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Determination of residue levels of the avicide 3-chloro-4-methylaniline hydrochloride in red-winged blackbirds (*Agelaius phoeniceus*) by gas chromatography-tandem mass spectrometry☆,☆☆,☆☆☆

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ABSTRACT

The avicide 3-chloro-4-methylanaline hydrochloride (chloro-*p*-toluidine hydrochloride, CPTH, DRC-1339) is used to control pest bird species that damage agricultural crops. A specific and sensitive gas chromatography-tandem mass spectrometry method was developed and validated for the determination of CPTH in avian breast muscle, GI tract, kidney, and liver. Tissue samples were extracted with a solution of acidified water and acetonitrile. The sample was made basic and cleaned up with a combination of liquid-liquid partitioning and solid phase extraction. Separation was achieved using a HP-5 ultra-inert GC column (15 M, 0.25 µm film) with detection on a triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode. The monitored transition for CPTH was *m/z* 140.9 → 106.2 for quantitation and 139.9 → 105.2 and 139.9 → 77.2 for confirmation. The linear range was 5 to 5000 ng/mL. The precision for the determination of CPTH in all tissues averaged 7.2% and the accuracy averaged 6.7%. The recovery of CPTH fortified at 5 different levels averaged 101% in liver, 98.8% in GI tract, 92.9% in breast muscle, and 87.9% in kidney. The established method was successfully used to determine CPTH residue levels in red-winged blackbirds exposed to three different doses of CPTH.

1. Introduction

3-Chloro-4-methylaniline hydrochloride (3-chloro-*p*-toluidine hydrochloride, DRC-1339, CPTH) is a slow-acting toxicant in avians. Because the time to death (1–3 days) is well after exposure, very few analyses of collected carcasses have found significant CPTH residues [1,2]. Public concerns surround the possibility for secondary hazard or non-target exposures from the use of CPTH. CPTH intoxication is often suspected when mysterious bird deaths are noted by members of the general public or conservation groups.

Detection of CPTH exposure in both target and non-target birds has been very problematic. Very early efforts to detect exposure were based on necropsies of the carcasses with examination to attempt to detect physiological indicators of exposure such as the accumulation of uric acid deposits [1]. Such deposits were thought to be indicative of CPTH exposure [3]. More recent advances have seen the use of deuterated standards and gas chromatography with mass spectrometry for the detection of CPTH residues at low levels in the tissues of exposed birds [4]. This newer technique has improved performance over older techniques with the added benefit of very good analytical sensitivity. The reported limit of detection was 12 ng/g for breast muscle and 25 ng/g for GI tract [4]. Despite these improvements, difficulties with noisy baselines and low extraction efficiencies led to very few verified exposures which were also prone to false positives.

In one study with grackles, the concentration present in the tissues was found to increase with increasing exposure levels [1]. Given that the anticipated exposure dose for a red-winged blackbird would be around 25 mg/kg [5], the odds of detecting exposure in tissues from these birds are very low. Once a bird is exposed to a lethal dose of CPTH, time to death is typically 1 to 2 days. The observed half-life of elimination for CPTH has been estimated to be 5.4 h in sensitive species [6]. Therefore, detection of a lethal exposure to CPTH is almost

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impossible to achieve as the toxicant has left the body of the bird prior to death. Use of tandem mass spectrometry coupled with improvements to the extraction techniques used could produce a more sensitive method which could detect these residues.

2. Materials and methods

2.1. Trapping of birds

Thirty-seven red-winged blackbirds (*Agelaius phoeniceus*) of mixed genders were trapped from wild populations in Colorado (USFWS Permit Number MB019065-3) and transported to the National Wildlife Research Center's Outdoor Animal Research Facility. The birds were held in quarantine for not < 7 days. They were maintained in group housing during quarantine and given free access to water and a maintenance diet through the study. All procedures involving animals were carried out with the approval of the NWRC Animal Care and Use Committee (NWRC protocol QA-2708).

2.2. Purification of CPTH

CPTH is sold as Compound DRC-1339 Concentrate in several formulated products for use by certified pesticide applicators. The purity limits for these products are 94.1 to 99.9% CPTH. Significant impurities within the material could confound the results of these dosing tests. Therefore, the material was purified before use in the *in-vivo* portion of the study.

All chemicals were obtained from Fisher Scientific (Waltham, MA, USA) unless otherwise specified. One half kilogram of Compound DRC-1339 Concentrate (PM Resources, Fort worth, TX, USA) was weighed into a 2-L beaker. Methanol (1.5 L) was added and heated to approximately 40 °C on a stirring hot plate. To this was stirred in 20 mL of a 1 N HCl solution in water and 30 g of activated charcoal. The solution was allowed to stir for approximately 1 h until it was nearly colorless. It was then filtered through a Whatman #1 filter paper in a Buchner funnel, reserving the liquid. The liquid was evaporated in a rotary evaporator under vacuum until a wet powder consistency was achieved. The powder slurry was filtered through a Whatman #1 filter on a Buchner funnel, with the liquid being reserved. The powder was covered with approximately 200 mL of acetonitrile and, after a 2 minute wait, vacuum was applied. The filtrate was discarded to waste. The process of rotary evaporation and filtering was repeated with the methanol solution until no more powder was obtained. The white, crystalline product was allowed to dry in a fume hood overnight. The purified CPTH powder was stored in a refrigerator at 4 °C until used. Purity was confirmed by HPLC-UV prior to the initiation of the *in-vivo* portion of the study and found to be 99.9%.

2.3. Synthesis of surrogate compound

A surrogate compound was used for all chromatographic analyses. As a deuterated material was not commercially available, it was synthesized. Deuterated d_6-CPTH was synthesized following the procedures in Hurlbut et al. [7] with modification of one step. The initial nitration reaction was accomplished using sodium mordenite (Zeolist International, Kansas City, KS, USA) following the procedures established by Smith and Fry [8]. The mordenite increased the yield of the *p*-nitrotoluene significantly over the original procedure. The reactions yielded a very pale yellow powder with a purity of > 99% by GC/MS.

2.4. Administration of dose

Red-winged blackbirds were randomly assigned to test or control groups and exposed to one of three doses of the purified CPTH (8 birds per treatment level) *via* oral gavage and held for three days. Oral gavage was accomplished by using a #9 gelatin capsule containing the appropriate mass of CPTH and packed with D-mannitol (an inert material) to fill the unused space in the capsule. The birds were held in a supine position and the capsule was delivered using a stainless steel dosing syringe. Dosage levels were approximately 12.5, 25, and 37.5 mg/kg based on an average weight of 60 g for a red-winged blackbird. A control group of 8 birds received a capsule containing only D-mannitol. Birds in both the test and control groups were held in individual cages for the remainder of the study. Twice daily, the condition of the birds was observed and noted. Deceased birds were removed; their weight and the time were recorded. No individuals from the control group expired during the exposure period. All birds that survived the full 3-day exposure period were euthanized with CO_2. All birds were euthanized humanely in accordance with American Veterinary Medical Association standards and practices.

2.5. Tissue collection

All birds, including the control group, were necropsied to remove tissue samples for analysis. An incision was made in the skin covering the abdomen, and the birds were skinned completely. A lateral incision was then made at the base of the breast bone and a pair of scissors used to cut the breast bone on each side. The kidneys, GI tract (from esophagus to cloaca with contents included), liver, and a portion of the breast muscle were removed from each bird and individually flash frozen with liquid nitrogen. The samples were ground to a fine powder with a chilled mortar and pestle in the case of kidney and liver samples or through the use of a freezer mill (SPEX CertiPrep, Metuchen, NJ, USA) and stored in individual containers. Kidney samples were stored at −80 °C. All other samples were stored at −30 °C.

2.6. Gas chromatography–mass spectrometry analysis

The GI tract, liver, kidney, and breast muscle were analyzed for CPTH content by gas chromatography/tandem mass spectrometry. During the extraction process the CPTH was converted from the hydrochloride to the free base form (CPT). Although CPT was the form detected by the mass spectrometer, all results were reported in terms of CPTH (Fig. 1).

The homogenized samples (0.2 to 0.5 g) were fortified with a surrogate compound (d_6-CPTH) and extracted with 2.5 mL of 20% acetonitrile/80% 1 M hydrochloric acid. The sample extract was made basic (pH approximately 10) with the addition of 2.5 mL of 2 M sodium hydroxide which converted the CPTH to CPT and extracted with three 3 mL aliquots of hexane. The hexane extract was cleaned-up using a silica solid phase extraction (SPE) cartridge (Biotage, Charlotte, NC). The CPT was eluted from the SPE with 2 mL of a 50 µg/mL solution of *p*-toluidine (Sigma Aldrich, St. Louis, MO, USA) in *n*-butyl acetate. Analysis was performed using an Agilent 7890A Gas Chromatograph attached to an Agilent 7000B QQQ Mass Spectrometer (Santa Clara, CA, USA). Separation was achieved using a 15 m × 0.25 mm ID, 0.250 µm, DB5-MS UI column (Agilent Technologies, Santa Clara, CA, USA) connected through a purged ultimate union to a 0.65 m length of 0.15 mm ID fused silica. This arrangement allowed the use of a post-run backflush to minimize contamination of the detector with high boiling temperature matrix components. Other instrument parameters are in Table 1.

2.7. Method validation

The method was validated by evaluating the following parameters: selectivity, limits of detection (DL) and quantification (QL), linearity, accuracy, recovery. When not specified, result values were tabulated and analyzed using Microsoft Excel 15.0 (Microsoft Corp, Redmond, WA).

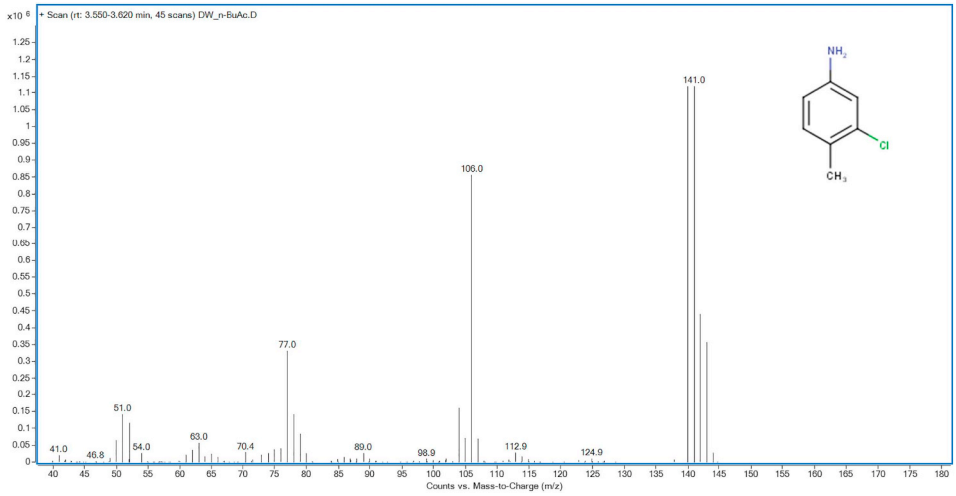

Fig. 1. Mass spectrum of CPTH.

Table 1
Gas chromatograph/mass spectrometer conditions.

Carrier gas:	Helium
Inlet liner:	Ultra-inert liner, splitless, single taper with wool
Injection port:	250 °C; pulsed splitless; 15.9 psi; constant-flow
Injector pressure:	Pulse at 70 psi for 1 min; split vent at 60 mL/min at 2.5 min
Injection volume:	1 µL
Temperature program:	

Time (min)	Temperature (°C)	Rate (°C/min)
0.00	70	–
2.00	70	–
7.25	175	20
8.50	300	100
12.00	300	–

Collision cell:	2.25 mL/min He quench gas; 1.5 mL/min nitrogen collision gas
Ion source:	Electron impact
Source temperature:	230 °C
Solvent delay:	4.5 min

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell (ms)	Collision energy (V)
d_6-CPTH	148.9	112.2	20	19
d_6-CPTH	146.9	112.2	40	15
d_6-CPTH	112.2	81.2	20	19
CPTH	140.9	106.2	50	15.5
CPTH	139.9	105.2	20	16
CPTH	139.9	77.2	20	19

2.7.1. Control tissues

The repeatability and performance of the method was assessed using a surrogate avian species. Coturnix quail (*Coturnix japonica*) were used as control matrix for the preparation of fortified quality control samples. Quail were chosen due to the ability to readily obtain large quantities of tissue from each individual. The red-winged blackbirds are much smaller and it would have taken a large number of individuals to provide enough matrix for quality control purposes. Both birds are granivores, and quail were an adequate representative of red-winged blackbirds. Quail were purchased from a hatchery in Colorado and their tissues were processed according to the procedures outlined above.

2.7.2. Selectivity

Selectivity of the method was evaluated by analyzing 7 replicate samples of breast muscle, GI tract, liver, and kidney tissue from commercially raised Coturnix quail. Potential interferences were assessed at the retention time of CPTH by comparing the analytical response in the control samples to samples fortified near the QL.

2.7.3. Detection and quantification limits

The detection limit (DL) for CPTH in red-winged blackbird tissues was estimated from the mean chromatographic response of control samples compared to control samples that had been fortified to 20 ng/g with CPTH for GI tract, liver, and breast muscle or 50 ng/g with CPTH for kidney. The DL was defined as the concentration of CPTH required to generate a signal equal to 3× the baseline noise (measured peak-to-peak) observed in the baseline at the retention time of CPT in the control samples. The quantitation limit (QL) for CPTH in red-winged blackbird tissues was estimated in a similar fashion to as the DL with the multiplier of 10× baseline noise used instead of 3×.

2.7.4. Linearity

Two sets of six calibration standard solutions were prepared ranging from 5.15 ng/mL to 5300 ng/mL. The standards were made basic by the addition of 2 M sodium hydroxide which converted the CPTH to CPT and extracted with a solution of 50 µg/mL *p*-toluidine in *n*-butyl acetate. Each standard was injected in triplicate; the response ratio of CPT area to d_6-CPT area was plotted against relative concentration. A weighted (1/x) quadratic regression was performed on the data set.

The accuracy of the calibration standards was determined for each data set by calculating the observed concentration of each standard in the calibration curve using the regression equation. This calculated observed concentration is then compared to the theoretical concentration of the standard to determine the accuracy as a percentage.

2.7.5. Recovery

A minimum of 7 replicates at each fortification level were prepared by adding an appropriate aliquot of a fortification solution to a 500 mg sample of control quail tissue. Due to the limited sample size of the RWBB kidneys, the mass of sample used for quality control purposes was reduced to 200 mg to more accurately reflect the sample masses of the study samples. Control quail liver, GI tract, and breast muscle were fortified at 20, 50, 100, 300, and 1000 ng/g. Control quail kidney was fortified at 50, 125, 250, 750, and 2500 ng/g.

The accuracy of the method was determined by calculating the relative error of the mean observed concentration at each fortification level. This value was calculated by determining the absolute value of the difference between observed concentration and target concentration and dividing by the target concentration.

2.7.6. Statistical methods

The statistical model used to evaluate the variance of the quality

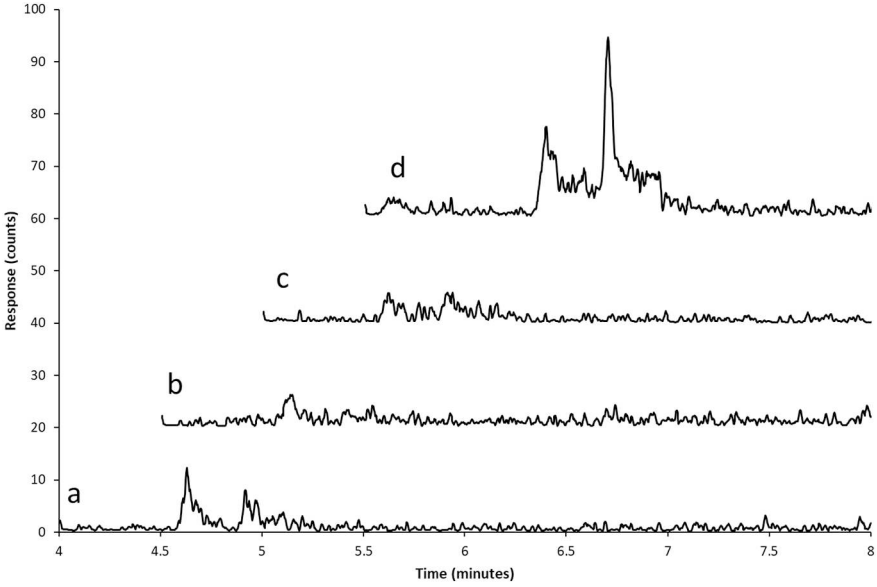

Fig. 2. Chromatograms of red-winged blackbird tissues from an untreated sample: breast muscle (a), GI tract (b), kidney (c), and liver (d). The m/z 140.9 → 106.2 MRM transition is shown.

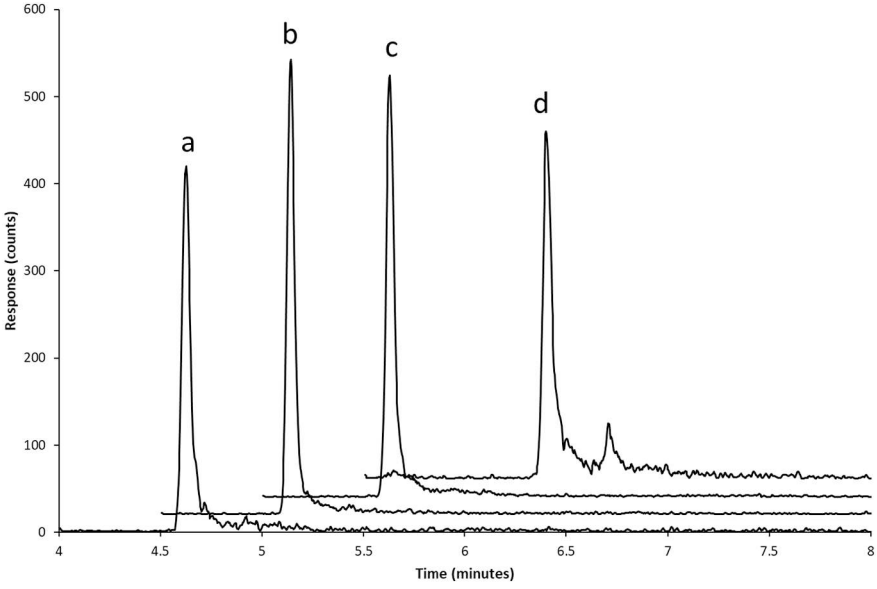

Fig. 3. Chromatograms of red-winged blackbird tissues from an untreated sample which were fortified with CPTH: breast muscle at 50 ng/g (a), GI tract at 50 ng/g (b), liver at 50 ng/g (c), and kidney at 125 ng/g (d). The m/z 140.9 → 106.2 MRM transition is shown.

control data was SAS's General Linear Model (GLM) (SAS Institute, Inc., Cary, NC). The GLM performs analysis of variance by using least squares regression to fit general linear models. The response was the percent recovery of the quality control samples. The fixed effects were the tissue matrix type and the fortification level. The interaction between fixed effects was also evaluated.

3. Results and discussion

3.1. Method development

A deuterated form of CPTH (d_6-CPTH) was selected as the internal standard (IS) for this method. Multiple reaction monitoring (MRM) transition pairs were selected for both CPT and d_6-CPT and optimized with respect to collision energy. The dwell values were weighted towards the quantitative transition for each compound. The quantitative transition for CPT was m/z 140.9 → 106.2 while the d_6-CPTH transition was m/z 146.9 → 112.2.

CPTH is the hydrochloride salt of the free base CPT. During method development, this chemistry was exploited to enable extraction of the CPTH as its free base CPT. This also served to remove a significant amount of water soluble matrix components and dissolved the CPT in a GC friendly solvent. The use of a normal phase SPE provided further clean-up of the sample extract. A solution of *p*-toluidine was used as the final sample solvent. CPT can react with active sites in the GC inlet. Use of a competing compound acted as an analyte protectant and produced

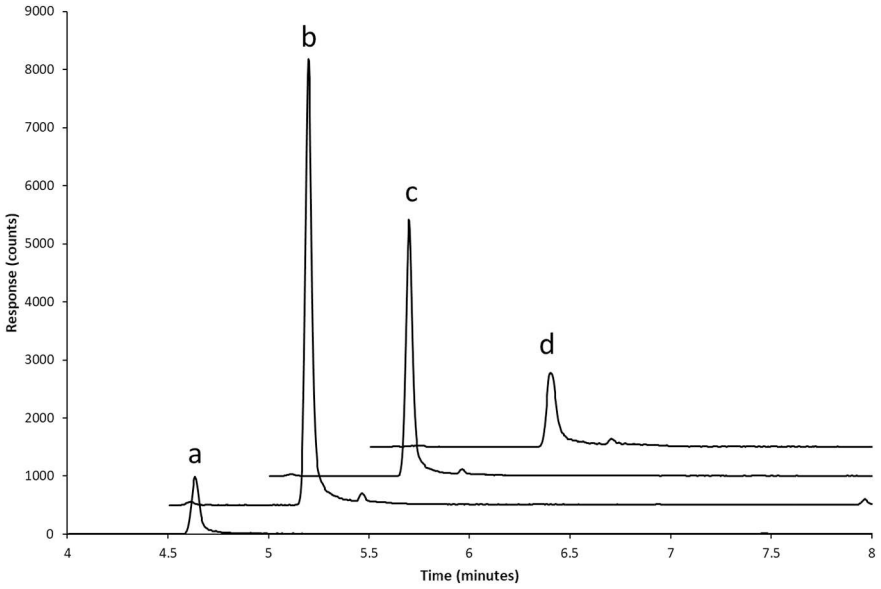

Fig. 4. Chromatograms of red-winged blackbird tissues from a bird dosed with 25/mg/kg CPTH: breast muscle (a), GI tract (b), liver (c), and kidney (d). The *m/z* 140.9 → 106.2 MRM transition is shown.

Table 2
Detection and quantitation limits for red-winged blackbird tissue analyzed using GC/MS/MS.

Tissue type	Detection limit	Quantitation limit
GI tract	4.3 ng/g	14 ng/g
Liver	2.6 ng/g	8.7 ng/g
Breast muscle	3.2 ng/g	11 ng/g
Kidney	5.9 ng/g	20 ng/g

stronger and more consistent sample responses. Use of *p*-toluidine also aided in the recovery of the CPT from the SPE cartridge by minimizing interactions of the CPT with any active sites in the cartridge.

3.2. Method validation

3.2.1. Selectivity

Typical MRM chromatograms of control tissues fortified at 50 ng/g for GI tract, liver, and breast muscle and 125 ng/g for kidney and from a red-winged blackbird dosed with 25 mg/kg of CPTH are presented in Figs. 3 and 4. There were matrix peaks observed near the retention time of CPT in most of the matrices tested (Fig. 2). In most cases, these peaks occurred after the retention time for CPT and did not interfere with the detection of CPT. In some cases (in particular for breast muscle samples) peaks were observed at the retention time of CPT. These peaks were attributed to either carry-over in the GC inlet or matrix peaks in the samples. Under the conditions specified, the retention times of d_6-CPT and CPT were 4.6 min.

3.2.2. Detection and quantification limits

The detection limit (DL) for CPTH in red-winged blackbird tissues was estimated from the mean chromatographic response of control samples compared to control samples that had been fortified to 20 ng/g with CPTH for GI tract, liver, and breast muscle or 50 ng/g with CPTH for kidney. The DL was defined as the concentration of CPTH required to generate a signal equal to 3× the baseline noise (measured peak-to-peak) observed in the baseline at the retention time of CPT in the control samples. The quantitation limit (QL) for CPTH in red-winged blackbird tissues was estimated in a similar fashion to as the DL with the multiplier of 10× baseline noise used instead of 3×. The DL and QL for GI tract, kidney, liver and breast muscle of red-winged blackbirds are shown in Table 2.

Table 3
Average regression coefficients (n = 18).

Coefficient	Mean
a	0.00156 ± 0.00051
b	2.08 ± 0.087
c	0.022 ± 0.032

3.2.3. Linearity

The average of all calibration curves was determined (n = 18) where Y represents the ratio of concentration of CPTH to d_6-CPTH and X represents the ratio of CPT area response to d_6-CPT area response (Table 3). Calibration was achieved with a 1/x weighted quadratic

Table 4
Precision and accuracy of CPTH in red-winged blackbird tissues.

Matrix	n	Target (ng/g)	Observed (ng/g)	Precision (RSD, %)	Accuracy (RE, %)
Breast muscle	7	20	21.3 ± 6.7	31.5	6.50
	9	50	43.1 ± 3.1	7.19	13.8
	7	100	87.0 ± 2.5	2.87	13.0
	7	300	294 ± 14	4.76	2.00
	7	1000	889 ± 42	4.72	11.1
GI tract	7	20	20.7 ± 0.74	3.57	3.50
	7	50	48.7 ± 3.9	8.01	2.60
	7	100	95.2 ± 2.0	2.10	4.80
	7	300	298 ± 4.1	1.38	0.67
	7	1000	988 ± 37	3.74	1.20
Kidney	7	50	39.4 ± 7.6	19.3	21.2
	7	125	86.1 ± 7.9	9.18	13.9
	7	250	219 ± 27	12.3	12.4
	7	750	697 ± 33	4.73	7.07
	7	2500	2341 ± 80	3.42	6.36
Liver	6	20	20.2 ± 1.3	6.44	1.00
	7	50	48.5 ± 3.4	7.01	3.00
	7	100	98.6 ± 1.3	1.32	1.40
	7	300	309 ± 11	3.56	3.00
	6	1000	1060 ± 66	6.23	6.00

Table 5
Mean CPTH residue levels (ng/g) in red-winged blackbirds following a single, oral dose.

Dose level		Kidney	GI tract	Liver	Breast muscle
12.5 ng/g	Mean ± sd =	78.9 ± 45.5	66.6 ± 30.1	85.1 ± 36.1	23.8 ± 14.9
	Range =	18–126	6.83–110	19.8–148	4.9–50
	RSD =	58%	45%	42%	63%
	n =	8	8	8	8
25 ng/g	Mean ± sd =	297 ± 199	585 ± 820	215 ± 125	42.2 ± 27.2
	Range =	55.2–676	102–2400	75.8–441	10.3–79.5
	RSD =	67%	140%	58%	66%
	n =	8	7	8	8
37.5 ng/g	Mean ± sd =	356 ± 124	559 ± 684	317 ± 145	49.5 ± 21.0
	Range =	203–570	107–2170	167–571	26.4–89.5
	RSD =	35%	122%	46%	42%
	n =	8	8	8	8

regression analysis ($Y = aX^2 + bX + c$) with correlation coefficients of 0.999 or better. The calibration curve was linear over the range of 5.15 ng/mL to 5300 ng/mL CPTH. The accuracy of all concentrations in the calibration curve was < 10%.

3.2.4. Precision and accuracy

The precision and accuracy values for recovery of CPTH from red-winged blackbird tissues demonstrated good method performance across all levels (Table 4). The samples were analyzed over the course of a 5 month period of time. The average precision of CPTH, represented by the relative standard deviation of the observed concentrations, was 7.2% and the average accuracy, represented by the relative error, was 6.7%. Therefore, the validated method provided acceptable precision and accuracy for the determination of CPTH in tissues.

ANOVA results from a GLM analysis (SAS General Linear Model (GLM); SAS Institute, Inc., Cary, NC) demonstrated a significant effect from tissue type ($F = 12.96$; $p \leq 0.0001$), fortification level ($F = 2.95$; $p = 0.0229$), and from the interaction of tissue and level ($F = 2.33$; $p = 0.0101$). Recovery levels for liver and GI tract were not significantly different from each other ($\alpha = 0.05$). Recovery from breast muscle tissue was lower than both liver and GI tract, but higher than that for kidney. Recovery from kidney tissue was lower than any other matrix.

3.2.5. Bird dosing study

Samples from the control exposure group had observable CPTH responses in several cases, although they were below the QL. These responses were likely due to the presence of matrix peaks in the chromatograms or from instrument carry over effects (Fig. 2). Values below the DL were reported as not detected. For statistical analysis of these samples, a value of one-half of the DL was used. Results between the DL and QL were considered to be qualitative only. Therefore, results between these two values were only reported to two significant digits where other results used three significant digits.

The residue levels observed in the *in-vivo* study (Table 5) were higher than anticipated, particularly in the GI tract. Given the rapid excretion observed in the results from a radio-tracer excretion study [6], there was an expectation that the GI tract residues would be quite low. There are two possible explanations for this discrepancy. Firstly, the dose administered in the radio-tracer study was 3.6 mg/kg while the low dose group in the current study was 12.5 mg/kg. Since the time to death is dose dependent for CPTH, the higher dose used in the current study led to all but one of the dosed birds being found dead by the morning following administration of the dose. The lone bird in the low dose group which survived the three days of the study had lower residues in all three tissues compared to other birds in the low group. This higher dose likely contributed to less of the CPTH being excreted prior to death.

4. Conclusion

A method for the quantitation of CPTH in bird tissues was developed and validated. The method was successfully used to quantitate residue levels of CPTH in red-winged blackbirds exposed to an oral dose at three levels. The method made some improvements on previous methods used for CPTH residue determinations. During a previous study at NWRC designed to mimic a field exposure in mourning doves (*Zenaida macroura*), the results for breast muscle samples were nearly all below the 70 ng/g QL reported for the method used [2]. The current method has a QL in breast muscle which is significantly lower (14 ng/g), demonstrating a clear improvement on previous methods. This was likely due to the use of tandem mass spectrometer rather than the mass selective detector (single quadrupole) used in the literature method. The results for GI tract samples in the 2006 research were above the QL for the method, but both tissues demonstrated RSD values near 50%. This was consistent with the results from the current study. Also similar between the current study and those in the literature was the difference in concentration of CPTH observed between GI tract and breast muscle. In all cases, the concentration in GI tract was much larger than that for breast muscle in an individual bird.

The method represents a significant improvement on previous methods. The DL for the current method represents a significant improvement on previous methods. The DL for previously reported methods [4,7] were between 12 and 25 ng/g for the matrices tested. The DL for the method described varies between 2.6 and 5.9 ng/g (Table 2). Previous methods relied on single ion monitoring (SIM) for detection of residues. The described method utilizes tandem mass spectrometry which provides added certainty of detected residues in the form of qualifier transitions. The lowered DL and use of quantifier ion confirmation provide added confidence to the low level residues often observed in field samples and provide a valuable tool in future studies to assess field exposures from the use of CPTH as a bird control agent.

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