Improved Method for Quantifying the Avicide 3-Chloro-*p*-toluidine Hydrochloride in Bird Tissues Using a Deuterated Surrogate/GC/MS Method

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A method using a deuterated surrogate of the avicide 3-chloro-*p*-toluidine hydrochloride (CPTH) was developed to quantify the CPTH residues in the gastrointestinal (GI) tract and breast muscle tissues in birds collected in CPTH-baited sunflower and rice fields. This method increased the range of a previous surrogate/gas chromatography/mass spectroscopy method from 0–2 to 0–20 μg/g in tissue samples and greatly simplified the extraction procedure. The modified method also sought to increase recoveries over a range of matrix effects introduced by analyzing tissues from birds collected in the field, where the GI tract contents would be affected by varying diet. The new method was used to determine the CPTH concentration in GI tract samples fortified with CPTH-treated rice bait to simulate the consumption of varying amounts of treated bait by two nontargeted bird species, pigeon (*Columbia livia*) and house sparrow (*Passer domesticus*). The new method was then used to examine the CPTH concentrations in the gizzard contents of the targeted bird species, red-winged black bird (*Agelaius phoeniceus*) and brown-headed cowbird (*Molothrus ater*), that were collected after feeding at a treated bait site. The method proved sufficiently sensitive to quantify CPTH in the breast muscle tissues and the gizzard contents of red-winged blackbirds and brown-headed cowbirds during an operational baiting program. The levels of CPTH determined for these birds in both tissue samples were determined to be highly correlated. The appearance of CPTH in the breast muscle tissue immediately after feeding was not anticipated. The potential secondary hazard posed by the targeted birds to potential scavengers and predators was also evaluated.

KEYWORDS: 3-Chloro-*p*-toluidine hydrochloride; CPTH; DRC-1339; secondary hazard

INTRODUCTION

The avicide CPTH (3-chloro-*p*-toluidine hydrochloride) is used to control 18 primary target species of pest birds and 3 secondary target species. Pest birds are controlled where they damage crops such as rice or sunflower (blackbirds, Icterinae); prey on young livestock or other important or protected species (gulls and corvids); or are a nuisance or a health risk (pigeons, *Columbia livia*), as in large roosts in metropolitan areas (Eisemann, personal communication). CPTH has been perceived to provide a degree of selectivity; it is more toxic to targeted species than nontargeted species (*1*, *2*). Early methods for characterizing CPTH exposure in birds were based on necropsying the carcass and looking for physiological characteristics of CPTH exposure, including the accumulation of uric acid deposits in the peritoneal and pericardial cavities (*1–3*). Early

analytical methods for assaying residues in tissues proved to be difficult, time-consuming, and had poor repeatability (*4*). Improvements in sensitivity and repeatability were made using a deuterated surrogate of CPTH (*5*). Continuing efforts in improving analytical methods for the detection of CPTH residues in bird tissues are being driven in part by Environmental Protection Agency registration requirements for the continued use of CPTH as an avicide. Of particular concern are the body levels of CPTH in target animals, the amounts of CPTH consumed by nontarget birds, and the risks these birds pose to scavengers or predators.

Blackbirds, brown-headed cowbirds (*Molothrus ater*), starlings (*Sturnus vulgaris*), and grackles (*Quiscalis* spp.) are commonly controlled through the application of a 2% CPTH (w/w)-treated rice bait mixed 1:25 with untreated rice. CPTH is slow-acting, requiring 4–160 h to result in the death of a bird that has consumed a sufficient amount of treated bait (*1–4*, *6*). Birds may not consume a sufficient amount of treated bait to be toxic due to an aversion from discoloration of the

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bait (*7*, *8*) or some taste, smell, or texture associated with CPTH. Establishing the effects of CPTH on birds in the field has proven problematic given the slow-acting nature of the toxicant and the ability of the birds to leave the treatment site after feeding on the bait (*6*, *9*).

This study sought to refine the method of Hurlbut et al. (*5*) for determining the CPTH residues in bird tissues and to apply this method to quantify CPTH residues in blackbirds collected at bait sites immediately after feeding. The refinements were required as the original method did not provide adequate repeatability across a large number of samples and diverse matrices. The residue data collected using the modified method would then permit us to ascertain the amount of treated bait consumed and the potential secondary hazards to predators consuming birds containing CPTH residues. The term "blackbirds" refers collectively to red-winged blackbirds (*Agelaius phoeniceus*) and brown-headed cowbirds; both species were collected for this study. The method was developed and validated using CPTH-fortified tissue samples from pigeons. This method was further evaluated using a blind treatment where treated bait was added as a fortification to gastrointestinal tract (GI) tissue samples from pigeons or house sparrows (*Passer domesticus*) that had been collected in a location with no history of CPTH use, which would simulate the samples collected from birds feeding at bait sites in the field. This method was then used to determine residues in the gizzard contents and breast muscle tissues from blackbirds collected at field bait sites.

MATERIALS AND METHODS

Reagents. Solvents used include hexane, J. T. Baker, HPLC grade; isopropyl alcohol (IPA), Mallinckrodt, analytical reagent; acetonitrile, Fisher, HPLC grade; *n*-butyl acetate, B&J, high purity; and H_2O, distilled. Chemicals used include CPTH, Purina Mills, technical grade; *p*-toluidine, Aldrich, analytical reagent; NaCl, Fisher; NaOH, Fisher, 50% w/w in H_2O; and HCl, Fisher, reagent grade. Deuterated CPTH was synthesized according to Hurlbut et al. (*5*).

Gas Chromatography/Mass Spectrometry (GC/MS). Standards and tissue extracts were analyzed with a Hewlett-Packard 5890 gas chromatograph and 5970 mass selective detector. A 1 μL sample was injected onto a 4 mm cyclosplitter (Restek, Siltek) deactivated liner at 200 °C. The analytes were separated on a DB-5-MS, 30 m × 0.25 mm (i.d.) column, 0.25 μm film. The head pressure on the column was 15 psi, with a split vent flow rate of 60 mL/min and a purge vent flow rate of 1 mL/min. The oven temperature was programmed with an initial temperature of 70 °C, held for 1 min, ramped to 160 °C at 15 °C/min, followed by a ramp to 300 °C at 70 °C/min. This temperature was held for 19 min to bake off any residual compounds retained by the column. The total run time was 27 min. Ionization was by electron impact (70 eV). Spectra were collected in single-ion monitoring mode with ions $m/z = 141$ monitored for CPTH and 147 for CPTH-D_6.

Standard Preparation and Quantification. Standards were prepared using an extraction procedure similar to that for the tissue samples. CPTH and/or CPTH-D_6 were partitioned as the free base into ethyl acetate from NaCl-saturated water solutions. Stock solutions containing 1000 μg/mL CPTH and 1350 μg/mL CPTH-D_6 were prepared in water and were diluted to 100 and 20 μg/mL. Standards containing both CPTH and CPTH-D_6 at concentrations of approximately 20.0, 15.0, 10.0, 1.0, 0.1, and 0.05 μg/mL CPTH and 1.0 μg/mL CPTH-D_6 were prepared by adding volumes of respective 100 and 20 μg/mL stock solutions to NaCl-saturated water for a final volume of 1.00 mL. To each solution, 1.0 mL of 2.0 M NaOH was added. The solution was extracted 3 times with 2.0 mL of 50 μg/mL *p*-toluidine in ethyl acetate. The solutions were shaken for 10 min on a platform shaker (Eberbach Equalpoise, Ann Arbor, MI). The ethyl acetate separated from the aqueous phase and was pipeted off. The ethyl acetate solutions were pooled in a 10.00 mL volumetric flask and brought to volume with ethyl acetate. The concentration of the CPTH and CPTH-D_6 was determined in each

solution using GC/MS as described above. To calculate the concentration, the ratio of peak area for $m/z = 141$ for CPTH was divided by the peak area for $m/z = 147$ for CPTH-D_6 in each standard. This was regressed against the CPTH concentration in the standard using SAS version 6.11. This equation was used to determine the CPTH concentration in extracts from tissue samples. During validation, two separate sets of standards were prepared from two sets of stock solutions.

Sample Extraction and Analysis. Three sample types, breast muscle tissue (1.0 g), GI tissue including contents (2.0 g), or gizzard contents alone(0.5 g), were analyzed. All samples were extracted 3 times with 3.0 mL of 80% 1 M HCl and 20% acetonitrile. At each addition, the samples were shaken for 10 min on a platform shaker (Eberbach) followed by centrifugation (Fisher Scientific) at 3600g for 5 min. The extracts were combined in a Teflon centrifuge tube containing 5.0 g of NaCl. To this, 10.0 mL of 2.0 M NaOH was added to convert the CPTH residues to the free base form (CPT). The CPT was partioned into hexane by extracting the acid/base solution 3 times with 5.0 mL of hexane. After each hexane addition, the samples were shaken for 10 min and centrifuged for 5 min at 3600g. After 50 μL of IPA was added, the extracts were eluted through a silica solid phase extraction (SPE) column (IST, 1 g solid phase; Jones Chromatography, Lakewood, CO). The SPE columns were pretreated with 2.0 mL of ethyl acetate, 2.0 mL of 50 μg/mL *p*-toluidine in ethyl acetate, and 2.0 mL of ethyl acetate, followed by 5.0 mL of hexane. The analytes were recovered by elution with 50 μg/mL *p*-toluidine in ethyl acetate into graduated test tubes that had been prerinsed with 2.0 mL of 50 μg/mL *p*-toluidine in *n*-butyl acetate and brought to a final volume of 2.00 mL. The CPTH and CPTH-D_6 were quantified by GC/MS of the final solution. To calculate the concentration of CPTH in the sample extracts, the ratio of peak area for $m/z = 141$ for CPTH was divided by the peak area for $m/z = 147$ for CPTH-D_6 in each sample. The concentration in samples was calculated from a linear regression equation using the ratio of peak areas for $m/z = 141$ and 147 from a set of standards. When the concentration was outside the linear range, samples were diluted and reanalyzed.

Method Development and Validation. *System Sensitivity and Linearity.* To establish the linear range of the method, two different standard stock solutions (956.8 and 1041 μg/mL) were used to prepare two sets of standards across the range of 0.048−20.8 μg/mL CPTH (20.8, 10.4, 1.04, 0.104, and 0.0502 from the 1041 μg/mL stock solution; 19.1, 9.59, 0.957, 0.957, and 0.0478 from the 956.8 μg/mL stock solution). Each solution also contained 1.0 μg/mL CPTH-D_6. Five concentrations of standard from each stock solution were prepared, and these were analyzed using the GC/MS method described above. Solvent blanks containing only 50 μg/mL *p*-toluidine in *n*-butyl acetate were also analyzed. Each solution was analyzed by GC/MS. The ratios for the peak area for $m/z = 141$ for CPTH were divided by the peak areas for $m/z = 147$ for CPTH-D_6 in each standard. This was regressed, using a least squares linear regression model, against the CPTH concentration in each standard using SAS version 6.11. The data were also log−log transformed and regressed to determine that the data were linear over the range of CPTH concentrations used. The instrument limit of detection (ILOD) was calculated during every set of runs from the peak heights for the lowest level standard (0.0502 and 0.0478 μg/mL) CPTH solutions using $m/z = 141$, where the ILOD was defined as a signal peak height 3 times the average baseline (peak to peak) determined from the replicate solvent blanks.

Extraction Validation. Dead pigeons were obtained from a local pigeon breeder in Fort Collins, CO. Pigeons were necropsied, and the GI tract, including contents, and breast muscle tissue were removed. These tissues were composited from 3 to 4 birds after grinding in a Warring blender. Immediately before extracting, 1.00 g (with a range of ±0.20 g) of GI tract samples was fortified with CPTH dissolved in water at concentrations of 0.51, 1.02, or 10.2 μg/g and 2 μg/g of CPTH-D_6. Two grams of breast muscle tissue samples was fortified with CPTH dissolved in water at 0.51 or 10.2 μg/g and 1 μg/g of CPTH-D_6. Seven replicates were fortified at each concentration for each tissue type. Six control samples with no CPTH or CPTH-D_6 for each tissue type were also extracted. All samples were vortexed following fortification. The fortified samples were allowed to sit for 15−60 min before they were extracted. The extracts were analyzed using the previously described

GC/MS method. The percent recovery was calculated for each sample at each fortification level. The method limit of detection (MLOD) was calculated from the peak heights for $m/z = 141$ for CPTH in the 0.5 μg/g fortified tissue samples and the unfortified controls. The MLOD was defined as the signal peak height required to be 3 times the baseline (peak to peak) in the unfortified controls. Five standards containing approximately 20.0, 15.0, 10.0, 1.0, 0.1, and 0.05 μg of CPTH and 1.0 μg/mL CPTH-D_6 were run every 10 samples to ensure system stability over the run. The concentrations were determined from a linear regression equation that was based on the peak areas for all of the standards run over the course of the analysis.

Blind Study. Three house sparrows and two pigeons were collected by U.S. Geological Survey field personnel to complement samples collected during an evaluation of nontarget species foraging in sunflower fields in North Dakota where CPTH-treated bait was being applied. The birds were collected on a farm near Chaseburg, Vernon County, Wisconsin on October 5 and October 11, 2000. The GI tracts were removed and placed in chemically clean glass vials. One GI tract from each bird species was fortified with three treated rice bait seed. The seed was inserted inside the GI tract. Another GI tract from each bird species was fortified with one treated rice seed. The third house sparrow GI tract was fortified with one treated rice bait that had been weathered in the sun for 3 days. Samples were frozen and stored at −28 °C at the Upper Midwest Environmental Sciences Center, La Crosse, Wisconsin until they were shipped to the United States Department of Agriculture (USDA), National Wildlife Research Center (NWRC), Fort Collins, Colorado on October 23, 2000. Samples remained in frozen storage until analyzed.

Two replicates of GI tract and breast muscle tissue samples from pigeon (the same tissue source used to validate the method) were fortified at 0, 0.51, or 10.2 μg/g CPTH and analyzed with the samples. This allowed for MLOD determination and a QA/QC check on the method.

Field Study. Blackbirds were collected by APHIS field personnel during a baiting operation conducted to control bird populations in fields in Vermillion Parish in Louisiana. The fields were baited with 2% CPTH-coated rice mixed 1:35 with untreated rice. The 2% treated rice was formulated at the USDA, Wildlife Services Program, Pocatello Supply Depot (Pocatello, ID). The bait was mixed with untreated rice in the field. On the day that the treated bait mixture was applied, birds were collected by shooting as they left the feeding sites. Bird carcasses were frozen and stored until necropsied. Breast muscle tissue samples and espohagus and gizzard contents were collected, placed in glass vials, and stored frozen until assayed. The entire GI tract was not collected for analysis. Control birds were collected on a day when the fields were prebaited with untreated bait.

Sample Preparation. From the red-winged blackbirds and the brown-headed cowbirds collected by shooting, two control birds and four treated birds of each species were randomly selected for analysis. From each bird analyzed in both the blind and the field study, 0.5 g of gizzard contents and 2.0 g of breast muscle tissue were collected. Each of these samples was chopped and homogenized prior to extraction. Each sample was fortified with a surrogate standard and deuterated CPTH (CPTH-D_6) at a level of 4 μg/g for the gizzard contents and 1 μg/g for the breast muscle tissue.

Blackbirds collected near Fort Collins, CO were used as QC controls. These birds were captured for another study but died in quarantine. Death was attributed to the stress of being handled during capture. The birds were frozen until necropsied. The breast muscle tissue and gizzard contents were removed and stored in glass vials and frozen until needed. The gizzard contents from each bird were combined with 0.4 g of brown rice to provide a matrix similar to that in the baited birds.

QC gizzard content samples and breast tissue samples were fortified at 0.5 and 10.0 μg/g CPTH and at a level of 4 μg/g for the gizzard contents and 1 μg/g for the breast muscle tissue with CPTH-D_6. Two untreated controls and two replicates at each fortification level were extracted and analyzed with the tissue samples.

The data from the field-collected birds were statistically analyzed to determine if there were interspecies differences in the amount of bait consumed and the concentration of CPTH residues in the gizzard contents or breast muscle tissue using a one-tailed Student's *t*-test with

$\alpha = 0.05$ (Microsoft Excel). The relationship between the CPTH residue concentration in gizzard contents and the breast tissue across all birds analyzed was determined using linear regression.

The data were also used to calculate risk quotients (*1*) for potential scavenger or arial predator species. This was done to assess the secondary hazards that these birds would potentially pose for selected predators. The residue concentrations of CPTH for the gizzard contents and breast muscle tissue were used to calculate total body residues for the birds, and these values were used to calculate possible exposures to various scavengers and predators based on the "worst case" assumption that the diet consisted entirely of birds that had ingested CPTH-treated rice bait with body burdens at the highest levels determined in the study.

RESULTS AND DISCUSSION

Method Validation. The method of Hurlbut et al. (*5*) was initially modified to allow for an increased detection range. The original procedure detected residues over a 0−2.0 μg/g level. This was perceived to be too narrow a range for the purposes of this study, as a single-treated rice grain should contain approximately 0.4 mg of CPTH (*10*). The weights for GI tracts removed from pigeons ranged from 17 to 40 g. If a pigeon consumed a single grain of treated bait, the expected concentrations of CPTH in the GI tract would range from approximately 10 to 24 μg/g.

We originally set out to use the method of Hurlbut et al. (*5*), but after analyzing a larger number of diverse samples than used in the original paper, a set of problems became apparent that could not be addressed without modifying the method. Upon casual inspection, the two methods appear very similar but differ in key ways. Our modified method used an acidic aqueous phase extraction of the tissue. The aqueous phase was separated from the tissue and was liquid/liquid extracted with hexane. The CPTH was concentrated on the SPE column and eluted in ethyl acetate.

In contrast, the method of Hurlbut et al. (*5*) started with a basic aqueous phase extraction of the tissue. Hexane was added to the aqueous phase−tissue mixture. Following the addition of IPA and centrifugation, the CPT (free base of CPTH) partitioned into the organic layer. CPT was retained on the sorbent upon eluting the organic layer through a silica SPE column. The CPT was recovered by eluting with *n*-butyl acetate. In our experience, when the hexane was added to the aqueous phase−tissue mixture, it coextracted considerable quantities of oils and lipids along with the CPT. These oils and lipids were observed to coat the silica in the SPE column and restrict the flow of both the hexane in successive extractions and the *n*-butyl acetate in the elution step. When large amounts of these coextracted compounds were observed on the SPE column, the recoveries for that sample tended to vary widely from the expected value for a fortified sample. Recoveries as low as 10% of the expected value were observed when this occurred.

Our modified method also incorporated changes to the temperature programming of the GC. A more gradual temperature ramp was used to improve resolution of the CPT from interferences that had apparently not been a problem in the original paper. After the ramp, the temperature was held at 300 °C to bake off recalcitrant compounds observed when analyzing samples from birds collected in the wild. The birds analyzed by Hurlbut et al. (*5*) had been fed a formulated feed. We had access to the same tissues analyzed by Hurlbut et al. (*5*) and observed that this step was not required for those samples.

Our modified method used only the parent ions (*m/z*) to calculate CPTH and CPTH-D_6 concentrations in the samples. The original method used a sum of areas for three ions for each
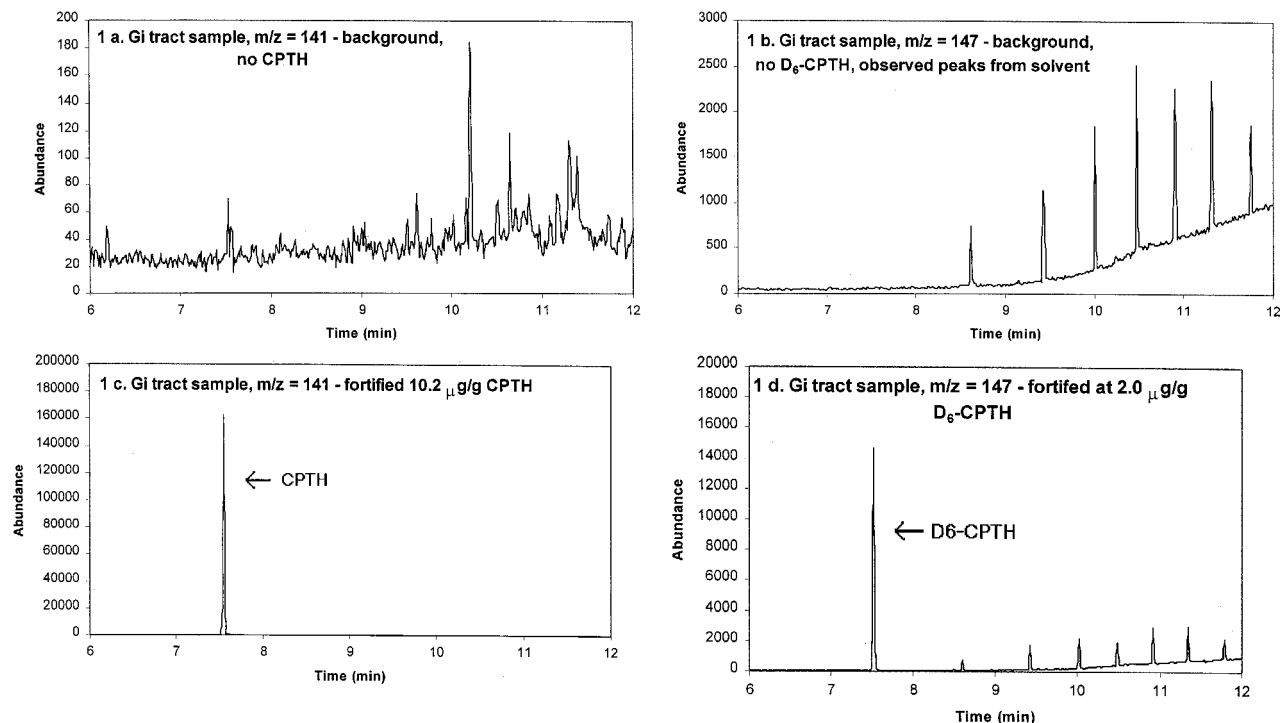
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Figure 1. GC/MS chromatograms for an unfortified pigeon GI tract sample and for a fortified pigeon GI tract sample at a level of 10.2 μg/g CPTH. Note that the scale for the *y*-axis differs across 4 orders of magnitude when comparing the figures.

compound to calculate concentration. When analyzing samples from birds collected in the wild, the relative peak area ratios reported by Hurlbut et al. (*5*) were not reproduced for CPTH and CPTH-D_6. The areas observed for the parent ions were not affected but those for ions with $m/z = 106$ and 140 for CPTH and ions with $m/z = 112$ and 149 for CPTH-D_6 varied widely. In addition to the elution problem discussed previously, this was thought to contribute significantly to the poor recoveries we observed.

A standard curve was generated across the range of 0.048–20.8 μg/mL CPTH. This corresponded to a 0.096–41.6 μg/g CPTH concentration in the GI tract and a 0.048–20.8 μg/g concentration in the breast muscle tissue, assuming 100% recovery. The equation describing the relationship between the CPTH concentration and the ratio of $m/z = 141/147$ using five concentrations of standard and two different standard stock solutions was $y = 0.876\,921x + 0.001\,35$, $r^2 = 0.9988$, where x is the CPTH concentration in the standards and y is the peak area ratios for ions (m/z) 141/147. Regressing log(ratio) vs log-(CPTH concentration) produced an equation with a correlation coefficient of $r^2 = 0.9998$. A correlation coefficient >0.999 for the log−log transformed data was interpreted as indicating that the method was linear over the range used.

The method was validated by determining recoveries of CPTH and CPTH-D_6 from seven replicate fortified tissue samples at 0.51, 1.02, and 10.2 μg/g CPTH levels for GI tract and 0.51 and 10.2 μg/g CPTH levels for the breast muscle tissues. The recovery results for CPTH from the tissue samples at the different treatment levels are presented in **Table 1**. Recoveries were required to be ±20% of the actual fortification level corrected from the CPTH-D_6 surrogate recovery data to be acceptable. The ILOD as estimated from the mean chromatographic response of three reagent blanks and the response of a 0.052 μg/mL CPTH standard (ion 141) was 0.004 μg/mL during the analysis of the breast tissue samples and 0.0054 μg/mL during the analysis of the GI tract samples.

Table 1. Percent Recovery Data for CPTH-Fortified GI Tract and Breast Muscle Tissue Samples[a]

CPTH concn (μg/g)	GI tract			breast muscle tissue		
	mean	s	CV	mean	s	CV
0.51	104	4.5	4.3	84.3	4.6	5.5
1.02	117	5.4	4.6	N/D	N/D	N/D
10.2	102	3.4	3.3	108	5.1	4.7

[a] CV is the coefficient of variation; CV = s/mean × 100. N/D is not determined.

The new method used an acid extraction of CPTH from the tissue. This improved the recovery of the CPTH at the higher concentrations and reduced the amount of coextractables that were carried into the hexane extraction. **Figure 1** depicts the chromatograms for CPTH ($m/z = 141$) and CPTH-D_6 ($m/z = 147$) in a GI tract fortified sample as well as a control with no fortification. There were no apparent matrix effects on the concentrations of CPTH or CPTH-D_6 in the fortified samples.

The MLOD was estimated from the mean MS response of seven unfortified control GI tract samples and the mean response of six control GI tract samples fortified at 0.51 μg/g CPTH. The MLOD was defined as the concentration of CPTH required to generate a signal equal to 3 times the baseline noise (measured peak to peak in the $m/z = 141$ chromatograms) observed in the control samples. Under the conditions stipulated in the method, the MLOD for CPTH in pigeon GI tract was 0.025 μg/g, and for the pigeon breast muscle, it was 0.012 μg/g. The breast muscle tissue was a much cleaner matrix and produced a less noisy baseline when compared to the GI tract samples.

Blind Study. The blind study consisted of two pigeon GI tracts and three house sparrow GI tracts that had been fortified by adding 2% CPTH-treated bait rice seed. The CPTH rice bait treatment and the corresponding results from the GC/MS are presented in **Table 2**. The house sparrows were all replicated twice, and the two pigeons were replicated 4 times to provide a more critical assessment of the method variability. As the

Table 2. Blind Study Results for CPTH-Treated Bait in GI Tracts from Pigeons and House Sparrows

individual no.	treatment	total GI tract mass (g)	replicate	CPTH (μg/g)
		House Sparrow Data		
A	3 rice bait seed	2.1	1	329
			2	201
			mean =	265
B	1 rice bait seed	3.0	1	104
			2	20.6
			mean =	62.3
C	1 weathered rice bait	2.1	1	19.2
			2	14.0
			mean =	16.6
		Pigeon Data		
D	3 rice bait seed	28.7	1	7.2
			2	24.8
			3	22.9
			4	40.2
			mean =	23.8
			s =	13.5
			CV =	56.8
E	1 rice bait seed	31.5	1	5.1
			2	1.7
			3	8.4
			4	2.0
			mean =	4.3
			s =	3.1
			CV =	72.9

Table 3. Total Sample Weights for the Breast Muscle Tissue and Gizzard Contents for Red-Winged Blackbirds and Brown-Headed Cowbirds

		mass (g)	
individual	CPTH treatment	breast muscle tissue	GI tract contents
		Red-Winged Blackbirds	
A	control	2.88	1.57
B	control	2.61	1.88
C	treated bait	2.25	2.76
D	treated bait	3.08	1.88
E	treated bait	3.13	1.55
F	treated bait	5.18	2.62
mean =		3.19	1.95
s =		0.67	0.55
		Brown-Headed Cowbirds	
G	control	5.95	0.67
H	control	7.16	1.88
I	treated bait	5.66	1.46
J	treated bait	5.66	1.74
K	treated bait	6.42	1.31
L	treated bait	7.06	1.07
mean =		6.32	1.36
s =		0.62	0.41

method required 1 g samples for extraction, the house sparrow GI tracts were too small to allow for more than two replicate samples. The samples were ground in a Warring blender, which shattered the rice seed bait into discrete fragments. Because the resulting sample was not homogeneous, there was considerable variability across replicates.

The blind study provided a semiquantitative assessment of the method. For example, while the bait was formulated to be 2% CPTH by mass, the variability of the coating on individual rice grains can be significant. Furthermore, the rice grains likely differed in mass. These factors can contribute to the variability in the fortification procedure. The CPTH level was not quantified for the treated rice on an individual grain basis during the blind study because of the perceived variability in the formulation as the CPTH-treated rice is dry formulated with a sticking agent (Hurley, personal communication). The bait was analyzed and characterized for 5 g samples. The coating was found, for the entire lot of bait produced, to be 2 ± 0.4%. The method used to analyze the bait used a larger sample size to mitigate the individual grain variability. The blind study addressed a wildlife management question: What would the analysis results look like if a bird, particularly a nontarget bird, ingested a single- or multitreated grain(s)? How might this be interpreted in a primary risk assessment, with regard to published LD$_{50}$ values for that species for CPTH? For the species evaluated, this appeared not to be a problem.

This study examined the relationship between the number of treated bait in the GI tract and the amount of CPTH recovered in a GI tract sample across two different bird species with different size GI tracts. In all cases, samples with more treated bait seed yielded higher levels of CPTH recovered. In the smaller GI tract samples, very large levels of CPTH were recovered. This study also allowed for the investigation of the effect of a natural diet on matrix effects on the method. The method had been developed using birds fed a commercially available feed. The results from the blind study indicated that the method provided meaningful data when used on birds consuming field-applied bait where the actual amount of bait and the corresponding level of CPTH that was consumed was

unknown. Such samples are commonly analyzed in our laboratory. For example, in forensic cases, we are often required to analyze a single bird with an uncertain history.

Field Study. We analyzed two species of bird that collectively are referred to as blackbirds, the red-winged black bird and the brown-headed cowbird. Both species were collected in Louisiana. There were fewer brown-headed cowbirds collected during the field study, reflecting the natural population distributions of the two species. To allow for interspecies comparisons, the number of individuals analyzed was limited to the number of brown-headed cowbirds. The red-winged blackbirds were randomly selected to match the number of brown-headed cowbirds. Two brown-headed cowbirds were collected prior to applying the treated bait, and two red-winged blackbirds were selected randomly as pretreatment controls. There were four brown-headed cowbirds collected during the treated rice bait application; therefore, four red-winged blackbirds were randomly selected for analysis. The birds were selected without regard for sex of the bird.

The GI tract contents and the total breast muscle tissue samples were insufficient to allow for replicate extractions on all of the samples, as was done in the previous studies. Comparisons of the total mass for the contents from the gizzard indicated that the red-winged blackbirds consumed a significantly larger amount of rice bait than the brown-headed cowbirds (**Table 3**; one-tailed Student's t-test, equal variance, $\alpha = 0.05$, df = 10, $t = 1.93$, $t_{critical} = 1.81$, $P(t_{critical} < = t) = 0.041$). Visually, the GI tract contents for all birds were predominately rice grains.

Fortified and blank QC samples were run with the field samples during extraction and analysis. There were two replicates of both breast muscle tissue and GI tract contents fortified with CPTH and CPTH-D_6. The control samples with no CPTH and CPTH-D_6 and the samples fortified at approximately 0.5 μg/g CPTH were used to calculate the MLOD values for these samples. The MLOD for the gizzard contents was 0.030 μg/g and 0.0063 μg/g for the breast muscle tissue.
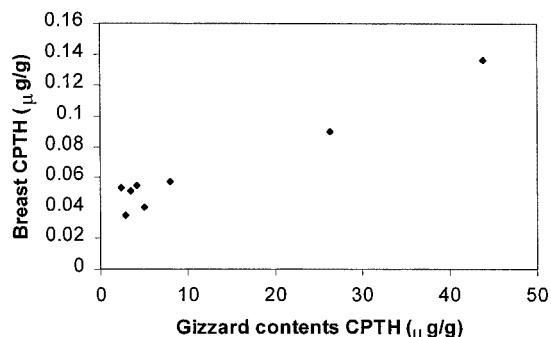
For both red-winged blackbirds and brown-headed cowbirds collected before applying the treated bait, the average concentrations of CPTH in the gizzard contents as well as the breast muscle tissue were less than the MLOD (**Table 4**). One of the

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Table 4. Concentrations of CPTH for the Breast Muscle Tissue and Gizzard Contents for Red-Winged Blackbirds and Brown-Headed Cowbirds

individual	CPTH treatment	CPTH concentration (μg/g) breast muscle tissue	GI tract contents
		Red-Winged Blackbirds	
A	control	0.0094	<MLOD
B	control	<MLOD	<MLOD
C	treated bait	0.058	8.0
D	treated bait	0.137	44.0
E	treated bait	0.041	5.0
F	treated bait	0.090	26.0
		Brown-Headed Cowbirds	
G	control	<MLOD	<MLOD
H	control	<MLOD	<MLOD
I	treated bait	0.035	2.8
J	treated bait	0.054	2.4
K	treated bait	0.055	4.1
L	treated bait	0.051	3.5

Figure 2. Relationship between CPTH concentration in gizzard contents and breast muscle tissue for red-winged blackbirds and brown-headed cowbirds.

prebaited birds had a tissue level of CPTH near the MLOD. The history of this bird is uncertain, and the bird may have been exposed to a treated bait site at another location and contained residues from this exposure.

The average gizzard contents for the birds collected at treated bait sites were 3.2 + 0.75 μg/g CPTH (mean $\pm$ 1 SD) for brown-headed cowbirds and 21 $\pm$ 18 μg/g for the red-winged blackbirds. The average breast muscle tissue contents were 0.049 $\pm$ 0.0091 and 0.081 $\pm$ 0.042 μg/g for brown-headed cowbirds and red-winged blackbirds, respectively. CPTH residues were analyzed using a one-tailed Student's *t*-test with a hypothesis that the red-winged blackbirds, having consumed more baited rice, would have higher gizzard content CPTH concentrations as compared to brown-headed cowbirds. This analysis indicated that CPTH concentrations in the gizzard contents were not significantly different in red-winged blackbirds (Student's *t*-test, unequal variance, one-tailed test, $\alpha = 0.05$, $t = 1.94$, $t_{critical} = 2.35$, P ($t_{critical} <\, = t$) = 0.049 64, df = 6). In addition, there were no significant differences between the two species for CPTH concentration in the breast muscle tissue ($\alpha = 0.05$, $t = 1.509$, $t_{critical} = 1.943$, $P(t_{critical} <\, = t) = 0.091$, df = 6). These results raise the question as to whether the two bird species differ in their ability to discriminate between treated and nontreated bait. This is a subject that deserves further study, although the interpretation is constrained by our sample size.

CPTH induced nephrotoxicity in susceptible bird species (*3*). As such, CPTH is a slow-acting toxicant, requiring days to exhibit toxicity. Because the birds shot on the bait sites were assumed to be collected shortly after feeding, the appearance of the CPTH in the breast muscle tissue so quickly after feeding was to some degree unexpected. However, there is a strong, positive correlation between breast muscle tissue CPTH and gizzard CPTH concentrations (**Figure 2**). The linear regression equation describing the relationship is breast muscle CPTH concentration (μg/g) = 0.039 + 0.002 15 $\times$ gizzard CPTH concentration, $r^2 = 0.95$. This suggests that the analysis of both matrixes is valuable for determining CPTH exposure in birds.

The data in **Tables 3** and **4** were used to perform secondary risk assessments for scavenger and predator species. The total body burden of CPTH for individual birds was estimated by multiplying the concentration of CPTH in a sample by the total sample mass. The values for the two sample types were then summed (μg CPTH in the gizzard contents + μg CPTH in the breast muscle tissue). The highest estimated body burden of CPTH for a brown-headed cowbird is 5.77 μg, and for a red-winged blackbird, it is 82.82 μg. Using average body masses of 49.0 g for a brown-headed cowbird and 41.5 g for a red-winged blackbird (*11*), these correspond to CPTH concentrations of 0.12 mg/kg for a brown-headed cowbird and 2.0 mg/kg for a red-winged blackbird.

The CPTH concentrations of 0.12 mg/kg for brown-headed cowbirds and 2.0 mg/kg for red-winged blackbirds were then used to assess the secondary hazard that these birds might have potentially posed to a predator (**Table 5**). The possible predators considered were the barn owl (*Tyto alba*), the northern harrier

Table 5. Toxicity Data and Risk Quotients for Selected Predator and Scavenger Species Assuming Total Dietary Intake Is Based on Either Brown-Headed Cowbirds or Red-Winged Blackbirds

species	estimated LD$_{50}$[a] (mg/kg)	avg. wt[b] (g)	ingestion rate[c] (g g^{-1} d^{-1})	CPTH consumed[d] (μg CPTH g^{-1} d^{-1})	risk quotient[e]	source[f]
barn owl	4.2	466	0.15	0.018	0.0043	bhcb
				0.3	0.071	rwbb
northern harrier	100	441	0.19	0.023	0.00023	bhcb
				0.38	0.0038	rwbb
American kestral	178	116	0.3	0.036	0.0002	bhcb
				0.6	0.0034	rwbb
Cooper's hawk	562	439	0.2	0.024	0.00004	bhcb
				0.4	0.0007	rwbb
coyote	100	15009	0.06	0.0072	0.00007	bhcb
				0.12	0.012	rwbb
dog	100	10000	0.06	0.0072	0.00007	bhcb
				0.12	0.012	rwbb

[a] 13 and 14. [b] 15–17. [c] 17–21. [d] CPTH consumed (μg CPTH g^{-1} d^{-1}) = CPTH concentration in food source $\times$ ingestion rate. [e] Risk quotient = CPTH consumed/estimated LD$_{50}$. [f] Source: bhcb is a brown-headed cowbird; rwbb is a red-winged blackbird.

(*Circus cyaneus*), the American kestral (*Falco sparverious*), the Cooper's hawk (*Accipiter cooperii*), the coyote (*Canis latrans*), and the common dog (*Canis familiariz*). All of these animals have been observed to feed on birds known to frequent bait sites or the carcasses of birds found in the area after baiting. The birds have estimated acute toxicities (LD_{50}) that range from 4.2 mg/kg for the barn owl to 562 mg/kg for the Cooper's hawk. Both of the mammals have an estimated LD_{50} of 100 mg/kg.

Worst case risk quotients, calculated as the ratio of estimated daily dose to the LD_{50} (*1*), were determined for each of the predators assuming the total daily diet, for a single day, consisted of either brown-headed cowbirds (0.12 mg/kg CPTH) or red-winged blackbirds (2.0 mg/kg) at the highest CPTH concentrations determined in this study. The quotient method is used by the U.S. Environmental Protection Agency to estimate pesticide exposure-related hazards to nontarget species (*12*). For general use purposes, a risk quotient less than 0.1 is considered to be an acceptable level of risk. Risk quotients greater than 0.1 pose an unacceptable level of risk for endangered species and may result in the imposition of restrictions on pesticide use where these endangered species occur. The calculation does not address multiple day exposures.

The secondary hazard risk based on the risk quotient was greatest for all of the predator species considered, when it was assumed that the entire diet consisted of red-winged blackbirds, as the CPTH concentration in the red-winged blackbirds was considerably greater than that of the brown-headed cowbirds and both bird species are similar in mass. The largest risk quotient calculated for the bird predators was 0.071 for the barn owl, and the lowest was for the Cooper's hawk at 0.0007. Both of these values are less than 0.1, indicating that these predatory birds are at little or negligible secondary risk from CPTH toxicity as used in this study. For both the dog and the coyote, the risk quotient was less than 0.1, again reflecting negligible secondary risk from CPTH toxicity as applied in this study.

As proposed by Hurlbut et al. (*5*), the use of a deuterated surrogate greatly facilitated the determination of CPTH residues in bird tissues. The modified method presented has been demonstrated to be effective in determining CPTH residue concentrations in GI tract, gizzard contents, and breast muscle tissues of a number of bird species, both captive and wild. The modified method requires less time for preparation and sample processing and is more robust than earlier methods. The method appears to be well-suited to monitoring wildlife damage management efforts focusing on the concentration of CPTH in the GI tract or breast muscle tissue of target or nontargeted bird species. The method was sensitive enough to quantify CPTH in the breast muscle tissue of birds collected in the field as they left a feeding site. The data generated by the method can be used in assessing secondary risk hazards for target and nontarget species.

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