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Refined Methodology for the Determination of Neonicotinoid Pesticides and Their Metabolites in Honey Bees and Bee Products by Liquid Chromatography−Tandem Mass Spectrometry (LC-MS/MS)†

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An analytical method was refined for the extraction and determination of neonicotinoid pesticide residues and their metabolites in honey bees and bee products. Samples were extracted with 2% triethylamine (TEA) in acetonitrile (ACN) followed by salting out, solid phase extraction (SPE) cleanup, and detection using liquid chromatography−tandem mass spectrometry (LC-MS/MS). The method was validated in triplicate at three fortification concentrations in each matrix. Good recoveries were observed for most analytes and ranged between 70 and 120% with relative standard deviations between replicates of <20% in most cases. The method limits of detection were 0.2 ng/g for the parent neonicotinoid pesticides and ranged between 0.2 and 15 ng/g for the neonicotinoid metabolites. This refined method provides lower detection limits and improved recovery of neonicotinoids and their metabolites, which will help researchers evaluate subchronic effects of these pesticides, address data gaps related to colony collapse disorder (CCD), and determine the role of pesticides in pollinator decline.

KEYWORDS: Honey bee; bee pollen; bee honey; colony collapse disorder (CCD); pollinator decline; neonicotinoids; QuEChERS; LC-MS/MS

INTRODUCTION

In recent years, colony collapse disorder (CCD) and pollinator declines in general have become serious environmental concerns that could ultimately threaten the production of many crops in the United States. The magnitude of loss suffered by some beekeepers in recent years has been highly unusual with losses of 30−90% of their hives (*1*). This phenomenon, which currently does not have a directly recognizable underlying cause, has been termed "colony collapse disorder." The main symptom of CCD is simply a complete absence or a low number of adult honey bees present in the hive without evidence of mortality. Often honey, the queen bee, and immature bees (brood) remain present in CCD hives after worker bees disappear. Although disease appears to be a factor most frequently associated with the recent pollinator declines, researchers suspect that the declines likely result from a combination of factors representing environmental stressors that compromise the immune system of bees and make them more susceptible to disease. Pesticide use has been identified as a potential contributing factor to these declines and may be one of the environmental stressors contributing to pollinator declines, along with other factors such as new and re-emerging pathogens, habitat loss, pests, and nutritional stress. However, the nature and extent of the relationship between pollinator declines and pesticide use has not been fully established.

In the United States, a CCD Steering Committee mandated by Congress was formed to examine potential causes of CCD and develop approaches to its mitigation (*2*). The California Department of Pesticide Regulation (CDPR) has begun a reevaluation of chemicals in the nitroguanidine insecticide class of neonicotinoids containing the active ingredients of imidacloprid, clothianidin, dinotefuran, and thiamethoxam (*3*). In Europe, similar efforts are underway in several countries: decisions have been made to suspend or temporarily suspend the use of imidacloprid and clothianidin as seed treatments in France, Germany, Italy, and Slovenia, although foliar uses are allowed. Some of the suspensions were recently lifted in 2008 (*4*).

Neonicotinoids are a relatively new group of insecticides recently registered in the United States. Neonicotinoids show good activity against pest insects resistant to other classes of insecticides such as organophosphates, carbamates, pyrethroids, chlorinated hydrocarbons, and several other classes of compounds (*5*). Members of neonicotinoid insecticides containing the nitroguanidine moiety such as imidacloprid, dinotefuran, thiamethoxam, and clothianidin (**Figure 1**) have very selective toxicity to insects. Spraying can contaminate nectar and poison honey bees either through direct contact with the product or through contact with its residue. Seed dressing can also poison honey bees through oral contact with the parent compound or metabolite compounds. Neonicotinoids are widely used on cucurbits such as cantaloupe, cucumber, and watermelon crops and other crops pollinated by bees.

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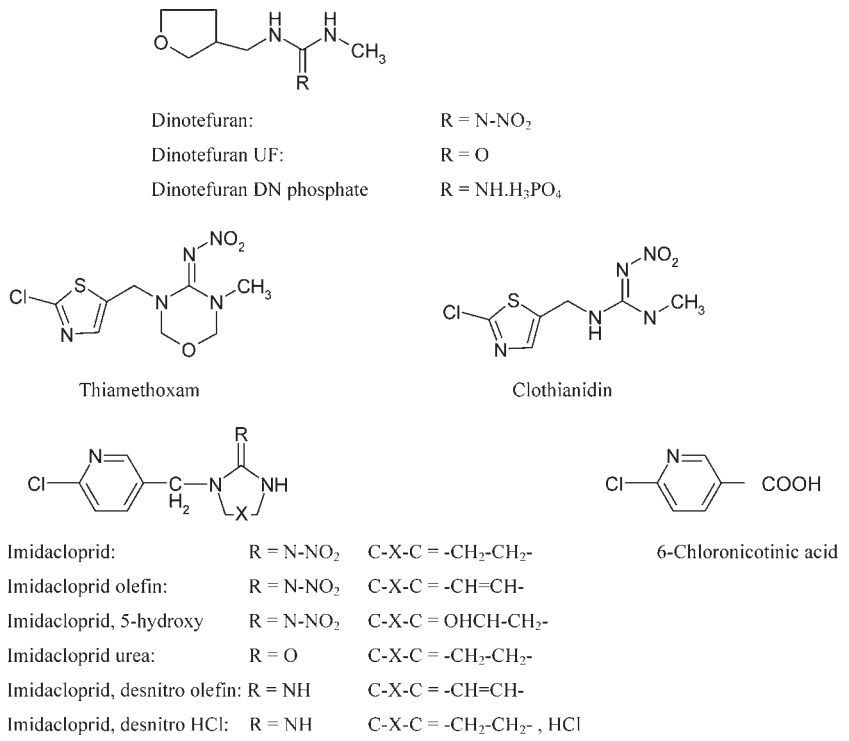


Figure 1. Chemical structures of the investigated neonicotinoids.

The nitro-substituted compounds imidacloprid, thiamethoxam, and dinotefuran are the most acutely toxic to the honey bee, with acute toxicity LD_{50} values as low as 18 ng/bee for imidacloprid, 30 ng for thiamethoxam, and 75 ng for dinotefuran (*6*). Other studies (*7*) have shown higher acute LD_{50} of imidacloprid to bees as high as 200 ng/bee. This difference can be due to a variation in detoxification capacity in honey bee colonies. The acute LD_{50} of the 5-hydroxy metabolite is similar to that of imidacloprid, whereas the acute toxicity of the olefin is higher than that of imidacloprid. The acute LD_{50} of the urea is almost half that of imidacloprid, whereas the desnitro metabolites and 6-chloronicotinic acid acute LD_{50} values are >1000 ng/bee (*8*).

In contrast to these acute LD_{50} findings, in a 10 day chronic toxicity study, all imidacloprid metabolites, including 6-chloronicotinic acid, revealed equal toxicity to bees, with a total dose ingested by the bees of about 3000−100,000 times lower than the doses needed to produce the same effect after acute intoxication. Mortality of 50% was obtained after 8 days of exposure to imidacloprid or any of its metabolites during which each bee cumulatively ingested as low as 0.1 ng/bee of the toxic substance (*8*). It was suggested that the high toxicity of imidacloprid and its metabolites, at very low doses, to honey bees could reflect the existence of binding sites with different affinities and would be more specific to the 2-chloropyridinyl moiety (*8*) (**Figure 1**). The study also showed that mortality rose with low doses, fell with intermediate doses, and rose again with high doses. The authors suggest that at high doses imidacloprid and the metabolites that resemble it (olefin, 5-hydroxy, and urea) fit into specific receptors binding to the guianidine ring differently from the high-affinity receptors binding to the 2-chloropyridinyl moiety, which all compounds may act on at very low doses (*8*).

Because neonicotinoids containing the nitroguanidine group may be harmful to bees at concentrations of <1 ng/g, many attempts have been undertaken to improve analytical methods for quantifying these insecticides. Most analytical methods have focused on the parent compounds and the metabolites of significant acute toxicity. Because chronic studies have shown that the metabolites may also be toxic to bees, a rugged and reliable analytical method for the analysis of parent neonicotinoids and all metabolites is needed. Unlike other methods based on liquid chromatography (LC), and coupled with ultraviolet (UV), diode array (DAD) (*9*), electrochemical and postcolumn photoactivation (*10*, *11*) or a fluorometric detector (*12*), liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has been shown to be one of the most sensitive techniques in pesticide residue analysis. Although other researchers have used LC with electrospray ionization mass spectrometry (ESI-MS) (*13*) or atmospheric pressure chemical ionization source−mass spectrometry (APCI-MS) (*14*), the reported methods did not include several neonicotinoid metabolites and the detection limits were >1 ng/g. Recently published extraction and cleanup methods used on honey bees, pollen, and bee honey included liquid−liquid partitioning and solid phase extraction (SPE) (*15*), micro solid phase dispersion (MSPD) (*16*), and dispersive solid phase extraction, known as QuEChERS (*17*). However, these methods have yielded poor recoveries for the imidacloprid desnitro olefin and HCl metabolites, 6-chloronicotinic acid, and the dinotefuran metabolites UF and DN.

The present work aims to refine the QuEChERS extraction approach and develop an analytical method for the analysis of neonicotinoid insecticides and their metabolites. The target recoveries are aimed to fall within 70−120% and to reach sub-parts-per-billion detection limits in bees, bee pollen, and bee honey to help risk assessors determine the role of these insecticides in CCD and pollinator decline, if any.

MATERIALS AND METHODS

Standards and Reagents. Analytical reference materials were certified from their respective manufacturers. They were imidacloprid (99.4%), imidacloprid olefin (97.9%), imidacloprid, 5-hydroxy (99.3%), imidacloprid urea (99.4%), desnitro imidacloprid olefin (99.9%), desnitro imidacloprid HCl (97.9%), 6-chloronicotinic acid (99.3%), clothianidin (99.6%) (Bayer Crop Science, Kansas City, MO), thiamethoxam (99.2%), clothianidin (thiamethoxam metabolite, 97.1%) (Syngenta Crop Protection, Inc.,

Table 1. MRM Precursor/Product Ion Transitions and Instrument Conditions

analyte	molecular formula	cone voltage (V)	ion transition (primary)	dwell time (ms)	collision energy (eV)	ion transition (secondary)	collision energy (eV)
imidacloprid	$C_9H_{10}ClN_5O_2$	20	256.13 > 175.03	15	18	256.13 > 209.10	14
imidacloprid olefin	$C_9H_8ClN_5O_2$	14	254.07 > 205.17	15	18	254.07 > 171.07	24
imidacloprid, 5-hydroxy	$C_9H_{10}ClN_5O_3$	22	272.14 > 190.99	15	14	272.14 > 225.08	20
imidacloprid urea	$C_9H_{10}ClN_3O$	24	212.10 > 128.10	15	18	212.10 > 78.01	40
imidacloprid, desnitro olefin	$C_9H_9ClN_4$	28	209.08 > 125.97	36	18	209.08 > 90.03	30
imidacloprid, desnitro HCl	$C_9H_{11}ClN_4$	32	211.10 > 126.00	36	22	211.10 > 90.03	36
6-chloronicotinic acid	$C_6H_4ClNO_2$	28	157.90 > 77.90	15	25	157.90 > 121.95	18
dinotefuran	$C_7H_{14}N_4O_3$	12	203.20 > 129.10	79	12	203.20 > 157.10	8
dinotefuran UF	$C_7H_{14}N_2O_2$	14	159.03 > 102.03	36	10	159.03 > 67.01	18
dinotefuran DN phosphate	$C_7H_{15}N_3O$	22	158.10 > 57.05	36	20	158.10 > 102.09	16
thiamethoxam	$C_8H_{10}ClN_5O_3S$	12	292.10 > 132.00	15	22	292.10 > 181.10	22
clothianidin	$C_6H_8ClN_5O_2S$	15	250.00 > 169.00	15	12	250.00 > 131.90	16
^{13}C-d_3-imidacloprid	$C_9H_7ClD_3N_5O_2$	20	261.20 > 180.10	15	18	261.20 > 214.10	18

Greensboro, NC), dinotefuran (99.6%), dinotefuran DN phosphate (99.1%), and dinotefuran UF (99.7%) (Mitsui Chemicals, Inc., Tokyo, Japan), as well as the internal standard ^{13}C-d_3-imidacloprid, all obtained from the U.S. EPA National Pesticide Standard Repository (Ft. Meade, MD). **Figure 1** shows the chemical structures of the investigated neonicotinoids.

Standard stock solutions of individual compounds were prepared by weighing about 50 mg of each analyte and dissolving in 50 mL of acetonitrile (ACN). The neonicotinoid metabolites were first dissolved in about 5 mL of water and diluted with ACN. Working standard solutions were prepared by mixing an appropriate amount of standard stock solutions and dilution in ACN. Calibration standards were prepared in each matrix by fortifying extracted control samples at the final stage, at four concentration levels ranging from the limit of detection (LOD) up to 10 times the limit of quantitation (10 × LOQ). Calibration curves were all linear with a correlation coefficient (r^2) of ≥0.98.

Matrix Fortification. The matrices used in this study were honey and bee pollen collected from organically grown farms purchased from a local store, as well as honeybees from bee hives unexposed to pesticide within a perimeter of 5 miles obtained from the U.S. Department of Agriculture (USDA), Agricultural Research Station (ARS), Bee Research Laboratory. Live bees were frozen at −80 °C overnight, then transferred to the laboratory, and kept frozen at the same temperature until used for analysis.

Fifteen grams of each matrix was fortified with standards of the three studied neonicotinoids and their nine metabolites in triplicate at three concentration levels equivalent to LOQ, 5 × LOQ, and 10 × LOQ. Quantitation was carried out using the internal standard ^{13}C-d_3-imidacloprid and calculated as the ratio between the responses of the analyte primary ion transition and the internal standard primary ion transition.

Sample Preparation. Extraction and cleanup were based on the QuEChERS method introduced by Anastassiades et al. (*17*) and modified as follows: Homogenized control samples (15 g) were fortified at appropriate concentrations and extracted with 12 mL of water and 15 mL of 2% triethylamine (TEA) in ACN with a tissuemizer for 3 min. To each sample were added 6 g of magnesium sulfate anhydrous ($MgSO_4$) and 1.5 g of sodium acetate (NaOAc) (UCT, Bristol, PA) and shaken using a Geno Grinder (SPEX CertiPrep Inc., Metuchen, NJ) at 1200 strokes per minute (spm) for 2 min. Samples were then centrifuged (Jouan Inc., VA) for 5 min at 2500 rpm. The organic supernatant was transferred to a 15 mL tube containing 0.5 g of $MgSO_4$ and shaken to remove moisture. Twelve milliliters of the extract was passed through a C_{18} SPE cartridge (1 g, Phenomenex, CA) preconditioned with 3 mL of 2% TEA in ACN with the aid of a vacuum or positive pressure and rinsed with an additional 10 mL of 2% TEA/ACN. The combined eluants were evaporated to dryness in a water bath under a stream of N_2. Samples were then reconstituted to 1 mL of water/methanol (75:25) by adding 1 mL of the internal standard solution (10 ng/mL in water/methanol (75:25)). Samples were filtered through 0.7 μm glass filter disks (GF/F, Whatman, Maidstone, U.K.) followed by 0.2 μm nylon filter discs (Pall Life Sciences, Ann Arbor, MI) into liquid chromatography (LC) vials.

Liquid Chromatography−Tandem Mass Spectrometry (LC-MS/MS) Instrument. The LC-MS/MS instrument consisted of a Waters Acquity ultraperformance liquid chromatograph (UPLC) equipped with a 10 cm × 2.1 mm (i.d.), 1.8 μm particle size, Acquity HSS T3 column (Waters, Milford, MA) or a 5 cm × 2.1 mm (i.d.), 1.9 μm particle size, Ultra II Aromax column (Restek) coupled to a Waters Xevo TQ triple-quadrupole mass spectrometer operated in the positive electrospray ionization mode. The LC was operated under gradient conditions with mobile phases of water/methanol (95:5) + 5 mM ammonium formate + 0.1% formic acid and (A) and water/methanol (5:95) + 5 mM ammonium formate + 0.1% formic acid (B) at a flow rate of 0.35 mL/min and 40 °C . The initial mobile phase composition for the HSS-T3 column was 95% A, which was held for 3 min, followed by a linear gradient to 60% A in 12 min, then 95% B was held for 2 min. The analytical column was then equilibrated at the initial conditions for 2 min for a total run time of 19 min. Conditions for the Aromax column were the same except that the initial conditions were held for 1 min instead of 3 min and the gradient was extended to 15 min. The injection volume was 5 μL. A third C_{18} column was also used (Acquity BEH C_{18}, 10 cm × 2.1 mm, 1.8 μm particle size, Waters), for comparison. The HSS-T3 column was used for the method validation.

The MS source temperature was set at 120 °C with nitrogen flow rates of 50 and 1000 L/h for the cone and desolvation gases, respectively. The desolvation temperature was 450 °C. Argon was used as the collision gas with a flow of 0.15 mL/min, which produced a pressure of 4×10^{-3} mbar in the collision cell. Optimization of cone voltage and collision energy (CE) for each analyte was achieved by infusing 100 ng/mL standards at a rate of 5 μL/min, combined with 0.35 mL/min of a 1:1 ratio of mobile phases A/B. The mass spectrometer was operated in multiple reaction monitoring mode (MRM) with monitoring of two precursor/products ion transitions for each analyte. The target ion transition with highest intensity (primary ion transition) was used for quantitation, whereas the second target ion transition was used for confirmation. Further confirmation was obtained through a product ion scan (PIC) for each peak, which was matched to a reference spectrum for each analyte. The instrument uses Target Lynx software version 4.1 (Waters Corp.) for quantitation and confirmation calculations. Ion transitions, cone voltages, and collision energies for the analytes are shown in **Table 1**.

RESULTS AND DISCUSSION

Extraction and Cleanup. The QuEChERS approach is composed of an extraction step with acetonitrile and partitioning using $MgSO_4$, followed by dispersive solid phase extraction using primary−secondary amine (PSA). The parent neonicotinoids were successfully recovered using QuEChERS method (*17*, *18*); however, when it was applied on bee or bee pollen samples, it yielded poor recovery of the metabolites. **Table 2** shows the average recovery percentages of bee samples fortified with imidacloprid and its metabolites in triplicate at three fortification levels and in bee pollen in seven replicates at 2 × LOQ using the QuEChERS approach. The average recoveries of the neonicotinoid metabolites from bees using QuEChERS ranged between 19 and 30% for desnitro imidacloprid olefin and between 12 and

15% for desnitro imidacloprid HCl; there were no recoveries for 6-chloronicotinic acid, compared to 68−73, 49−68, and 72−93%, respectively, for the same metabolites when the refined method was applied. A similar trend of recovery was obtained for bee pollen. The imidacloprid metabolite 6-chloronicotinic acid was completely unrecovered because it was bound to the PSA, which is known to retain pesticides containing carboxylic acid groups. Modifications to this approach were made to successfully recover most of the neonicotinoid metabolites. PSA was replaced with C_{18} SPE cartridges, eluted with 2% TEA, a strongly polar base, which aids the elution of the metabolites bound to the C_{18} sorbent. The polarity of the eluting solvent is a critical step to recover the neonicotinoid metabolites. We found that using either 1−5% acetic acid (AcOH) or 1% TEA in ACN was insufficient to efficiently desorb the metabolites from the C_{18} cartridge and that 5% TEA in ACN gave the same recovery as 2% TEA in ACN. It was necessary to use SPE cartridges rather than dispersion of the C_{18} in the matrix extract, because loss of some of the analytes was noted if vacuum or positive pressure was not used during elution.

Table 2. Average Recovery Percentages (± Standard Deviation) of Bee and Bee Pollen Fortified Samples with Imidacloprid and Its Metabolites Using the QuEChERS Approach[a]

	bees $n = 3$			bee pollen $n = 7$
analyte	LOQ	5 × LOQ	10 × LOQ	2 × LOQ
imidacloprid	100.7 ± 18.1	95.1 ± 16.0	103.7 ± 15.7	97.6 ± 24.0
imidacloprid olefin	79.9 ± 12.2	95.1 ± 11.5	103.2 ± 7.5	69.6 ± 10.5
imidacloprid, 5-hydroxy	84.2 ± 17.2	95.1 ± 11.4	99.7 ± 12.9	75.4 ± 12.1
imidacloprid, urea	78.7 ± 11.7	90.0 ± 11.3	95.8 ± 9.3	na
imidacloprid, desnitro olefin	18.9 ± 6.4	25.6 ± 3.5	29.9 ± 0.9	na
imidacloprid, desnitro HCl	14.8 ± 2.7	12.5 ± 1.5	14.0 ± 2.6	na
6-chloronicotinic acid	nd	nd	nd	nd

[a] n = number of replicates. na, not analyzed; nd, not detected.

Table 3. Effect of Salting-out Agents (Average ± Standard Deviation, $n = 3$) on the Recovery of Neonicotinoid Insecticides and Their Metabolites from Fortified Bee Pollen[a]

analyte	NaOAc	NaCl	sodium citrate
imidacloprid, desnitro olefin	107.3 ± 13.2	26.2 ± 9.8	21.5 ± 2.8
imidacloprid, desnitro HCl	48.6 ± 14.5	28.6 ± 5.2	27.9 ± 8.6
dinotefuran	87.2 ± 9.7	46.6 ± 18.2	26.3 ± 4.1
dinotefuran UF	74.0 ± 8.7	nd	nd
dinotefuran DN phosphate	132.6 ± 27.4	40.2 ± 18.3	86.0 ± 26.0
thiamethoxam	84.1 ± 8.4	55.7 ± 10.4	45.8 ± 8.1

[a] n = number of replicates. nd, not detected.

The final dried extract was reconstituted in 1 mL of a mixture of H_2O/MeOH (75:25), which was filtered through 0.7 μm glass membrane filter disks followed by a 0.2 μm nylon filter disk. The strongly aqueous solvent and the hydrophilic membrane disks helped eliminate undesired matrix material through filtration, which might cause interferences in the chromatography and in the mass spectrometer. Other filter disk types such as the hydrophobic polyvinylidene fluoride (PVDF) retained the polar metabolites, especially imidacloprid desnitro HCl.

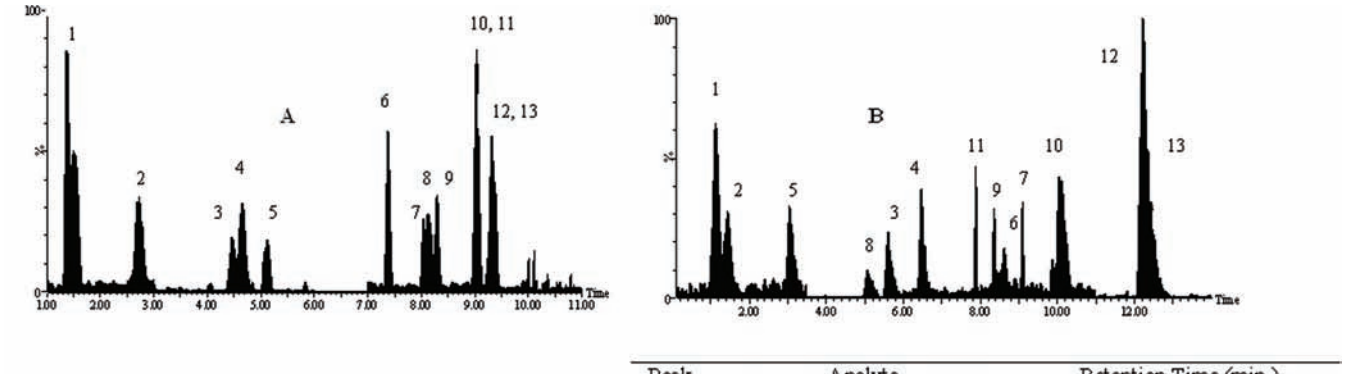
Effect of Salting-out Agents on the Recovery of the Analytes. The effect of salts in the partitioning step was also investigated in bee pollen, one of the complicated matrices. A comparison between adding combinations of NaCl, NaOAc, and sodium citrate (1.5 g of trisodium citrate dehydrate + 0.75 g of disodium hydrogen citrate sesquihydrate) to $MgSO_4$ showed that there were insignificant differences when using any of the salts on imidacloprid, imidacloprid olefin, 5-hydroxy imidaclorprid, imidacloprid urea, 6-chloronicotinic acid, and clothianidin. However, a significant effect of the type of salt used was observed with the remaining neonicotinoids. The lowest recovery of imidacloprid desnitro olefin (22−26%), imidacloprid desnitro HCl (28−29%), dinotefuran (26−47%), dinotefuran UF (unrecovered), dinotefuran DN (40%), and thiamethoxam (46−56%) was obtained when NaCl or sodium citrate was added to $MgSO_4$, although dinotefuran DN was successfully recovered by the addition of sodium citrate (86%). NaOAc, on the other hand, yielded the highest recovery for these compounds when added to $MgSO_4$, although imidacloprid desnitro HCl was not completely recovered (47%). These salts play an important role in "salting out" the organic compounds from the aqueous layer into the organic layer in the partitioning step of the extraction. **Table 3** shows the average recovery percent and standard deviation after the addition of each of NaOAc, NaCl, and sodium citrate to $MgSO_4$. In addition, we found that analyte recoveries were reduced if the extract was not dehydrated with 0.5 g of $MgSO_4$ before it was applied onto the C_{18} SPE cartridge.

Recovery Studies. The three matrices, bee carcass, bee pollen, and honey, were fortifed with 12 neonicotinoid parents and metabolites in triplicate at levels equivalent to LOQ, 5 × LOQ, and 10 × LOQ. The LOQ was calculated as the lowest concentration of each analyte's response to the most abundant ion transition (quantitation ion transition) which yielded a signal-to-noise (S/N) ratio of at least 10:1 and for which the secondary ion transition (confirmation ion transition) yielded a S/N ratio of at least 3:1. The LOQ is therefore equivalent to 3.33 times the LOD, the response of which yields a S/N of at least 3:1 for both ion transitions. The LOD, average recovery percent, and

Table 4. Average Recovery Percentages (± Standard Deviation) of Fortified Bee, Bee Pollen, and Bee Honey Samples[a]

		bees			bee pollen			bee honey		
analyte	LOD (ng/g)	LOQ	5 × LOQ	10 × LOQ	LOQ	5 × LOQ	10 × LOQ	LOQ	5 × LOQ	10 × LOQ
imidacloprid	0.2	89.2 ± 7.7	101.6 ± 13.5	96.0 ± 17.5	108.2 ± 13.2	109.2 ± 10.6	122.8 ± 9.9	53.5 ± 7.5	67.7 ± 8.5	78.1 ± 3.0
imidacloprid olefin	3.6	98.6 ± 9.4	107.1 ± 4.1	104.2 ± 16.7	112.1 ± 10.6	107.8 ± 2.3	113.2 ± 11.4	90.0 ± 17.8	75.9 ± 12.3	93.6 ± 12.0
imidacloprid, 5-hydroxy	2.4	103.7 ± 6.9	99.6 ± 5.7	99.4 ± 16.9	99.6 ± 12.0	100.7 ± 13.9	109.7 ± 4.1	124.2 ± 32.7	85.4 ± 10.1	85.7 ± 5.3
imidacloprid, urea	0.2	92.1 ± 19.5	99.8 ± 6.2	96.6 ± 6.5	112.1 ± 8.1	103.7 ± 17.1	122.8 ± 4.9	97.3 ± 9.6	85.6 ± 2.3	88.4 ± 4.7
imidacloprid, desnitro olefin	0.2	71.6 ± 9.1	68.1 ± 7.9	72.9 ± 2.7	70.0 ± 8.8	67.1 ± 5.0	72.8 ± 8.0	101.9 ± 12.4	97.4 ± 2.3	99.3 ± 7.5
imidacloprid, desnitro HCl	0.2	67.9 ± 2.0	48.8 ± 4.2	50.2 ± 5.2	43.7 ± 0.8	41.7 ± 5.1	46.4 ± 6.8	49.6 ± 14.1	35.3 ± 2.6	39.4 ± 3.1
6-chloronicotinic acid	15	93.0 ± 10.7	72.2 ± 7.6	82.9 ± 2.8	81.9 ± 11.9	79.8 ± 10.1	91.7 ± 19.4	76.6 ± 8.6	64.1 ± 1.5	64.1 ± 3.6
dinotefuran	0.2	69.7 ± 2.3	61.7 ± 15.3	92.1 ± 3.8	87.2 ± 9.7	78.4 ± 12.1	119.2 ± 23.9			
dinotefuran UF	1	88.5 ± 9.6	85.4 ± 12.0	91.5 ± 2.6	99.5 ± 6.1	95.8 ± 5.5	107.8 ± 12.3			
dinotefuran DN phosphate	2	na	na	na	132.6 ± 27.4	na	na			
thiamethoxam	0.2	80.6 ± 20.7	69.3 ± 12.6	89.4 ± 4.5	110.5 ± 12.8	91.1 ± 14.8	109.2 ± 15.5			
clothianidin	1.2	94.2 ± 11.3	101.2 ± 16.9	98.9 ± 2.0	111.9 ± 5.5	119.4 ± 24.9	144.7 ± 9.2			

[a] na, not analyzed.



Peak	Analyte	Retention Time (min.)		
		HSS-T_3 (A)	Aromax (B)	BEH C_{18} (C)
1	dinotefuran DN	1.38	1.10	1.1
2	dinotefuran UF	2.74	1.45	1.67
3	imidacloprid, desnitro olefin	4.45	5.60	2.74
4	imidacloprid, desnitro HCl	4.65	6.45	2.84
5	dinotefuran	5.12	3.04	4.05
6	thiamethoxam	7.37	8.60	4.74
7	imidacloprid olefin	8.03	9.09	7.39
8	chloronicotinic acid	8.14	5.04	n.a.
9	imidacloprid, 5-hydroxy	8.29	8.39	8.04
10	imidacloprid urea	9.04	10.02	6.41
11	clothianidin	9.09	7.87	6.38
12	^{13}C, d_3-imidacloprid	9.34	12.11	n.a.
13	imidacloprid	9.38	12.30	6.61

n.a. = not analyzed

Figure 2. Overlaid total ion chromatograms of ion transitions monitored for the neonicotinoids and metabolites on three different columns: (**A**) Acquity HSS T_3; (**B**) Ultra II Aromax; (**C**) Acquity BEH C_{18}. Retention times for each analyte and for each column are given in the included table.

standard deviation from the three replicates for each analyte are shown in **Table 4**.

Recovery percent of each analyte was calculated as the concentration found divided by the concentration added multiplied by 100. In general, the accuracy of the method (recovery percent) and its precision (standard deviation between replicates) were acceptable. Recoveries obtained for all analytes ranged from 70 to 120% with relative standard deviations of $<20\%$, with few exceptions. Imidacloprid desnitro HCl was the least recovered analyte despite the modifications made to the extraction solvent and salting-out agent and yielded a recovery of 49−68% in bees, 42−46% in bee pollen, and 35−50% in bee honey. The refined method significantly improved recoveries of both of the desnitro imidacloprids and 6-chloronicotinic acid. Recoveries of the dinotefuran and thiomethoxam metabolites ranged between 85 and 101% in bees and between 96 and 145% in pollen. Dinotefuran DN phosphate was analyzed in only bee pollen.

Liquid Chromatography−Mass Spectrometry (LC-MS/MS). Ultraperformance liquid chromatography (UPLC) provided good peak resolution and increased sensitivity, leading to more accurate residue determinations of the amounts of analytes. The T_3 bonded phase in the HSS-T_3 column was adequate to overcome the early elution and peak broadening of the dinotefuran metabolites UF and DN phosphate as well as the desnitro imidacloprid metabolites in the strongly aqueous mobile phases. T_3 bonding utilizes a trifunctional C_{18} alkyl phase bonded at a ligand density that promotes polar compound retention and aqueous mobile phase compatibility. The Aromax column is also a reversed phase material that exhibits retention and selectivity for aromatic and/or unsaturated compounds compared to conventional alkyl and phenyl phases. This column is a great alternative to C_{18} or bonded phase when increased retention is required. The peak shapes of the dinotefuran metabolites as well as the desnitrozimidacloprid metabolites were greatly improved and better retained on this type of stationary phase; however, peak tailing was observed, especially for late eluters, at high percentage organic mobile phase conditions. **Figure 2** shows the overlaid ion chromatograms of the monitored ion transitions of all the analytes injected into the three chromatographic columns as well as their retention times in each column.

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