting that the testis
447 accumulated a significant amount of IMI in the current experimental model. Meanwhile, the
448 tissue-blood concentration ratios of IMI obtained for the other target chemicals in tissues

449 were all below 1.0 (Table 2). This suggests that the tissue distributional patterns of IMI
450 observed in the current mice model was mainly instigated by a high exchange of blood
451 between the target organs.

452 The high IMI accumulation trend in testis observed in the current study agrees with findings
453 from a previous study which demonstrated significant distribution of IMI residues within
454 lizard gonads [3, 30]. However, the brain accumulation pattern of IMI observed in the current
455 rodent model contradicts previous findings from a reptile model [3]. Whereas the current
456 study revealed a significant accumulation levels of IMI in mice brains, findings from Wang
457 *et al.*'s study [3] showed very low residual levels of IMI in brains of lizards, after a single
458 oral exposure to IMI. This contrasting observation might be due to specie-specific differences.

459 In the present study, IMI was not detected in the liver of the exposed mice probably because
460 of its rapid metabolic transformation within the liver. Wang *et al.*, [3] also detected very low
461 residues of IMI in liver of lizards, following a one-time oral exposure to IMI. Meanwhile,
462 the current experimental model recorded appreciable amounts of IMI in mice adipose tissues
463 (iWAT, gWAT, mWAT, *Fig. 6A*). These observations indicate that, although IMI is less
464 lipophilic, significant amount of its residues may remain distributed in various adipose depots
465 within the mammalian system, especially under conditions of long-term persistent exposures.
466 In contrast, Wang *et al* [3] detected insignificant levels of IMI in fat, following a single oral
467 exposure of IMI to lizards. This contrasting observation was probably because of the short-
468 term exposure duration employed in their study and/or because of species-specific
469 differences.

470 **3.4.2. Biodistribution of IMI metabolites**

471 Imidacloprid is primarily metabolized in mammalian liver either by CYP450-mediated
472 imidazolidine hydroxylation or via aerobic nitroreduction by the molybdo-flavoenzyme
473 aldehyde oxidase (AOX) [4, 5 31, 43]. In the present study, key metabolites of IMI such as
474 4OH-IMI, 5OH-IMI, IMI-ole, dn-IMI and dn-dh-IMI were detected in blood and tissues of
475 the IMI exposed mice (*Figs. 5 and 6A; Table S3*). IMI-ole occurred as the most predominant
476 metabolite followed by 4OH-IMI, then dn-IMI, with 5OH-IMI and dn-dh-IMI being the least
477 recalcitrant in the tissues (*Figs. 5*).

478 **3.4.2.1. Biodistribution of IMI metabolites within the hydroxylation pathway**

479 The imidazolidine hydroxylation pathway is known to be more prolific in IMI metabolism;
480 and its activation transforms IMI into more water-soluble moieties such as 4OH-MI and
481 5OH-IMI. In the present study, 4OH-IMI and 5OH-IMI metabolites were highly detected in
482 blood, kidney, lung and testis, but their levels in liver were very low (*Fig. 5*). The momentous
483 detection of 4OH-IMI and 5OH-IMI in the present study confirms the CYP450-mediated
484 imidazolidine hydroxylation pathway as a prominent rout of IMI metabolism in mammalian
485 species. Interestingly, very low residual amounts of 4OH-IMI and 5OH-IMI metabolites
486 were detected in liver, compared to other tissues (*Figs. 5 and 6A*). This tendency might be
487 due to the spontaneous non-metabolic transformation of 4OH-IMI and 5OH-IMI into the
488 IMI-ole metabolite.

489 Imidacloprid-olefin is a desaturated secondary metabolite of 4OH-IMI and 5OH-IMI; and it
490 occurs as the commonest metabolite of IMI found in mammalian species [5, 25]. This

491 explains why IMI-ole was recalcitrant metabolite in blood and most tissues considered in the
492 present study (testis, brain, lung, iWAT, gWAT and mWAT; *Figs. 5 and 6A*). Wang *et al* [3]
493 also found IMI-ole as the predominant metabolite of IMI in lizard tissues. In current
494 experimental model IMI-ole detected with the least concentration in liver (*Fig. 6A*). This
495 might be due to the metabolic conversion of IMI-ole into dn-dh-IMI in the liver. The dn-dh-
496 IMI metabolite of IMI is considered as a terminal product of the IMI hydroxylation pathway,
497 which is formed as a result of hepatic nitroreduction of IMI-ole (*Fig. 1*). The levels of dn-dh-
498 IMI in the liver was found to be far higher than its levels detected in all the other tissues (*Figs.*
499 *5 and 6A*), confirming dn-dh-IMI as a terminal metabolite within the IMI hydroxylation
500 pathway. The dn-dh-IMI metabolite specifically accumulated in liver, lung, testis and kidney
501 of the current mice model (tissue to blood concentration ratios were 3.8, 3.1, 2.0 and 1.7
502 respectively, *Table 2*)

503 **3.4.2.2. Biodistribution of IMI metabolites within nitroreduction pathway**

504 Nitroreduction of IMI presents an alternative pathway for IMI metabolism in mammalian
505 species. Hepatic activation of this pathway by AOX triggers series of metabolic events which
506 eventually yields the dn-IMI metabolite of IMI [5, 31, 43]. In the present study, dn-IMI
507 showed significant accumulation in liver, brain, testis, lung and kidney of mice (tissue to
508 blood concentration ratios were 1.8, 1.5, 1.4, 1.3 and 1.2 respectively; *Table 2, Fig 5*). From
509 a toxicological point of view, the specific accumulation of dn-IMI in organs such as brain
510 and testis, generate serious concerns, in that, the dn-IMI metabolite is known to elicit a
511 nicotinic-type action, with a markedly higher toxicity to mammals than the parent IMI.

512 The dn-IMI metabolite is known to serve a major precursor for prominent IMI metabolites
513 such as 6-CNA and its glycine conjugates; thus, 6-CNA-glycine (*Fig.1*, [5]). In the current
514 study, the 6-CNA and 6-CNA-glycine were not detected in the mice model presumably
515 because of the limited feasibility of the nitroreduction metabolic pathway of IMI.

516 **3.4.3. Cumulative levels of IMI and its metabolites in mice tissues**

517 Total accumulation levels of the IMI compounds in mice tissues were estimated by the
518 summation of mean concentrations of all the detected IMI compounds ($\Sigma 6$ IMI compounds)
519 in each tissue (*Fig.6B*). The cumulative levels of the target IMI compound ($\Sigma 6$ IMI
520 compounds) in blood and tissues occurred in the decreasing order; blood > testis > brain >
521 kidney > lung > iWAT > gWAT > mWAT > liver > pancreas (*Fig.6B*). This result suggests
522 that organs such as testis, iWAT and kidney may be most susceptible to cumulative effects
523 of IMI and its metabolites, after a chronic low-dose exposure to IMI in mammalian species.

524 **3.4.4. Interplay between the current mice study and human studies**

525 Despite the widespread use of IMI, its detected levels in most human studies are relatively
526 low, compared to levels of other NNs reported in biomonitoring studies [17, 37, 44, 45, 46,
527 47, 48]. In a pharmacokinetic study, Harada *et al.* [29] detected only a small percentage of
528 IMI in human urine (about 12.7%), after intentional ingestion of micro doses of deuterium-
529 labeled IMI by human subjects. These observations suggest that, a greater proportion of IMI,
530 probably undergoes transformation into other metabolite forms in the human body. Results
531 from the present study has given a clear evidence that, most metabolites of IMI produced in
532 human system, may accumulate in different organs and tissues within the body.

533 Many biomonitoring studies have already detected IMI metabolites such as 5OH-IMI and 6-
534 CNA in human urine [44, 45, 46, 47, 48, 49, 50]. However, the detection frequency of 5OH-
535 IMI reported in human subjects was very low (about 19.7%) [44]. Similarly, the
536 accumulation levels of 5OH-IMI detected in the present study were found to be relatively
537 low, compared to the levels of most of the other IMI-compounds detected in the current
538 experimental model.

539 Although 6-CNA has been detected in some human studies [45, 47, 49, 50], its accumulation
540 levels in the current mice model were below LOQ. The inconsistency may be attributed to
541 the species-difference in IMI metabolism. This, however, brings to bear, the limitations that
542 might be associated with extrapolation of mice study to humans. Nonetheless, recent
543 biomonitoring studies have suggested that the 6-CNA metabolite may not be a dependable
544 biomarker of IMI exposure in mammalian species, due to its frequent occurrence in metabolic
545 pathways of several chemicals, including IMI, ACE, THI, NIT and cyclozaprid [47].

546 Recently, Wang *et al.* [48] detected dn-IMI and IMI-ole in human urine with high detection
547 frequencies, high concentrations, high specificities and good inter-day reliabilities. Based on
548 these findings, Wang *et al.* [48] projected IMI-ole as potential biomarker of IMI exposure in
549 human populations. In the present animal model, the IMI-ole metabolite of IMI was
550 predominately detected in blood and most of the target tissues. This probably validated Wang
551 *et al's* projection of IMI-ole as a potential marker of IMI exposures in human subjects.

552

553 4. Conclusion

554 We developed an accurate, precise and a sensitive LC-MS/MS-based method for routing
555 analysis of multiple IMI-related compounds in tissue specimens. We thence, applied the
556 method to determine the tissue-specific accumulation trends of IMI and its metabolites in
557 C57BL/6J male mice, following a long-term exposure to a less than the NOAEL (the no-
558 observed adverse-effect level) level) of IMI. To the best of our knowledge, this is the first
559 study to establish an analytical protocol for detection and quantification of multiple IMI
560 compounds in tissue specimens. Moreover, this study is the first to provide a basic tissue
561 distribution and/or accumulation data associated with chronic low-dose exposures to IMI in
562 mammalian species. In the current study, the Biodistributional trends IMI compounds in
563 organs such as the heart, adrenal glands, stomach, etc. were not elucidated. We therefore
564 recommend that further studies should be done to evaluate the fate of IMI in these organs,
565 under chronic low-dose exposure situation.

566

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584

585 **Disclaimer**

586 The authors declare no conflict of interest in the present study.

587 **Data Accessibility**

588 Data, associated metadata, and calculation tools are available by contacting the
589 corresponding author (y_ikenaka@vetmed.hokudai.ac.jp).

590

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741 [ca.gov%2Fdocs%2Frisk%2Frcd%2Fimidacloprid.pdf&usg=AOvVaw2wFLrnlwK3](https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&ved=2ahUKEwjO_pK1m-_vAhVWc3AKHYACDwkQFjAAegQIBRAD&url=https%3A%2F%2Fwww.cdpr.ca.gov%2Fdocs%2Frisk%2Frcd%2Fimidacloprid.pdf&usg=AOvVaw2wFLrnlwK39QbaYxwrv8YY)
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785 **Table 1:** Precision, Freeze-thaw stabilities, Linearity, limit of quantification (LOQ) and Limit of detection (LOD) of the
 786 analytical procedure

	Intra-day precision (%RSDs)			Inter-day precision (%RSDs)	Freeze-thaw Stability (%RSDs)		Linearity	LOD (ng/mL)	LOQ (ng/mL)
	2.5	5	10		2.5	10			
	ng/mL	ng/mL	ng/mL		ng/mL	ng/mL			
IMI	4.7	4.3	3.5	7.8	3.4	9.1	0.999	0.12	0.37
IMI-d4	7.4	6.9	7.4	7.3	4.8	5.6	0.997	***	***
dn-dh-IMI	2.8	4.7	3.7	3.4	23.2	28.6	0.999	0.07	0.22
dn-IMI	4.2	3.6	4.7	4.9	25.9	26.5	0.999	0.06	0.17
IMI-ole	4.0	3.1	5.9	5.6	21.1	16.3	0.999	0.13	0.41
4OH-IMI	6.6	3.3	7.4	7.0	5.8	3.5	0.999	0.28	0.85
5OH-IMI	3.4	3.4	3.9	8.6	0.9	7.2	0.999	0.10	0.29
6-CNA	3.7	4.7	3.5	13.0	29.8	27.2	0.999	0.21	0.64
6-CNA-13C6	3.5	7.7	5.1	10.0	19.5	19.8	0.999	***	***
6-CNA-glycine	5.8	6.9	5.4	9.7	2.8	8.8	0.997	0.47	1.43

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791 **Fig.1:** Metabolic pathways for imidacloprid [5,42].

792 **Fig. 2A&B:** (A) Recoveries and, (B) matrix effects of imidacloprid and its metabolites
793 obtained from sample purification with various SPE sorbents. The InertSep SCX cartridge is
794 a silica-based sorbent modified with benzene sulfonyl propyl functional groups; InertSep
795 CBA cartridge is a silica-based sorbent modified with carboxyl ethyl functional groups;
796 InertSep PSA is a silica-based sorbent modified with an ethylene-diamine-N-propyl
797 functional groups; InertSep GC/PSA is a two-layer cartridge, packed with graphite carbon
798 for removing pigments and NH₂ or PSA sorbent for sample cleanup of organic extracts;
799 InertSep Pharma is a copolymer-based sorbent comprised of nitrogen-containing
800 methacrylate and SDB.

801 **Figs. 3A & B:** (A) Recoveries and (B) matrix effects of imidacloprid compounds obtained
802 by InertSep CBA purification of various tissue matrices.

803 **Figs. 4:** Chromatogram showing peaks of imidacloprid and its metabolites in matrix-
804 matched standard working solutions containing all the compounds. Tissue extracts were
805 purified with InertSep CBA. LC Agilent 1290 Infinity II, MS: Agilent 6495 Triple Quad
806 LC/MS, Column: Kinetex 1.7µm Biphenyl (2.1×150 mm) (Phenomenex); Mobile phase A:
807 0.1% Formic acid + 10mM Ammonium acetate in DW, Mobile phase B: 0.1% Formic acid
808 + 10mM Ammonium acetate in MeOH, Flow: 0.35ml/min, Oven: 60°C, gradient: 0-
809 1.5min, B conc 20% -> 1.5-6min, B conc 95% -> 6-7min, B conc 95% -> 7-7.01min, B
810 conc 20% -> 9min end

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812 **Fig. 5:** Bioaccumulations trends of IMI and its metabolites in mice. Figure was plotted from
813 mean contraptions of the target chemicals in mice (n=4). iWAT means inguinal white adipose
814 tissue, mWAT means mesenteric white adipose tissue, gWAT means gonadal adipose
815 tissue.

816 **Fig. 6A&B:** (A) Tissue-specific accumulation pattern of IMI and its metabolites in mice
817 (Graph was plotted from mean contraptions of the target chemicals in mice, n=4). (B) Sum
818 of mean concentrations of 6 IMI compounds (Σ 6 IMI compounds). iWAT means inguinal
819 white adipose tissue, mWAT means mesenteric white adipose tissue, gWAT means gonadal
820 adipose tissue.

821 **Fig. S1:** Optimization of SPE elution solvent. Elution efficiencies of solvents were judged
822 based on recoveries of matrix-matched standards containing the target IMI compounds. 20%
823 MeOH; 20% methanol in distilled water, 50% MeOH; 50% methanol in distilled water, 20%
824 ACN; 20% acetonitrile in distilled water, 50% ACN; 50% acetonitrile in distilled water.

825 **Fig. S2:** Estimation of Method accuracy in three different concentrations (low, medium,
826 and high concentrations of target chemicals).

827 **Fig. S3:** Tissue weight effects on the method accuracy (Recoveries and matrix effects of
828 the target chemicals were estimated using three different tissue weights)

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838 **Supplementary Data**839 **Table S1:** Compound-specific mass spectrometer setting.

Compound	Fragmentor (v)	Collision Energy (eV)	Precursor ion (m/z)	Product ion (m/z)	Retention time (min)
Imidacloprid	380.0	16.0	256.1	175.1	5.3
Imidacloprid-d4	380.0	24.0	260.1	179.1	5.3
4OH-Imidacloprid	380.0	16.0	272.1	191.2	4.6
5OH-Imidacloprid	380.0	20.0	272.1	191.2	4.7
6-Chloronicotinic acid	380.0	20.0	158.0	122.3	3.9
6-Chloronicotinic-13C6	380.0	68.0	164.1	55.0	3.9
6-Chloronicotinic-glycine	380.0	20.0	215.2	169.1	3.5
dn-Imidacloprid	380.0	24.0	211.2	126.1	3.9
dn-dh-Imidacloprid	380.0	20	209.0	126.2	3.7
Imidacloprid-ole	380.0	16	208.2	126.1	4.8

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846 **Table S2:** Common name, IUPAC names, CAS numbers and molar masses of the target chemicals

Common name	IUPAC name	CAS number	Molar mass (g/mol)
Imidacloprid	1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine	105827-78-9	255.66
Desnitro-imidacloprid	1-[(6-chloropyridin-3-yl)methyl]imidazolidin-2-imine	-	210.67
Desnitro-dehydro-imidacloprid	N-desnitro-4,5-dehydro-imidacloprid	-	208.67
Imidacloprid-olefin	1-[(6-Chloro-3-pyridinyl)methyl]-N-nitro-1H-imidazol-2-amine	115086-54-9	253.65
4OH-imidacloprid	4-hydroxy-imidacloprid	-	271.66
5OH-imidacloprid	5-hydroxy-imidacloprid	-	271.66
6-CNA	6-Chloronicotinic acid	5326-23-8	157.55
6-CNA-glycine	6-chloronichotinic acid-glycine	-	214.55

847 - Not applicable

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857 **Table S3:** Mean concentrations of (ng/g or ng/mL) or imidacloprid and its metabolites in mice tissues (n=4)

	Pancreas	Liver	mWAT	gWAT	iWAT	Lung	Kidney	Brain	Testis	Blood
Imidacloprid	BDL	0.06±0.01	0.54±0.20	1.10±0.10	1.47±0.25	2.30±0.41	1.70±0.43	3.38±1.15	3.73±0.69	3.69±0.67
dh-dn-Imidacloprid	0.02±0.00	0.12±0.04	0.03±0.00	0.02±0.00	0.03±0.01	0.10±0.07	0.05±0.01	0.03±0.01	0.06±0.05	0.03±0.00
dn-Imidacloprid	0.04±0.01	0.31±0.02	0.08±0.01	0.06±0.01	0.13±0.02	0.22±0.17	0.21±0.04	0.26±0.17	0.24±0.04	0.17±0.04
Imidacloprid-olefin	BDL	BDL	0.62±0.15	0.64±0.13	1.44±0.41	0.04±0.00	0.97±0.37	0.71±0.10	2.69±0.58	4.60±0.69
4OH-Imidacloprid	0.10±0.00	BDL	0.36±0.02	0.21±0.02	0.35±0.08	0.28±0.05	1.33±0.30	0.28±0.09	0.60±0.20	1.10±0.28
5OH-Imidacloprid	0.02±0.00	0.02±0.00	0.05±0.02	0.04±0.02	0.10±0.02	0.83±0.19	0.17±0.00	0.06±0.03	0.27±0.10	0.21±0.05

858 mWAT; mesenteric white adipose tissue

859 gWAT; gonadal white adipose tissue

860 iWAT; inguinal white adipose tissue

861 BDL: below detection limit

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Simultaneous Quantification of Imidacloprid and its Metabolites in
Tissues of Mice upon Chronic Low-dose Administration of Imidacloprid

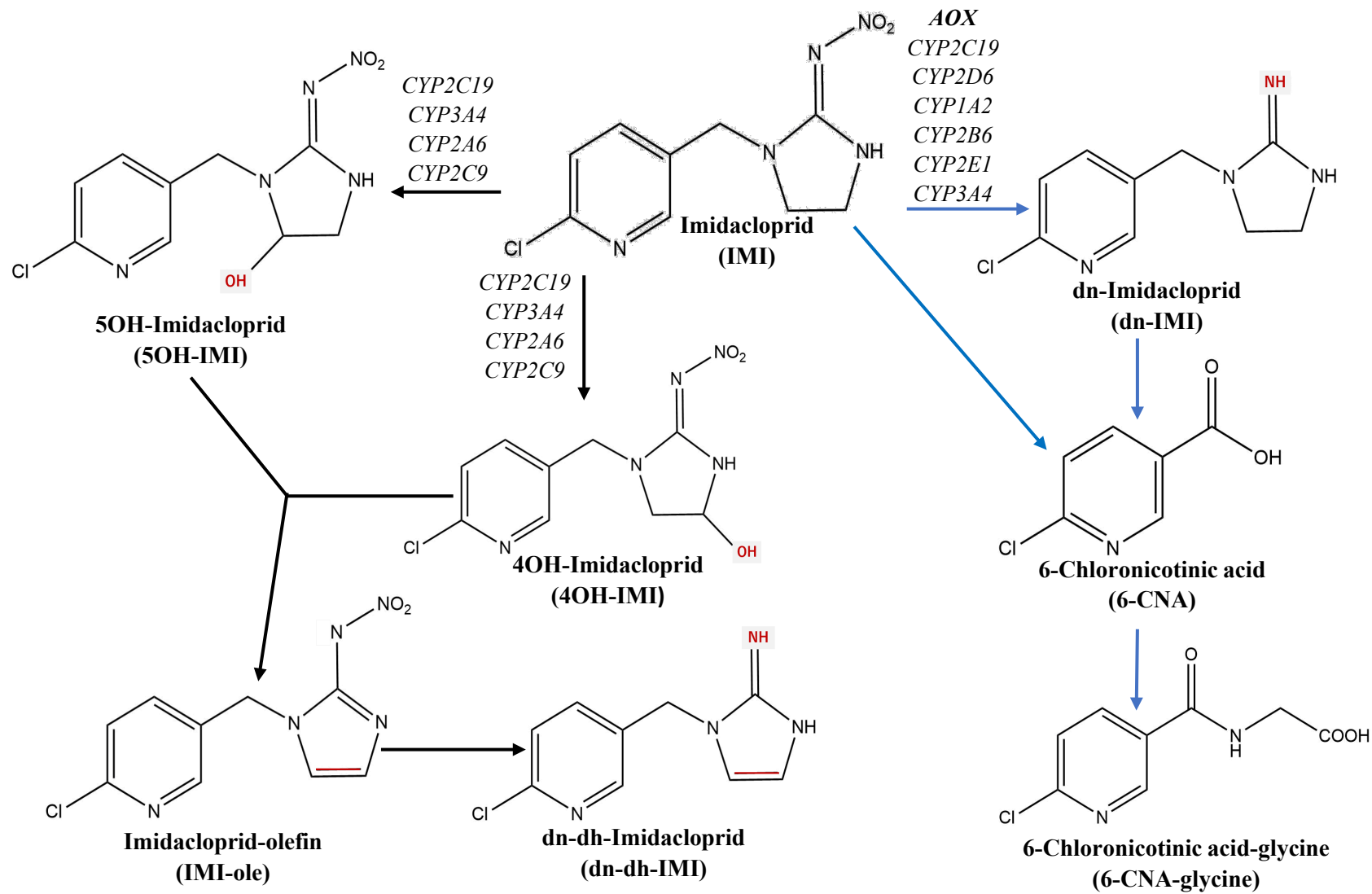


Fig.1

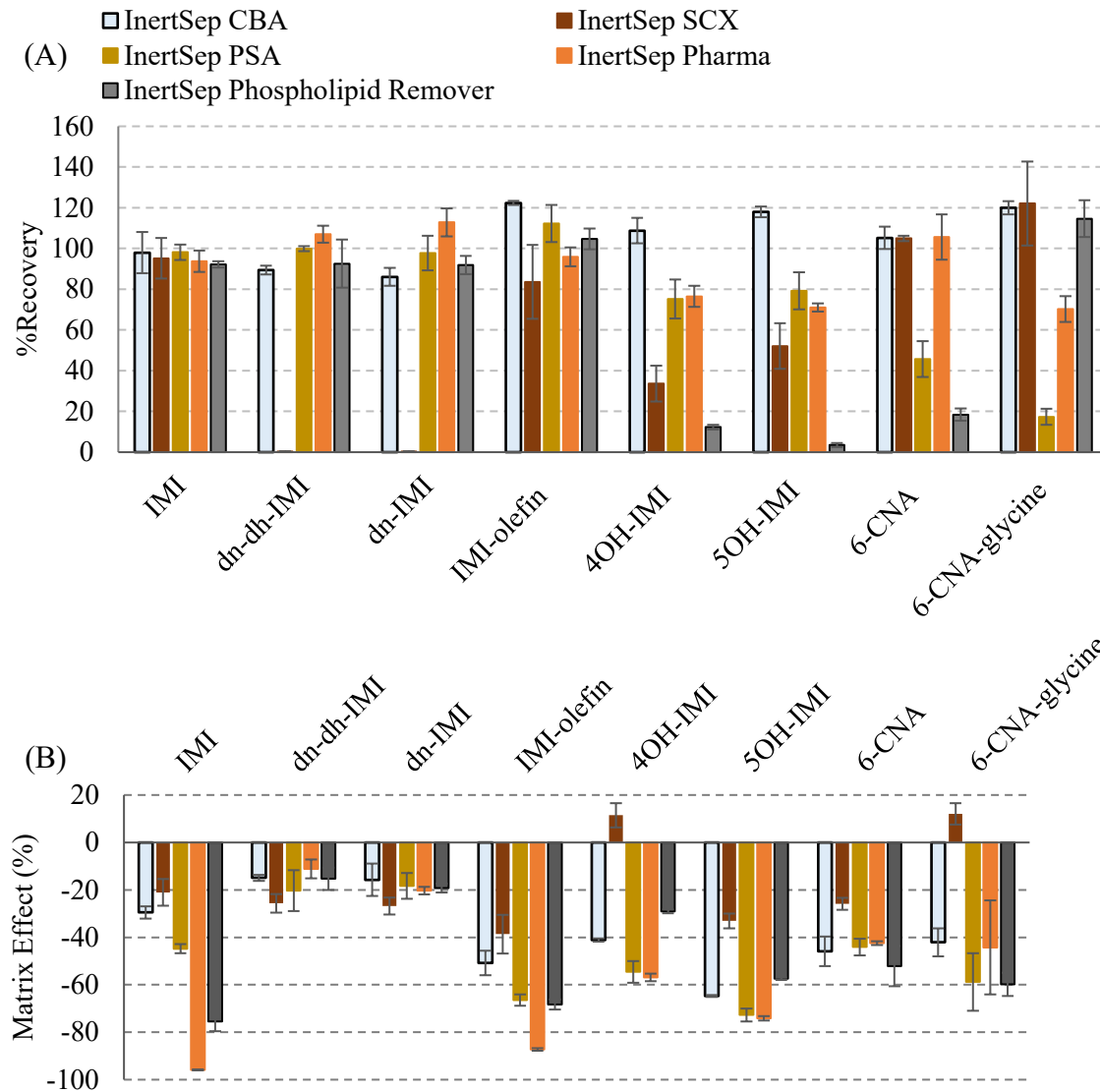


Fig.2 A&B

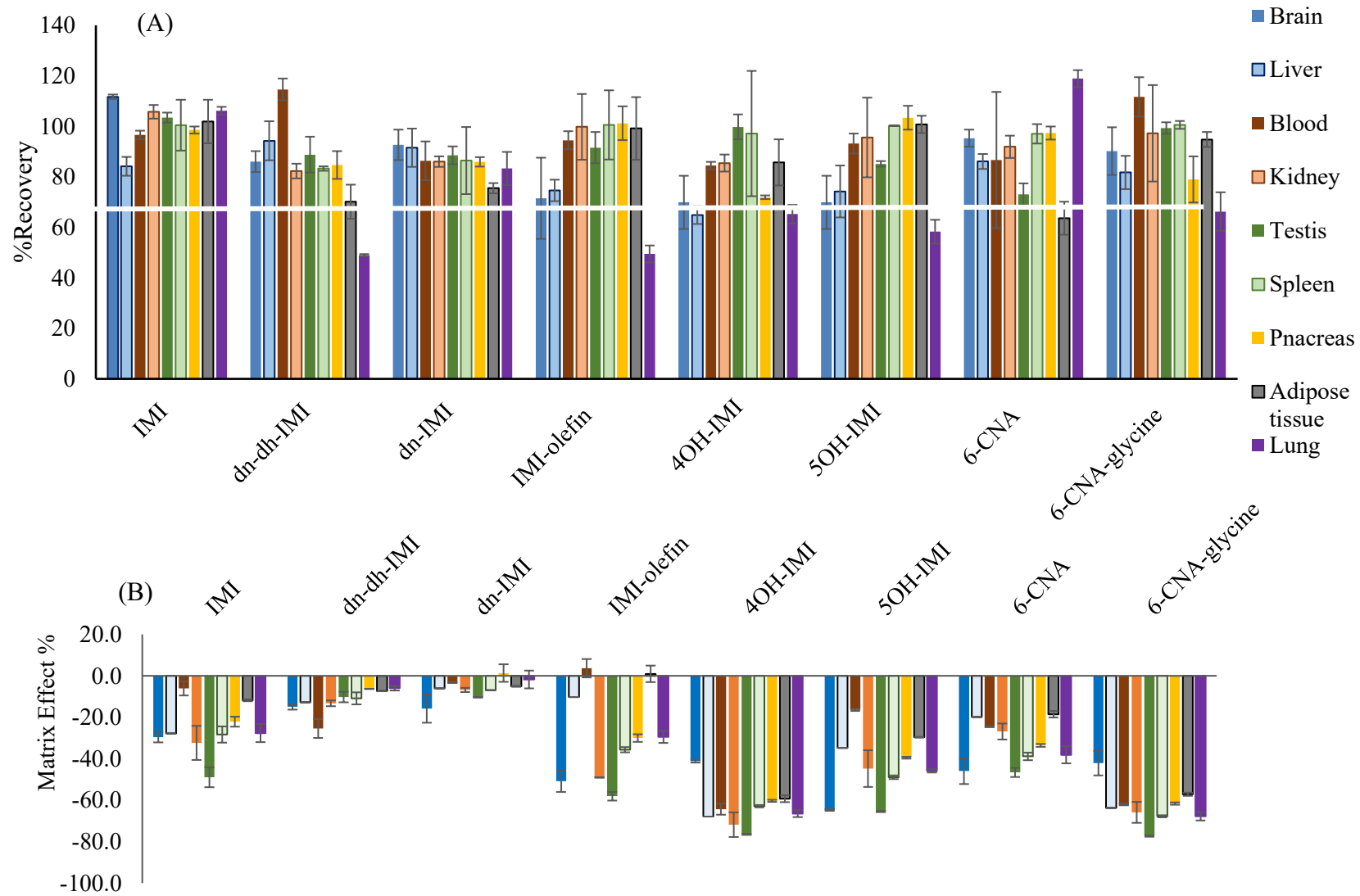
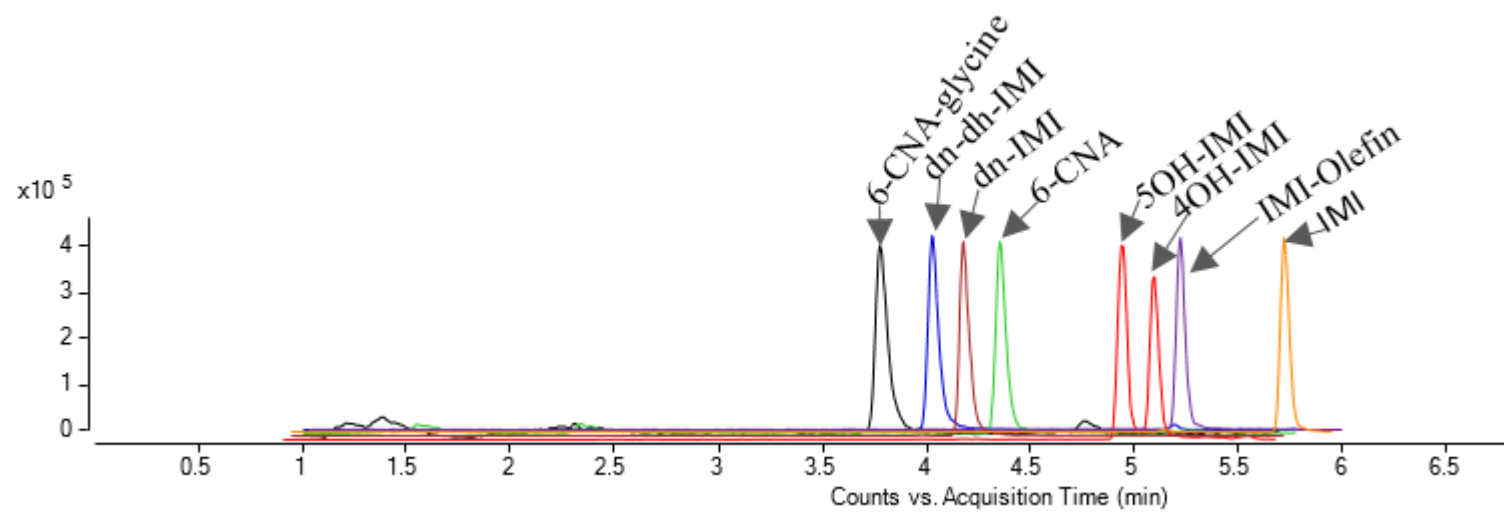


Fig.3 A & B



Figs. 4

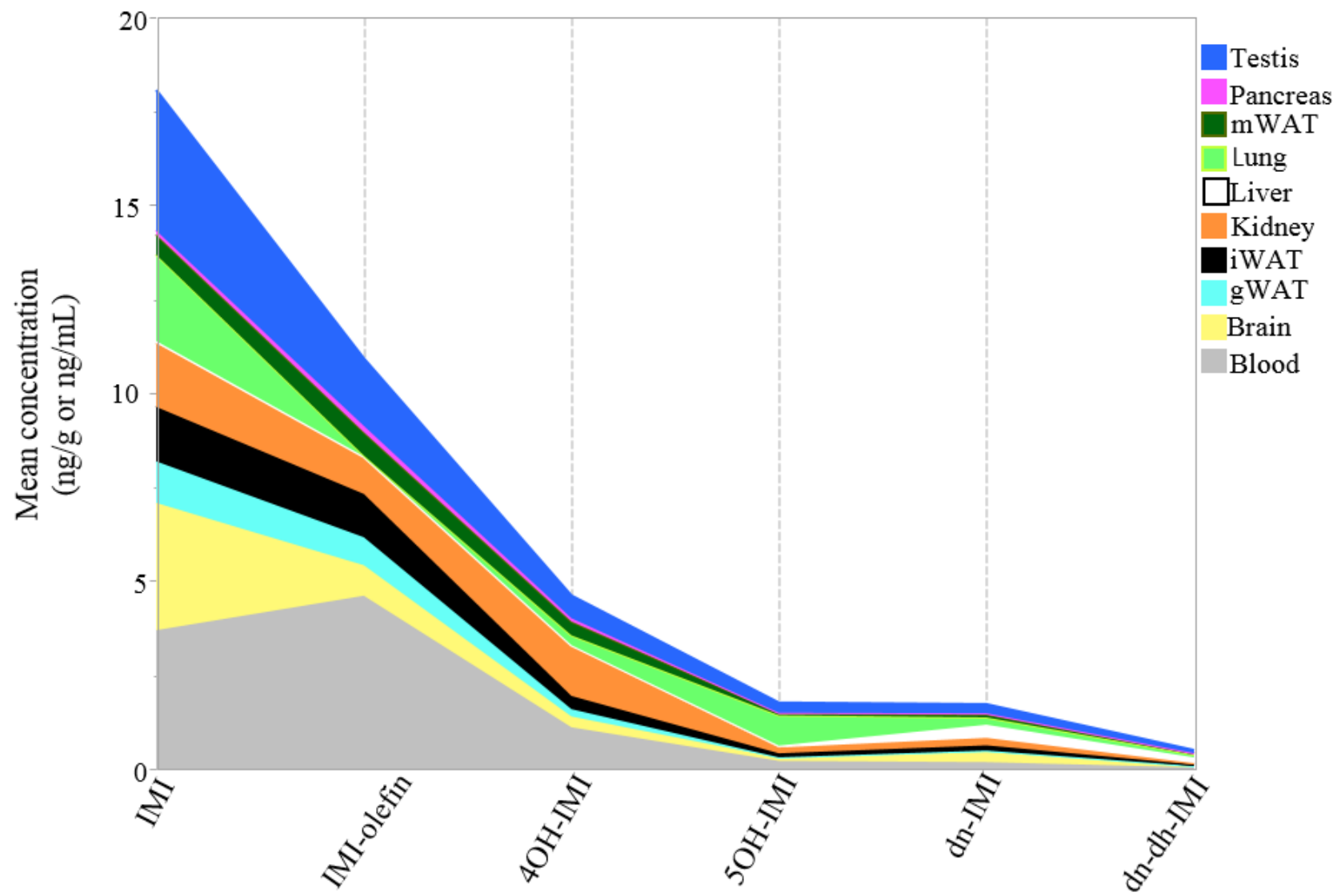


Fig. 5

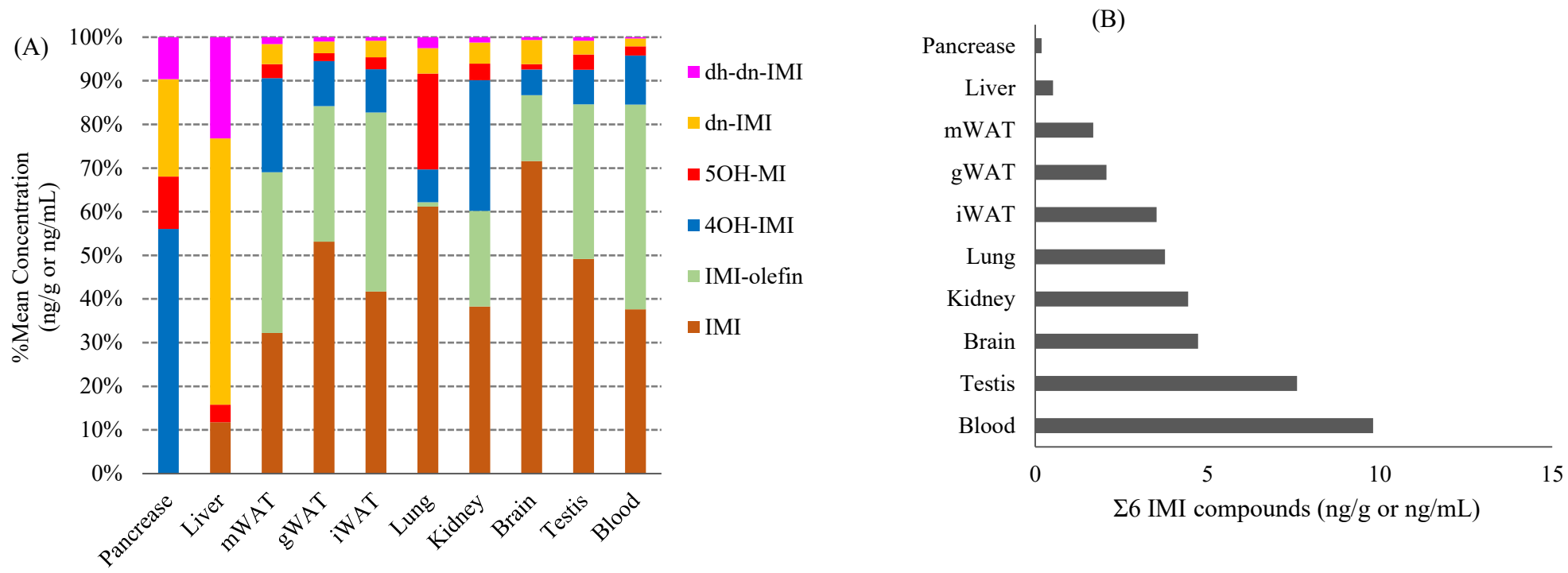


Fig.6 A&B