Part-per-trillion LC-MS/MS determination of neonicotinoids in small volumes of songbird plasma

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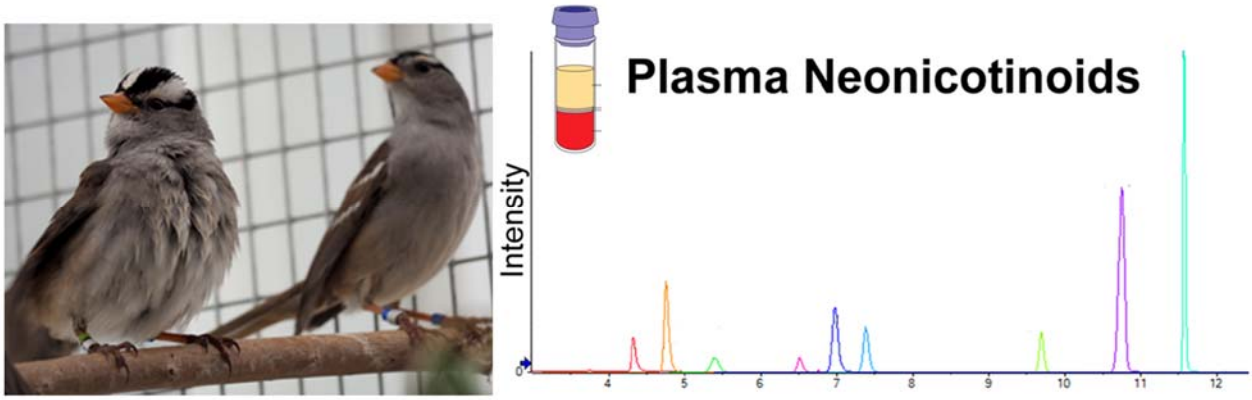


Highlights

- Non-lethal part-per-trillion LC/MS-MS method developed for the detection of neonicotinoids in songbird plasma
- 50 µL plasma samples extracted using simple precipitation and dilution procedure
- First study to confirm widespread neonicotinoid exposure in free-living songbirds
- Method confirmed imidacloprid concentration increase in plasma after dosing

Abstract:

Neonicotinoids are the most widely used class of insecticides in the world, and there are increasing concerns about their effects on non-target organisms. Analytical methods to diagnose exposure to neonicotinoids in wildlife are still very limited, particularly for small animals such as songbirds. Blood can be used as a non-lethal sampling matrix, but the sample volume is limited by body size. Neonicotinoids have a low bioaccumulation potential and are rapidly metabolized, therefore, sensitive assays are critically needed to reliably detect their residues in blood samples. We developed an efficient LC-MS/MS method at a part−per−trillion (pg/mL) level to measure eight neonicotinoid related insecticides (acetamiprid, clothianidin, dinotefuran, flonicamid, imidacloprid, nitenpyram, thiacloprid and thiamethoxam) plus one metabolite (6-chloronicotinic acid) in small volumes (50 µl) of avian plasma. The average recovery of target compounds ranged from 95.7 to 101.3%, and relative standard deviations were between 0.82-2.13%. We applied the method to screen blood samples from 36 seed-eating songbirds (white-crowned sparrows; *Zonotrichia leucophrys*) at capture, and detected imidacloprid in 78% (28 of 36), thiamethoxam in 22% (8 of 36), thiacloprid in 11% (4 of 36), and acetamiprid in 11% (4 of 36) of wild-caught sparrows. Six hours after capture, birds were orally dosed with 0 (control), 1.2 or 3.9 mg of imidacloprid/kg bw, test results using this method indicated that plasma imidacloprid was significantly elevated (low 26-times, high 316-times) in exposed groups. This is the first study to confirm neonicotinoid exposure in small free-living songbirds through non-lethal blood sampling, and to demonstrate that environmentally realistic doses significantly elevate circulating imidacloprid concentrations. This sensitive method could be applied to characterize exposure to neonicotinoids in free-living wildlife and in toxicological studies.

Keywords: Neonicotinoids, 6-Chloronicotinic Acid (6-CAN), Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS), Multiple Reaction Monitoring (MRM), Blood Plasma

1. Introduction

Neonicotinoids are a class of insecticides with nicotine-like molecular structure that have been used widely in the last two decades. They are registered for use in more than 120 countries and are the largest class of insecticides sold worldwide [1]. These insecticides are employed dominantly in insecticidal seed treatments. Neonicotinoids are considered less toxic to vertebrates and relatively safer for the environment [2, 3] due to their specific mode-of-action as insect-nicotinic acetylcholine receptor (nAChR) inhibitor [4]. However, there is increasing concern that the high availability of neonicotinoids in the environment could have negative direct or indirect effects on non-target invertebrate and vertebrate wildlife, including aquatic invertebrates [5, 6,7], bee colonies [8, 9, 10], insectivorous birds [11], and seed eating birds [12, 13, 14, 15]. Several regulatory agencies have restricted the use of certain neonicotinoids, and Canada, the United States, and Europe are currently re-evaluating clothianidin, imidacloprid, thiamethoxam and their associated products [16, 17, 18].

Wildlife that use agricultural landscapes have the potential to be directly exposed to neonicotinoids through several exposure routes, including consumption of treated seeds. The timing of seeding for many crops in the northern mid-latitudes coincides with the spring migration in birds [19], and thus seed-eating birds that use cropland for refueling may be particularly susceptible. Evidence of direct exposure to neonicotinoids in seed-eating birds comes from post-mortem analysis of tissue residues and gastrointestinal contents [13, 20, 21] and from field observations [14]. There is a need for sensitive and effective analytical methods to non-lethally determine neonicotinoid concentrations in small birds. Blood is a common sample media to evaluate pesticide exposure [22], and can be safely used for sampling purposes, as long

as total volume extracted is limited to less than 10% blood volume (approx. 1% of body mass) per sample and less than 15% in a 14 day period for repeated sampling [23]. An increasing number of methods have been published for the determination of neonicotinoids in agricultural, food products and environmental samples [24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34] with liquid chromatography-tandem mass spectrometry (LC-MS/MS) as the dominant analytical methodology [35, 36, 37, 38, 39, 40, 41]. However, very little method development work has focused on non-lethal biotic matrices in wildlife. We only found one previous study that described an analytical procedure to determine neonicotinoids in bird blood samples with limits of quantification from 2000 to 10000 pg/mL, and requiring 500 µL of blood [42]; however, considering the low bioaccumulation potential of neonicotinoids in mammals [43, 44], more sensitive methods are needed to reliably detect exposures, using smaller volumes suitable for animals such as songbirds.

We previously developed a direct aqueous injection LC-MS/MS method [45] for the analysis of eight neonicotinoids, including acetamiprid, imidacloprid, nitenpyram, thiacloprid (first generation neonicotinoids), clothianidin, thiamethoxam (second generation), dinotefuran (third generation) and flonicamid, a new systemic pesticide that is often included in the neonicotinoid group [46] in environmental water samples. A common metabolite of the first generation neonicotinoids, 6-chloronicotinic acid (6-CNA) [47, 48], was also included in the method as it had been detected in bees after exposure to neonicotinoids [35]. Compared to water samples, biological fluids like blood usually contain more complicated matrix components that may cause matrix effects during electrospray ionization [49]. Solid phase extraction with 96-well plates are often used to concentrate and cleanup this type of small volume biological samples [50]. QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) technique is another

alternative where sugars, lipids, proteins, pigments, excess water, etc. can be removed by dispersive solid phase extraction (dSPE) after extraction [51]. The dilute-and-shoot approach coupled with LC-MS has also been applied to deal with blood plasma samples [52], and is the simplest approach as it uses direct aqueous injection; however, detection limits suffer from the at least 100-fold dilution required to reduce the matrix effect. Here, we combined quick deproteinization and small dilution (≤10-fold) to develop a simple method with good sensitivity to measure neonicotinoids and 6-CNA in avian plasma

The overall objectives of our current work were 1) to develop an efficient and sensitive LC-MS/MS approach to measure eight neonicotinoid related pesticides in bird plasma, 2) to apply the method to assess concentrations of neonicotinoids in wild-caught migrating seed-eating songbirds (Eastern white-crowned sparrow; *Zonotrichia leucophrys*) at capture, and 3) to verify expected increases in plasma concentrations in the same birds following controlled oral exposure to environmentally relevant levels of imidacloprid.

2. Material and Methods

2.1. Chemicals & Reagents

Neat standards of native and isotope-labelled neonicotinoids, American Chemical Society (ACS) reagent grade ammonium acetate (CH_3COONH_4), formic acid (HCOOH), distilled in glass grade methanol (CH_3OH), and plasma from chicken and bovine (3.8% trisodium citrate as anticoagulant) were purchased from Sigma-Aldrich (Oakville, ON, Canada). High purity water was produced by passing reverse osmosis water through a Barnstead NANOpure^{TM} water purification system (Mississauga, ON, Canada). The isotope-labelled internal standards (ILISs)

104 acetamiprid-d_3, clothianidin-d_3, imidacloprid-d_4 and thiamethoxam-d_3 were used to correct
105 extraction loss, matrix effects and instrument variability and to monitor method performance in
106 each sample. Working solutions for native target compounds and ILISs in methanol were
107 prepared from neat standards as described previously [45]. A custom-mix stock solution of eight
108 native neonicotinoids in acetonitrile was purchased from AccuStandard Inc (New Haven, CT,
109 USA) for quality control. All standard solutions were stored at 5 ± 3°C and allowed to reach
110 room temperature before use.
111
112 **2.2. Bird Blood Sample Collection, dosing, and captive housing**
113 White-crowned sparrows are seed-eating migratory songbirds that have the potential to
114 be directly exposed to insecticides primarily through consumption of treated seeds or granules or
115 by coming in contact with foliage and soils sprayed with neonicotinoids. In May 2017 as part of
116 a companion study, a total of 36 Eastern white-crowned sparrows (*Z. l. leucophrys*) were
117 captured in mist nets or sparrow traps at Long Point Bird Observatory, Ontario (42.5829° N,
118 80.3985° W). Birds were blood sampled at capture, and held overnight in cages (66 cm L x 46
119 cm W x 50 cm H) on site in an animal housing room within a mobile research laboratory, with 2
120 to 3 birds per cage. Birds were weighed and dosed between 09:00 to 11:00, then weighed and
121 blood sampled between 15:00 to 17:30. Mean (±SE) time between dosing and blood sampling
122 was 6.1 ± 0.1 hours. Birds were randomly assigned to treatment groups, and dosing was through
123 oral gavage directly into the crop using 20G curved stainless steel tube feeders at a volume of 10
124 ml/kg bw, with either the low dose (1.2 mg IMI/kg bw; n = 12), high dose (3.9 mg IMI/kg bw; n
125 = 12), or vehicle control (sunflower oil; n = 12). Avian plasma samples used as blanks for quality
126 control were collected from Gambel's white-crowned sparrows (*Z. l. gambelii*) captured in

Saskatchewan in May 2016 for a previous study [12]. These birds had been held in captivity for ≥ 14 days in the Facility for Applied Avian Research at the University of Saskatchewan. Blood-samples were taken from the brachial vein following puncture with a 26G needle. Blood was collected into heparinized tubes, centrifuged to separate plasma from red blood cells, and plasma was then stored frozen at -20°C until analysis. Birds were provided with water and a mixture of millet, black oil sunflower seeds, and poultry starter crumbles (Proform 26%) *ad libitum*. Research protocols were in compliance with the Canadian Council on Animal Care guidelines and approved by the University of Saskatchewan Animal Care Committee (AUP 20110043), and conducted under Canadian Wildlife Service Scientific Permits 15SKSC005 and SC00008.

2.3. Dosing solution composition and analysis

Dosing solutions were made by dissolving technical grade imidacloprid (Sigma Aldrich 37894) in a small volume of acetone, then diluting with food-grade organic sunflower oil (Compliments brand, Sobeys Canada). Nominal concentrations were 0 mg IMI/ml (control), 0.10 mg IMI/ml (low), and 0.41 mg IMI/ml (high). Solutions were stirred overnight to evaporate off acetone, and stored in amber glass bottles in the dark for the duration of the study. Nominal concentrations were selected to be equivalent to 2.5% (low) and 10% (high) of the house sparrow LD50 (41 mg/kg bw) [53], where the high dose (4.0 mg/kg bw) was below that used in a previous captive study in white-crowned sparrows (10.25 mg/kg bw) [12]. These doses are environmentally realistic, with the mass consumed of imidacloprid in the low dose being equivalent to the mass of imidacloprid on ~1.1 treated canola seeds or 1 wheat kernel, and the high dose is equivalent to the mass on ~3.5 treated canola or 3.2 treated wheat kernels, according to current US application rates (Table S1).

Dosing solution concentrations of imidacloprid were confirmed by LC-MS/MS analyses at the National Hydrology Research Centre, Environment and Climate Change Canada, Saskatoon, SK. Solutions were diluted 100x into an intermediate solvent (acetone), then further diluted into water (20x for the control and low solutions, 100x for the high solution). Diluted aqueous samples were directly injected into the mass spectrometer (Waters 2695 Alliance HPLC system; Waters Corp., Milford, MA), using the same instrumentation and calibration methods described in Eng et al. 2017 [12]. Measured concentrations of imidacloprid were 0.12 mg/ml (low) and 0.39 mg/ml (high). Vehicle control oil and all blanks had no detectable levels of imidacloprid.

2.4. Sample Preparation and Instrument Analysis

Plasma samples were warmed up to room temperature, 50 to 200 µL of each sample was put into a 1.8 mL amber glass HPLC vial, then 20 uL of ILIS spiking solution in methanol, 20 uL of methanol and 2.5 uL of formic acid were added. The vial contents were mixed well and the whole content was blown down to dryness with light nitrogen flow at 30 ºC. Then 500 µL of 20:80 methanol:water was added into the vial to reconstitute, and the vial was centrifuged at 4000rpm for 20 minutes. The acid, organic solvent and blow down precipitated out proteins and other larger biomolecules in the plasma, a process referred to as deproteinization. Each vial was carefully removed from the centrifuge and ~200 µL of supernatant was transferred to a clean HPLC vial. Blank plasma samples were processed exactly the same way. For each fortified plasma sample, 20 µL of native spiking solution was added instead of pure methanol.

Dilution factors (10, 5 and 2.5 with 500 µL final volume) and temperature before blowdown (-20 or 45ºC) were optimized to reduce matrix effects using commercial bovine and

chicken plasma samples fortified with target compounds. The recoveries of fortified isotope-labelled neonicotinoids in 50, 100, 200 µL of bovine plasma and 200 µL of chicken plasma were assessed.

A dilution factor of 10 (50 µL plasma in 500 µL final volume) was used for validation of the method with fortified sparrow plasma, and for unknown sparrow samples. The optimized method was validated for songbird samples using pooled plasma from Gambel's white-crowned sparrows that had been held in captivity for ≥14 days. Plasma pools contained no detectable neonicotinoids. Target compound concentrations were calculated based on calibration curves established with 5-level calibration standards, which were plotted using a 1/x weighting and linear regression with internal standard correction.

The instrument analysis was carried out on a Sciex (Concord, ON, Canada) QTRAP®5500 mass spectrometer coupled with a Shimadzu Prominence/20 series (Columbia, MD, USA) LC system. 90 µL of instrument ready solutions were injected into a Phenomenex Kinetex Biphenyl 2.6 mm 100 x 4.6 mm LC column. Multiple reaction monitoring (MRM) data was acquired and processed using electrospray ionization mode with the Analyst 1.6.3 software and the Scheduled MRM™ (sMRM) algorithm with a target cycle time of 800 ms and a detection window of 60 s for each transition. Instrument conditions and parameters used were described in detail previously [45]. The two MRM transitions and corresponding collision energies, the ILISs used for quantitation, and the calibration ranges for each target compound are listed in Table 1. A control standard of a different source from calibration standards was analyzed with each batch of samples. The accuracy of each compound in the control standard had to be within 70 to 130% range. The accuracy (recovery) of each spiked replicate was calculated by Analyst Software by comparing the calculated concentration with the spiked

concentration. The average recovery (Avg Rec) and the relative standard deviation (RSD) of the spiked replicates were calculated by Excel formulae. The method detection limits (MDLs) were calculated according to the U.S. Environmental Protection Agency protocol [54].

2.5. Statistical analysis

Statistical analysis was completed using SAS 9.4. Imidacloprid concentration was log transformed to meet assumptions of normality. For samples that were below the MDL, random values between zero and the MDL were substituted into the dataset for statistical analysis. Comparisons of imidacloprid concentrations between dose groups over time (pre-dosing at capture vs post-dosing) were made with a linear mixed model (proc MIXED), with bird ID as a repeated subject effect, and fixed effects of dose, time, and dose*time. Tests for differences between means were adjusted for multiple comparisons using the Tukey-Kramer method. Significance level was set at $\alpha = 0.05$.

3. Results and Discussion

3.1 Analytical Method Optimization

We developed a sensitive part-per-trillion LC-MS/MS method to analyze small volumes of avian plasma for neonicotinoids and the metabolite 6-CNA using an easy (approximately one hour) sample preparation approach combining quick deproteinization and dilution. Plasma samples were mixed with methanol solution(s) and acidified with formic acid, and blown down to dryness to denature biomolecule components in the plasma as much as possible, then neonicotinoids were re-dissolved in 500 µL 20:80 methanol:water and separated from the

219 precipitates by centrifuge. It was found that an increase or decrease in temperature before blown
220 down in an attempt to help with the denaturation process did not improve recovery.
221 Based on recoveries of fortified isotope-labelled neonicotinoids in commercial bovine
222 and chicken plasma with different dilution factors (2.5 to 10), we found that dilution helped
223 reduce matrix effects. At a dilution factor of 2.5 (200 µL plasma in 500 final volume), signals for
224 all four isotope-labelled neonicotinoids were extremely suppressed for bovine plasma samples as
225 shown in Figure 1. This signal suppression was also observed for acetamiprid-d3, clothianidin-
226 d3, and thiamethoxam-d3 from chicken plasma with the same dilution factor, but the level of
227 suppression was much less. For imidacloprid-d4, its signal was actually enhanced in the 200 µL
228 chicken plasma sample. These results indicate that method performance can vary for plasma
229 samples from different animal species. Dilution of bovine plasma helped to reduce matrix effects
230 and the suppression caused by matrix components was improved significantly at the dilution
231 factor of 10 (50 µL plasma in 500 µL final volume), which we used for all further analysis.
232 It is worth pointing out that imidacloprid was detected at ~13 pg/mL in the commercial
233 chicken plasma sample during the analysis, which indicated that there was exposure to this
234 commonly used insecticide.
235
236 **3.2 Songbird Plasma Method Development and Validation**
237 Following method optimization, captive white-crowned sparrow plasma pool samples
238 were confirmed to contain no detectable neonicotinoids, and were then used for final validations
239 (Table 2). The average recoveries for target compounds in spiked sparrow plasma ranged from
240 95.7 to 101.3% and the recoveries for the four isotope-labelled neonicotinoids were in the range
241 of 58.0 to 82.6%. Clothianidin-d_3 suffered the worst ion suppression among the four isotope-

242 labelled neonicotinoids in fortified samples while acetamiprid-d_3 was affected the least. It is still
243 not clear why certain target compounds such as clothianidin suffer more from matrix effects, but
244 this type of ion suppression observed during LC-MS/MS analysis of neonicotinoids in biological
245 samples could be minimized by solid phase extraction, cleanup and ultra performance liquid
246 chromatography (UPLC) separation [55]. Internal standard correction was used in this method to
247 compensate for sample preparation loss, matrix effects and instrument variability. The method
248 detection limits are in the low pg/mL range for all parent neonicotinoids and ~178 pg/mL for 6-
249 CNA. Only 50 µL of blood plasma was needed for the analysis and the sample preparation work
250 of deproteinization and dilution was minimal (approx. 1 hour). The isotope-labelled internal
251 standards (ILISs) acetamiprid-d3, clothianidin-d3, imidacloprid-d4 and thiamethoxam-d3 were
252 used to correct extraction loss, matrix effects and instrument variability and to monitor method
253 performance in each sample. Relative standard deviation was around 5% for the four labelled
254 internal standards, and less than 2.5% for all target compounds after the internal standard
255 correction (Table 2). As mentioned earlier, there was only one study published before on the
256 measurement of neonicotinoids in bird blood samples [42]. The performance data demonstrate
257 that the recoveries of target compounds and the relative standard deviation of the recoveries are
258 improved compared to the previous study, and more importantly, the method would provide
259 precise and accurate results at low part-per-trillion (pg/mL) levels for neonicotinoid pesticides
260 monitored in bird plasma.

261 **3.3 Evaluation of Neonicotinoid Exposure in Free Living Songbirds**

262 The analytical procedure developed was applied to analyze plasma samples from free-
263 living white-crowned sparrows captured in southern Ontario, Canada during spring migration in
264 May 2017, to assess their exposure to neonicotinoid insecticides. We detected four

neonicotinoids, acetamiprid, imidacloprid, thiacloprid and thiamethoxam (Table 3). One sample (sample ID # 148) contained all four neonicotinoids. Figure 2 shows the chromatograms of the quantitation ion of each analyte in this sample, alongside the ion chromatograms in middle level calibration standard for comparison. Imidacloprid was above the MDL in 28 of the 36 plasma samples (78%), with the highest concentration of 177 pg/mL. The other eight samples also contained trace amount of imidacloprid but their levels were less than the MDL of 4.6 pg/mL. Eight of the 36 samples (22%) showed positive results for thiamethoxam with the highest concentration of 33.7. Trace amount of thiamethoxam was detected in another three samples at less than MDL (4.5 pg/mL) levels. Four samples showed positive results for acetamiprid (11%) and thiacloprid (11%), respectively, but all were below 5 pg/mL. Six samples showed trace amount of clothianidin (as demonstrated in the ion chromatogram in Figure 2A), a commonly used neonicotinoid in North America; however, there were none higher than the MDL level of 7.4 pg/mL. We caution that this might be attributed to clothianidin having the worst ion suppression encountered during LC-MS/MS detection. No dinotefuran, flonicamid, nitenpyram or 6-CNA was detected in the blood samples of the wild-caught white-crowned sparrows.

Studies of neonicotinoid residues in avian blood are rare; the only other available study was done in Eurasian eagle owls (*Bubo bubo*), which are top predators and residues would most likely be through secondary exposure from consuming contaminated seed-eating prey (e.g. rabbits *Oryctolagus cuniculus*, partridges *Alectoris ruffa* and pigeons *Columba spp*). Thirty owls were tested for nine neonicotinoids, and only one owl had detectable residues for IMI only [42], although limits of detection were about three orders of magnitude above those in the present study.

The detection of circulating neonicotinoids in the majority of wild-caught sparrows in this study suggests that free-living seed-eating birds are being exposed to neonicotinoids at high enough concentrations and/or frequent enough exposures for the parent compounds to be routinely detected in circulation. The birds measured in this study were caught on a northerly migratory stopover between their wintering and breeding grounds. Eastern white-crowned sparrows overwinter from central Texas to the lower Ohio River Valley, and breed in boreal shrub habitat in north eastern Canada. Their diet is > 90% plant based, and includes a variety of seeds as well as small grains (e.g. oats, wheat, barley, corn) [56]. The timing of spring migration overlaps with spring seeding for many agricultural crops, and birds may be exposed through direct consumption of neonicotinoid treated seeds. It is also possible that exposure comes from consumption of contaminated water, soil or seeds produced by plants that have taken up neonicotinoids systemically. Neonicotinoids are taken up into plant tissues and can be translocated to the flowers and seeds [57]. Residues have been detected in crop plants, as well as in non-crop plants from field margins that are exposed to neonicotinoids through agricultural runoff or drift [58, 59]. Most plant residue analysis has focused on pollen and nectar with respect to bee exposure, while seed concentrations are rarely reported. In addition to seed coatings, there could be other usage such as spray applications of neonicotinoids to both agricultural and urban areas. Other possible routes of avian exposure include inhalation and dermal exposure to environmental residues or consuming neonicotinoid contaminated surface water. Further work is needed to determine exposure pathways in birds including field surveys of treated seed availability and consumption by wildlife, characterization of systemic seed concentrations, and comparison of circulating neonicotinoid concentrations in non-seed eating birds (e.g. aerial insectivores) that use agricultural areas.

3.4 Controlled Imidacloprid Dosing Study

Under controlled dosing via oral gavage, white-crowned sparrows were exposed to environmentally realistic concentrations of imidacloprid and concentration changes in blood levels were monitored. There was no significant difference in pre-dosing plasma concentrations among treatment groups ($p \geq 0.815$). There was a dose-dependent increase in plasma imidacloprid, and a significant interaction between time and dose ($F_{2,33} = 19.15$, $p < 0.0001$). Plasma imidacloprid concentrations significantly increased between pre- and post-dosing in the low ($p < 0.0001$) and high dose ($p < 0.0001$) birds, but not the control birds ($p = 0.418$) (Fig. 3). There was a large variation in the plasma imidacloprid concentration at 6 hours post-dosing (low range = 100 to 3,210 pg/mL; high range = 840 to 35,100 pg/mL), suggesting individual variation in uptake, biotransformation and/or elimination; however, all birds dosed with imidacloprid had higher concentrations post-dosing than pre dosing, indicating that imidacloprid can be detected if a blood sample is taken within the 6-hour time frame after ingesting a similar mass of imidacloprid as found on a single treated seed. Interestingly, the geometric mean plasma concentration from the high dose was 24x higher than the low dose (5558.2 pg/mL vs 234.2 pg/mL), despite the difference in administered dose being only 3.25x higher (3.9 mg/kg bw vs 1.2 mg/kg bw). This pattern suggests that at concentrations in the high dose range, the rate of biotransformation and elimination is more limited. The timing and concentration of exposures are likely difficult to establish from blood residues; however, plasma concentrations in the range detected here (0.1 to >100 pg/mL) would likely indicate recent exposure. The majority of sparrows had pre-dosing concentrations below this level, but three birds had plasma

concentrations >100 pg/mL at capture. In comparison, the study of neonicotinoid residues in Eurasian eagle owl blood reported 3280 pg/mL of imidicloprid in one individual [42].

Improved knowledge of neonicotinoid toxicokinetics in birds, including a time-course analysis of plasma following controlled exposures combined with quantification of metabolites, could help further characterize whether residues detected from wild bird samples are due to recent low-level exposures or to reduced levels from past exposures. The metabolite 6-CNA was not detected in any samples pre- or post-dosing. Other metabolites not included in this study, such as 5-OH-imidacloprid, imidacloprid-olefin, and imidacloprid-nitrosoimine could be useful to quantify in the future to help understand the toxicokinetics of neonicotinoids in songbirds.

4. Conclusion

An efficient deproteinization and "dilute and shoot" LC-MS/MS approach for the determination of eight neonicotinoid insecticides and metabolite 6-CNA in bird plasma are described in this study. Two MRM transitions with each target compound were monitored for their unambiguous identification in complex biological samples and four isotope-labelled internal standards were employed to improve data quality. The ease of sample preparation, the small 50 µL volume of plasma required and the low MDLs in the ranges of 2.3 to 15.9 pg/mL for the parent compounds make it a promising method for assessing exposure to neonicotinoid insecticides in songbirds or other small animals by non-lethal blood sampling. This sensitive method also provided a useful tool to characterize toxicologically and environmentally relevant exposures of neonicotinoids in avian wildlife even at low concentrations. Using this new method, this is the first study to confirm migratory songbirds have been exposed to four of the registered neonicotinoids: acetamiprid, imidacloprid, thiacloprid and thiamethoxam in North America. This

354 novel method will greatly complement future ecotoxicological research on avian wildlife
355 exposure and effects to neonicotinoid insecticides, given their prevalence in agriculture
356 worldwide.
357
358 **Acknowledgements**
359 Funding was provided by an NSERC Discovery grant to C.A.M., and a Mitacs Accelerate
360 fellowship in partnership with Bird Studies Canada to M.L.E. Support from John Headley and
361 Kerry Peru for dosing solution analysis at National Hydrology Research Centre, Environment
362 and Climate Change Canada was greatly appreciated. We also thank Amy Wilson for field
363 assistance, and Bird Studies Canada and Long Point Bird Observatory for field site access and
364 logistical support.
365

Table 1. Multiple reaction monitoring (MRM) transitions, corresponding collision energies (CEs), isotope-labelled internal standards (ILISs) used, and calibration range for each target compound (a, b denote two MRM transitions monitored for a given target compound, MRM a was used for quantitation and MRM b was used for confirmation).

MRM Transition ID	Q1 Mass	Q3 Mass	CE (eV)	ILIS	Calibration Range (ng/L)
6-CNA a	158	122	25	imidacloprid-d_4	40-4000
6-CNA b	158	78	33	imidacloprid-d_4	40-4000
acetamiprid a	223	126	29	acetamiprid-d_3	2-200
acetamiprid b	223	90	46	acetamiprid-d_3	2-200
clothianidin a	250	169	18	clothianidin-d_3	2-200
clothianidin b	250	132	19	clothianidin-d_3	2-200
dinotefuran a	203	129	15	clothianidin-d_3	2-200
dinotefuran b	203	114	15	clothianidin-d_3	2-200
flonicamid a	230	203	25	clothianidin-d_3	4-400
flonicamid b	230	174	25	clothianidin-d_3	4-400
imidacloprid a	256	209	23	imidacloprid-d_4	2-200
imidacloprid b	256	175	25	imidacloprid-d_4	2-200
nitenpyram a	271	126	38	clothianidin-d_3	2-200
nitenpyram b	271	224	20	clothianidin-d_3	2-200
thiacloprid a	253	126	29	acetamiprid-d_3	1-100
thiacloprid b	253	90	50	acetamiprid-d_3	1-100
thiamethoxam a	292	211	19	thiamethoxam-d_3	2-200
thiamethoxam b	292	181	33	thiamethoxam-d_3	2-200
acetamiprid-d_3	226	126	29		
clothianidin-d_3	253	172	19		
imidacloprid-d_4	260	213	23		
thiamethoxam-d_3	295	214	19		

Table 2. Spiking levels in 50 µL fortified plasma pool samples (n = 12) from captive white-crowned sparrows, final concentrations in reconstituted solutions for instrument analysis, average recovery (Avg Rec%), standard deviation (SD), relative standard deviation (RSD%) and calculated method detection limits (MDLs) for target analytes.

Compound Name	Spiking Level ng/L	Final Concentration ng/L	Avg Rec %	RSD %	MDL ng/L
6-CNA	3200	320	101.3	2.0	177.7
acetamiprid	160	16	96.9	0.8	3.6
clothianidin	160	16	98.5	1.7	7.4
dinotefuran	160	16	95.7	2.1	9.1
flonicamid	320	32	99.4	1.8	15.9
imidacloprid	160	16	99.4	1.0	4.6
nitenpyram	160	16	101.2	1.9	8.8
thiacloprid	80	8	97.0	1.1	2.3
thiamethoxam	160	16	98.9	1.0	4.5
acetamiprid-d_3	400	40	82.6	4.5	
clothianidin-d_3	400	40	58.0	4.3	
imidacloprid-d_4	400	40	61.9	5.3	
thiamethoxam-d_3	400	40	70.2	4.7	

Table 3. Calculated analyte concentrations (pg/ml) of acetamiprid, imidacloprid, thiacloprid and thiamethoxam in 36 plasma samples of white-crowned sparrows at capture. Clothianidin, dinotefuran, flonicamid, nitenpyram and 6-CNA were non-detected (ND) in all samples.

Sample ID	Sampling Date	Acetamiprid	Imidacloprid	Thiacloprid	Thiamethoxam
189	8-May	ND	7.2	ND	21.6
869	8-May	ND	<MDL	ND	7.2
937	8-May	ND	9.9	ND	10.7
8154	8-May	ND	5.0	ND	33.7
195	9-May	ND	5.1	ND	10.4
204	9-May	ND	8.7	ND	ND
216	9-May	3.7	23.1	2.6	ND
220	9-May	ND	9.5	ND	7.4
842	9-May	ND	7.4	ND	<MDL
858	9-May	ND	<MDL	ND	ND
888	9-May	ND	36.1	ND	ND
889	9-May	ND	<MDL	ND	ND
943	9-May	ND	6.4	ND	ND
944	9-May	ND	16.5	ND	ND
949	9-May	ND	8.1	ND	ND
950	9-May	ND	<MDL	ND	ND
955	9-May	ND	<MDL	ND	ND
148	10-May	3.9	34.7	2.6	11.6
151	10-May	ND	129.0	ND	ND
114	11-May	ND	105.0	<MDL	<MDL
150	11-May	ND	177.0	ND	ND
155	11-May	3.9	83.0	2.5	ND
117	12-May	3.8	55.8	3.1	ND
575	12-May	ND	58.7	ND	5.1
180	13-May	ND	20.3	<MDL	ND
553	13-May	ND	61.2	ND	ND
560	13-May	ND	12.8	ND	ND
569	13-May	ND	7.2	ND	ND
573	13-May	ND	<MDL	ND	ND
152	14-May	ND	22.1	ND	ND
283	14-May	ND	63.9	ND	ND
411	14-May	ND	<MDL	ND	ND
551	14-May	ND	15.6	<MDL	ND
571	14-May	ND	5.7	ND	ND
576	14-May	ND	<MDL	ND	<MDL
3154	14-May	ND	8.1	ND	ND
	# Samples ≥ MDLs	4	28	4	8
	Max conc.	3.9	177	3.1	33.7

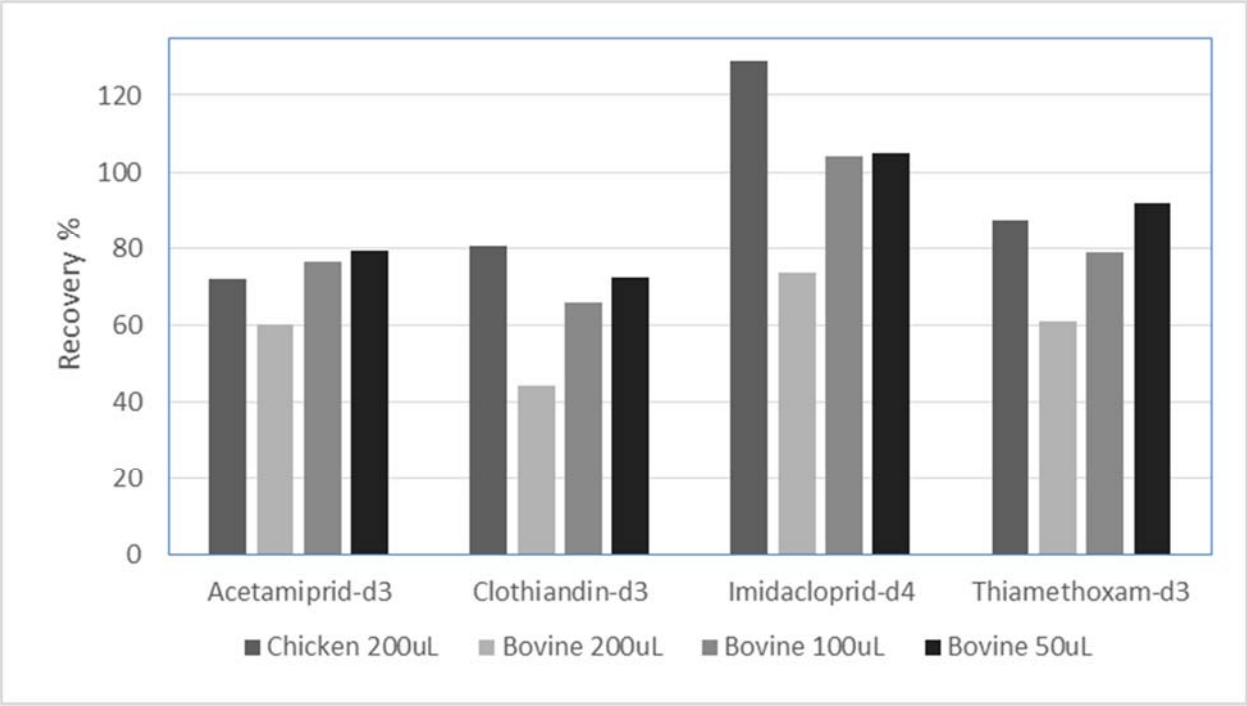
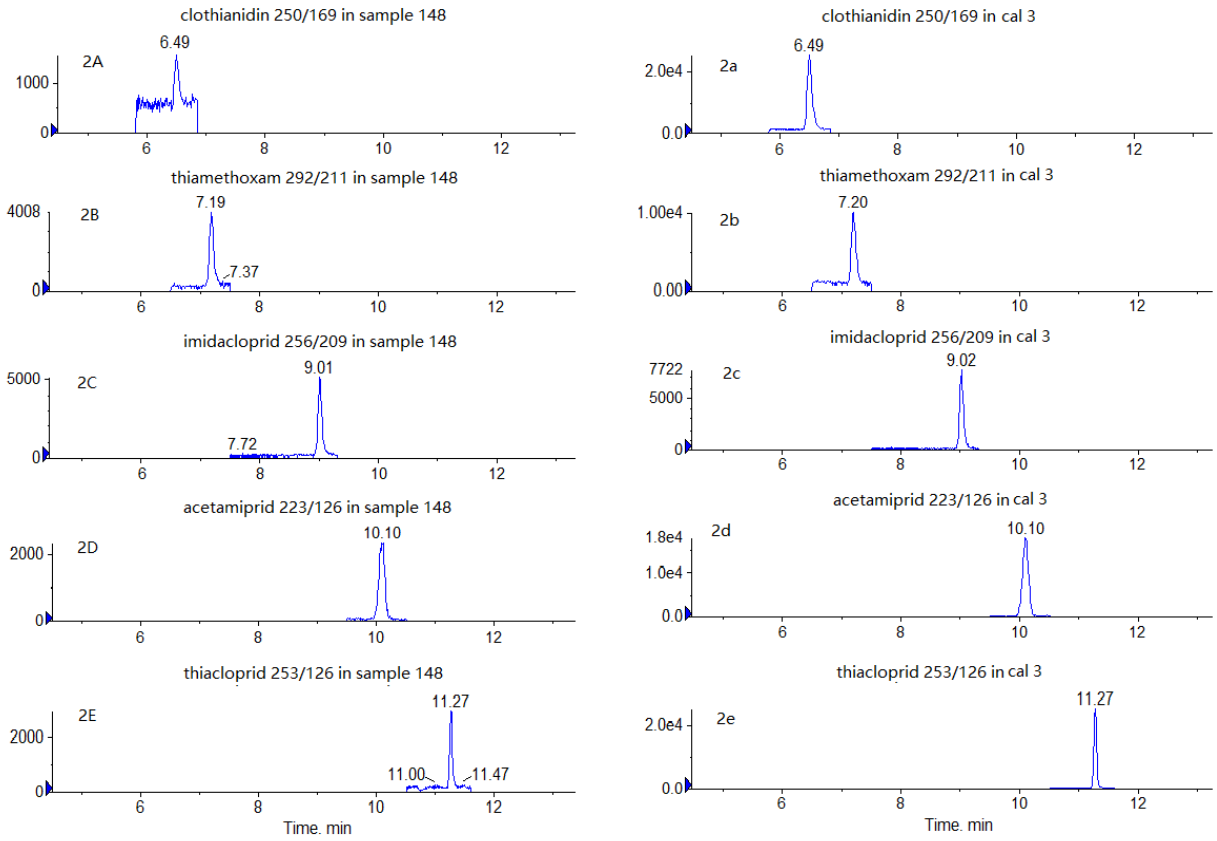


Figure 1. Recoveries of four isotope-labelled neonicotinoids in fortified samples with dilution factor of 2.5 (200 µL) for chicken plasma, dilution factors of 2.5 (200 µL), 5 (100 µL), and 10 (50 µL) for bovine plasma.



392

393

394 **Figure 2**. Ion chromatograms of clothianidin 250/169 in sample 148 (A left) and middle level

395 calibration standard solution cal 3 (a right), thiamethoxam 292/211 in sample 148 (B left) and

396 middle level calibration standard solution cal 3 (b right), imidacloprid 256/209 in sample 148 (C

397 left) and middle level calibration standard solution cal 3 (c right), acetamiprid 223/126 in in

398 sample 148 (D left) and middle level calibration standard solution cal 3 (d right), and thiacloprid

399 253/126 in sample 148 (E left) and middle level calibration standard solution cal 3 (e right).

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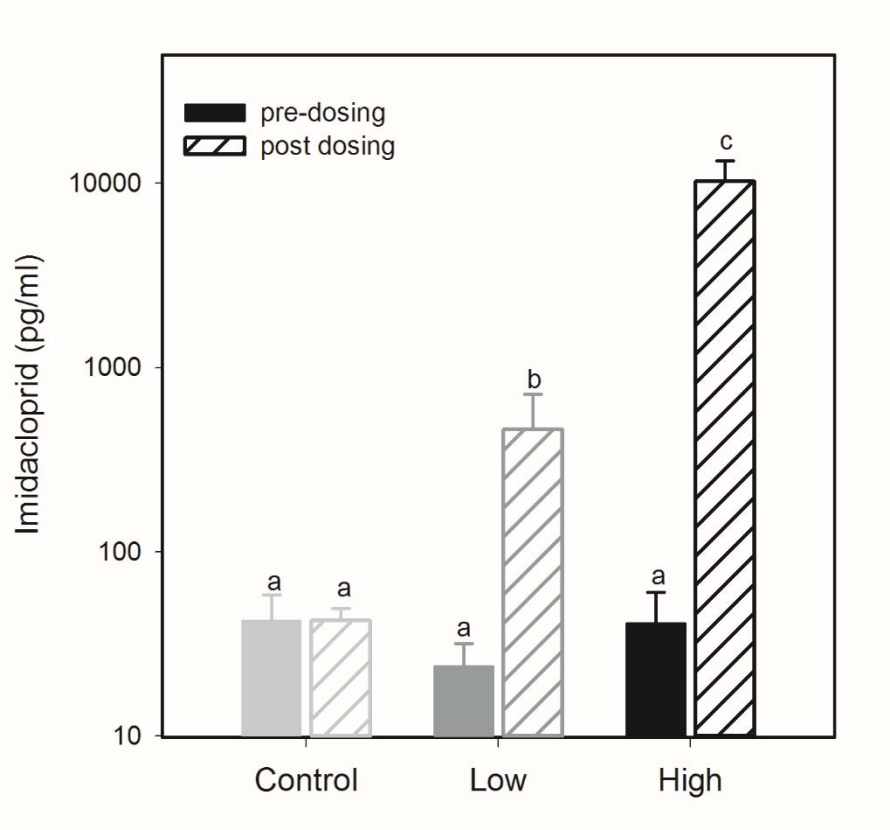

403

404 **Figure 3.** Mean (±SE) measured plasma imidacloprid concentrations (log_{10} scale) at capture (pre-
405 dosing) and 6 hours after oral exposure (post-dosing) to either the vehicle control (sunflower oil),
406 1.2 (low) or 3.9 (high) mg imidacloprid per kg body weight. Significant differences ($\alpha < 0.05$)
407 between groups indicated with different lower case letters.

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Table S1. Comparison of Imidacloprid low (1.2 mg IMI/kg bw/day) and high (3.9 mg IMI/kg bw/day) doses to potential exposure through seed consumption based on current application rates in Canada[a] and the US[b]. Estimates are based on an average white-crowned sparrow body mass of 27 g, with average seed consumption 7.9 g/day[c]

	Cereals[e] US	Cereals Canada	Canola US	Canola Canada	Corn US	Corn Canada	Sunflower US
Average mass of individual seed (g)[d]	0.035	0.035	0.003	0.003	0.38	0.38	0.05
Application rate	up to 2.4 fl oz/ 100lbs	up to 30 g a.i./ 100kg seed	up to 25.6 fl oz/ 100lbs	802 g a.i./ 100kg seed	up to 1.34 mg/ kernal	48 g a.i./ 80,000 seeds	up to 0.5 mg a.i./ seed
mg active ingredient (a.i.)/g seed	0.93	0.3	9.97	8.02	3.53	1.58	10
mg a.i. per individual seed	0.033	0.011	0.03	0.024	1.34	0.6	0.5
Number of seeds consumed per day (if 100% of diet)	226	226	2633	2633	21	21	158
mg IMI per day if diet 100% treated seeds	7.5	2.5	79	63.2	28.1	12.6	79
Number of seeds that contain equivalent to high dose (0.105 mg for a 27 g bird)	3.2	9.6	3.5	4.4	0.1	0.2	0.2
% of diet made up of treated seeds to consume equivalent to high dose	1.42	4.25	0.13	0.17	0.48	0.95	0.13
Number of seeds that contain equivalent to low dose(0.032 mg for a 27 g bird)	1	2.9	1.1	1.4	0.02	0.1	0.1
% of diet made up of treated seeds to consume equivalent to low dose	0.44	1.28	0.04	0.05	0.1	0.48	0.06

[a]Health Canada, Imidacloprid: Proposed Re-evaluation Decision. Pest Management Regulatory Agency PRVD2016-20 appendix IIa.
[b]Gaucho 600 flowable, US EPA reg 264-968
[c]food consumption values from M. Ramenofsky, R. Agatsuma, T. Ramfar, Environmental conditions affect the behavior of captive, migratory white-crowned sparrows. The Condor, 2008, 110:658-671.
[d]Seed masses from P. Mineau, C.Palmer, 2013. The impact of the nation's most widely used insecticides on birds. American Bird Conservancy
[e]wheat, barley, oats, rye, triticale