Characterizing 3-Chloro-*p*-Toluidine Hydrochloride on Rough-Hulled Rice and Ethyl-Cellulose-Coated Rice Baits Using High-Performance Liquid Chromatography

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Abstract

Methods are developed to extract and quantitate the avicide 3-chloro-*p*-toluidine hydrochloride (CPT HCl) from rough-hulled rice and ethyl-cellulose-coated rice baits using high-performance liquid chromatography. The mobile phase used in the ethyl-cellulose-coated rice matrix method is an acetonitrile(ACN)–phosphate buffer (60:40) at pH 8, and the rough-hulled rice matrix method uses an ACN–phosphate (70:30) buffer at pH 2. Increased retention time is observed for CPT HCl at the higher pH. The two methods have been useful in characterizing different bait formulations in an ongoing pesticide formulation improvement program.

Introduction

Roosting populations of red-winged blackbirds (*Agelaius phoeniceus*) and brown headed cowbirds (*Molothrus ater*) commonly cause significant damage, seasonally, to both sprouting rice seedlings in Louisiana in the spring and ripening sunflower in North and South Dakota in the fall. These roosting populations can be controlled by baiting fields with rice bait containing 3-chloro-*p*-toluidine hydrochloride (CPT HCl) (Figure 1). The baits are commonly formulated to contain 2% CPT HCl (the salt form). The treated bait is mixed 1:25 with untreated rice. CPT HCl is highly toxic to red winged blackbirds and brown-headed cow birds but is less toxic to nontargeted species (1).

CPT HCl is water soluble, dimerizes in the presence of light, and as a primary aromatic amine is fairly reactive (2,3). For example, CPT HCl has been observed to undergo a Millard reaction in the presence of simple sugars to form gluconurides (4). To prevent the loss of CPT HCl during baiting, efforts have been made to evaluate various coatings to prevent loss in the field, particularly following a rainfall event. Two rice baits were devoloped for evaluation. The first used ethyl cellulose as a water-resistive coating. The second bait was based on applying CPT HCl to rough-hulled rice because it is perceived that birds may prefer rice with the hull on the grain. Traditionally, bait has been produced from hulled rice seed [where the seed coat (caryopsis) is removed]. As part of this effort, it was necessary to evaluate the effect of pH on the analysis of CPT HCl on rice grain baits. Historically, CPT HCl has been extracted in acetonitrile (ACN) and quantitated in the extract by high-performance liquid chromatography (HPLC) using an isocratic mobile phase of ACN and water on a C8 or C18 analytical column (3). This method proved unreliable when it was attempted for use in the quantitation of CPT HCl on either rough-hulled rice or ethyl-cellulose-coated rice matrices.

CPT HCl has a pK_a of 3.7 (2). Given that the CPT HCl can exist in the protonated or free-base form, it was important to determine whether there were advantages to analyzing extracts at a low (pH 2) or high (pH 8) pH using HPLC. Two different methods were developed: the first was to extract CPT (the free base form) from ethyl-cellulose-coated rice baits, using a high pH, and the second was to extract CPTH (the protonated form) from rough-hulled rice at a low pH. As a point of semantics three acronyms were used to refer to the different forms of the avicide depending on whether it is the salt form (CPT HCl), free base form (CPT), or protonated ion (CPTH).

Experimental

Materials and equipment

Solvents used included methanol, hydrochloric acid, NaOH 50% (w/w), and HPLC-grade ACN from Fisher Scientific

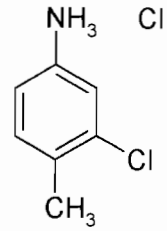

Figure 1. The structure of CPT HCl.

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(Pittsburgh, PA). Acetone was obtained from EM Science–Merck, KGaA (Darmstadt, Germany). Powder reagents obtained from Fisher Scientific included potassium phosphate monobasic and sodium hydroxide. The technical-grade CPT HCl used to treat the seed was obtained from Purina Mills, LLC (St. Louis, MO) and in-house certified 94.9% pure. Sodium hydrogen sulfite, used to deactivate the rough-hulled seed coat, was obtained from Aldrich (Milwaukee, WI). Ethocel, an ethyl-cellulose polymer with an ethoxyl content in the range of 48–49.5%—applied as a water repellent, was obtained from Dow Chemical Corp. (Midland, MI). Acetyltributylcitrate (ATBC) was obtained from Morflex (Greensboro, NC). Kollidon, a polyvinylpyrolidine used as an excipient with the CPT HCl, was obtained from BASF (Parpsippany, NY), Alcolec S. was obtained from American Lecithin Company, Inc. (Woodside, NY). Soybean oil was obtained from Hain Pure Foods Company, Inc. (Uniondale, NY).

HPLC

Extracts from ethyl-cellulose-coated rice bait matrices

Extracts were analyzed on a Hewlett Packard 1090 HPLC system with a diode array detector (Agilent, Willmington, DE). A 5-µL sample was injected onto a Phenomenex (Phenomenex, Torrence, CA) Luna C-18 (2) 250- × 3.0-mm column with 5-µm diameter packing and a Phenomenex Luna C-18 (2) 2.0- × 4.0-mm guard column. The mobile phase was 60% ACN–40% 0.01M KH_2PO_4 buffer (pH 8.0) with a flow rate of 0.5 mL/min. The elution was performed isocratically under ambient temperature conditions. The CPT was detected at λ = 241 nm.

Extracts from rough-hulled rice bait

As in the previous method, extracts were analyzed on a Hewlett Packard 1090 HPLC system (Agilent) with a diode array detector. A 5-µL sample was injected onto a Phenomenex Luna C18 (2) 250- × 3.0-mm column with 5-µm diameter packing, and a Phenomenex Luna C18 (2) 2.0- × 4.0-mm guard column. The mobile phase was 70% ACN–30% pH 2 KH_2PO_4 buffer with a flow rate of 0.5 mL/min. The elution was performed isocratically under ambient temperature conditions. The CPTH was detected at λ = 241 nm.

Rice seed samples

For the ethyl-cellulose-coated rice matrix method, medium grain (hulled, no seed coat) brown rice was obtained from one commercial supplier in each of the following states: Louisiana, Missouri, and California. For the rough-hulled rice matrix method, cocodrie (a cultivar) rough-hulled rice (intact seed coat) was obtained from a single commercial supplier in each of the following states: Louisiana, Mississippi, and Texas.

Preparation of ethyl-cellulose-coated CPT HCl-treated rice bait

To prepare the bait, sufficient ethocel for a 4% coating on the rice bait was dissolved in a 1:1 mixture of acetone and methanol at approximately 8% solids, and 15% ATBC (based on ethocel) was added. A 2% solution of kollidon in methanol was prepared and sprayed onto the rice in a mixer. This was stirred until only partially sticky and the CPTH powder was added with stirring. The ethocel solution was sprayed onto the rice in 1/10 increments at approximately 5-min intervals. The coated bait was spread on foil-covered trays and placed in 60°C oven for 2 h to cure the coating.

Preparation of CPT HCl rough-hull-treated rice bait

To produce the bait, the rough-hulled rice was placed in a sealable container and mixed with sufficient solution containing sodium hydrogen sulfite (7.5%, based upon the rice weight) to cover the rice. Additional water was added as required to maintain coverage (some solution is absorbed by the rice) and allowed to soak overnight (minimum 12 h). The liquid was drained and the rice was spread onto trays to dry. A solution of CPT HCl was prepared (4%, based on the rice) and, again, the rice was soaked overnight. This solution was drained and the rice was dried on foil sheets. After analysis for CPTH, any shortages were supplemented using the Alcolec S: soybean oil adhesive at 1.5% and the required CPT HCl powder. The adhesive is applied to minimize powder loss on this bait even if the CPT HCl concentration is within limits.

Preparation of primary, calibration, and working standards and fortified samples

The primary standard of CPT HCl (~ 1000 mg/mL) was prepared in deionized water. The standards for both the rough-hulled rice matrix method and the ethyl-cellulose-coated rice matrix methods were prepared by diluting the stock solution into the appropriate mobile phase. The standards used to establish linearity for the rough-hulled rice matrix method were prepared at: 1, 10, 25, 50, 75, and 100 µg/mL in 70% ACN–30% 0.01M KH_2PO_4 buffer (pH 2). For the ethyl-cellulose-coated rice matrix, the standards were prepared at 1, 5, 10, 25, 50, 75, and 100 µg/mL in 60% ACN–40% 0.01M KH_2PO_4 buffer (pH 8). Standards at approximately 50 µg/mL were prepared in the appropriate mobile phase and analyzed during sample analysis. Concentrations of analyte in the sample extracts were calculated from this external standard.

Both the rough-hulled rice and ethyl-cellulose-coated rice matrices were dry fortified with the salt: CPT HCl at either 1% or 3% (w/w). For a 1% fortified rice sample, approximately 10 mg of CPT HCl was added to an approximate 1-g rice sample. For a 3% fortified rice sample, approximately 30 mg of CPT HCl was added to an approximate 1-g rice sample. To assess the importance of $NaHSO_3$ treatment, the rough-hulled rice seed, both $NaHSO_3$ washed and unwashed seeds, were fortified with approximately 20 mg of CPT HCl.

Extraction

Extraction from ethyl-cellulose-coated CPT HCl treated rice bait

A 1.0-g sample of treated rice was weighed into a plastic centrifuge tube. The ethyl cellulose was dissolved by adding 6.0 mL of ACN, followed by sonication of the mixture for 10 min and then agitation on a mechanical shaker for 10 min. To facilitate the dissolution of the CPT (free base) that might be sorbed to the seed, 4.0 mL of 0.01M HCl was added, and this mixture was agitated for 10 min on a mechanical shaker. The mixture was centrifuged for 2 min and the supernatant decanted into a 50-mL volumetric flask. The extraction was repeated twice more by adding 10.0 mL of 0.01M HCl and then 5.0 mL of 0.01M HCl. All extracts were combined. The pH was adjusted by adding 10 mL of 0.02M KH_2PO_4 buffer (pH 8) to the flask. The solution was then brought

to volume with ACN and 1.00 mL of this was diluted 1:10 in mobile phase (60% ACN–40% 0.01M KH_2PO_4 buffer, pH 8). An aliquot was filtered through a 0.45-µm pore Teflon filter into an LC vial and capped.

Extraction from CPT HCl-treated rough-hulled rice bait

A 1.0-g sample of treated rice was weighed into a plastic centrifuge tube. The CPTH was extracted by adding 10.0 mL of 70% ACN–30% (pH 2) KH_2PO_4 buffer and then shaking on a mechanical shaker for 10 min. The mixture was centrifuged for 2 min and the supernatant decanted into a 25-mL volumetric flask. The extraction was repeated once more, and all extracts were combined in a 25-mL volumetric flask. The solution was then brought to volume and 1.00 mL of this was diluted 1:20 with 70% ACN–30% (pH 2) KH_2PO_4 buffer. An aliquot was filtered through a 0.45-µm pore Teflon filter into an LC vial and capped.

Results and Discussion

The two methods were developed sequentially, with the method for ethyl-cellulose-coated rice developed first. Both methods are similar in that they use an acid to protonate the CPTH to aid in its release from sorption sites. This was based on prior experience with CPTH in bird tissue and β-cyclodextrin sorbed CPTH formulated baits (5,6). The ethyl-cellulose-coated rice method used ACN to dissolve the ethyl cellulose coating. The concentration of CPTH in the final solution in both methods for a rice bait sample (containing ~ 2%) CPTH was approximately 40 µg/mL.

Chromatography of CPT, CPTH, and method instrument detection limit

In the pH 8 mobile phase, CPT eluted at 5.7 min with the first nonretained peak, which is used to indicate column void volume that elutes at 0.47 min, and in the pH 2 mobile phase, CPTH eluted at 3.4 min, with the first nonretained peak eluting at 0.37 min. The peak width at half height for a working standard (~ 50 mg/mL) differed slightly for the two mobile phases with a peak width at half height for the pH 2 mobile phase of 0.080 min, compared with a value of 0.110 min for the pH 8 mobile phase. The two methods differed markedly in retention factor, theoretical plate number, and response factor (Table I).

The instrument detection limit (IDL) is defined as the concentration of CPTH that would produce a peak height five times the base line noise that is measured peak to peak in a mobile phase blank (7). The IDL for the ethyl-cellulose-coated rice matrix method was 0.12 µg/mL, and for the rough-hulled rice matrix method it was 0.08 µg/mL.

Table I. Chromatographic Characteristics of the Two Methods

	Ethyl-cellulose-coated rice matrix (pH 8 mobile phase)	Rough-hulled rice matrix (pH 2 mobile phase)
Retention factor (k')	11.0	8.1
Theoretical plates (N)	14946	10121
Response factor (peak area/std. conc.)	17.3	12.9

Unfortified control rough-hulled rice and ethyl-cellulose-coated rice obtained from the Louisiana supplier produced no significant chromatographic interferences at the time of retention of CPT(H) (Figure 2). There were no peaks in either matrix that eluted close to the CPTH peak (Figure 2). Chromatograms from the analysis of extracts of the rough-hulled rice obtained from suppliers in Mississippi and Texas using the rough-hulled rice matrix method did not contain any interfering peaks at the time of elution (data not shown). Chromatograms from the analysis of extracts of rice obtained (from suppliers in Missouri and California) using the ethyl cellulose coated rice matrix method did not contain any interfering peaks at the time of elution (data not shown).

Assay linearity, method dection limit, limit of quantitation, and recovery

Linearity for the rough-hulled rice matrix method was determined across the range of 5 to 120 µg/mL. Linearity for the ethyl-cellulose matrix method was established from 1 to 100 µg/mL. Regression equations were calculated for CPTH concentration versus peak area using SAS version 8.01 (SAS Institute Inc., Cary, NC). For both methods, two sets of standards from separate stock solutions were prepared and injected in replicate. Both methods were determined to be linear over their respective ranges, with the ethyl-cellulose-coated rice matrix method having an $R^2 = 0.9988$ and the rough-hulled rice matrix method having an $R^2 = 0.9999$.

The method limit of detection (LOD) and method limit of quantitation (LOQ) for both methods were determined by extracting and analyzing seven replicate unfortified control samples and then two sets of fortified samples, which were fortified at 1% CPT HCl (w/w). For both matrices, the LOD was calculated as the concentration of CPTH that would produce a peak height 3.14 times the standard deviation (3.14 s) of the seven replicates of the

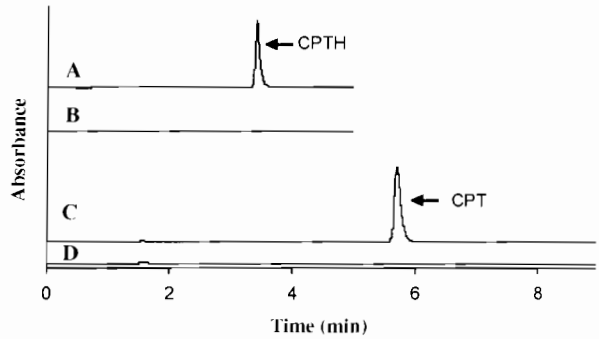

Figure 2. Chromatogram for a rough-hulled rice control sample fortified at 3% (A). Chromatogram for a rough-hulled rice control sample (B). Both chromatograms (A,B) were collected with a mobile phase of 70% ACN–30% 0.01M KH_2PO_4 buffer (pH 2.0). The chromatographic traces end before 9 min because the analysis run time was only 5 min. Chromatogram for an ethyl cellulose coated rice control sample (C). Chromatogram for an ethyl cellulose coated rice a sample fortified at 3% (D). Both chromatograms (C,D) were collected with a mobile phase of 60% ACN–40% 0.01M KH_2PO_4 buffer (pH 8.0).

sample above the baseline in a blank sample (7). The LOQ was calculated as the concentration of analyte that would produce a signal 10 times the standard deviation of the mean of the seven replicates above the baseline in a blank sample (7). The LOD was 1.8 mg/g and the LOQ was 6.3 mg/g for the ethyl-cellulose-coated rice matrix method. The LOD was 1.5 mg/g and the LOQ was 5.1 mg/g for the rough-hulled rice matrix method. Use of the 1% (~ 10 mg/g) fortified rice to estimate the MDL was considered acceptable because the LODs were approximately ⅓ the level of fortification (7).

Recoveries were assessed using rice-sample replicates fortified at both 1% and 3%, and their concentrations were determined using a single point working calibration standard (not extracted) prepared in the appropriate mobile phase. Analyte recovery was calculated as a percentage from the measured amount of analyte divided by the mass of the analyte added to the sample. The mean percent recoveries for the 1% and 3% fortified samples in the rough-hulled rice matrix method were 92.0% ± 1.1% and 94.0% ± 0.8%. The percent recoveries for the same levels of fortification in the ethyl-cellulose-coated rice matrix method were 102% ± 6% and 101% ± 2%, respectively. Percent recovery values in the range of 80–120% were considered to be acceptable.

The rough-hulled rice was washed with $NaHSO_3$ to prevent the CPT HCl from reacting with the surface of the caryopsis. Upon wetting, the caryopsis turned bright orange in the presence of CPT HCl when the wash step was not included. CPTH was extracted from both washed and unwashed rough-hulled rice fortified with 20 mg/g CPT HCl. For (n = 3) the unwashed rough-hulled rice, the percent recovery was 95.5% ± 3.5%; and for the $NaHSO_3$ washed rough-hulled rice, the recovery was 83.6% ± 6.4%. These values were not significantly different when compared using the Student's t-test (α = 0.05) in Excel (Microsoft, Redmond, WA). However, the coloration of the seed was considered unacceptable because birds may visually select against the treated seed in a bait mixture with untreated seed.

Accuracy and precision

Intraday accuracy and precision were determined for both methods on 3 separate days by dry-spiking control rough-hulled rice or ethyl-cellulose-coated rice at approximately 10 and 30 mg CPT HCl (as shown in Table II). For the replicates at each level accuracy (%D) was within ± 15%. Precision as expressed by the relative standard deviation (RSD) was less than 15% for both methods. Interday accuracy and precision were determined using the mean concentrations for the analyte on each of the 3 days and are presented below the individual day data in Table II. Both interday accuracy and precision were within ± 15%.

To assess the effect of time on the stability of extracts, the day 2 extracts for each method were allowed to sit at ambient temperature for 24 h and reanalyzed. These data are identified as "aged extracts" in Table II. For both methods there was little or no effect of time on the amount of analyte measured as the accuracies, and precision of these data are of the same magnitude as those determined on the day of extraction.

Conclusion

The two methods had adequate precision and accuracy for the purpose of analyzing the CPT HCl fortified bait matrices that were examined. The principal effect of analyzing for CPTH at pH 2 compared with analyzing for CPT at pH 8 was to decrease the retention time for the elution of the analyte and the associated chromatographic performance parameters calculated from retention time. The two methods compliment one another and have proven useful in an ongoing bait development program at the U.S. Department of Agriculture/Animal and Plant Health Inspection Service/Wildlife Services/National Wildlife Research Center. Both methods have been used to support bait development for use in studies to assess efficacy in feeding trials.

Table II. Accuracy and Precision Data for Three Different Days of Extraction for Rice Dry Fortified with CPT HCl at Approximately 10 or 30 mg/g of Rice

	Ethyl-cellulose-coated rice matrix				Rough-hulled rice matrix			
	CPT HCl added (mg ± s)	CPT HCl measured (mg ± s)	RSD	%delta	CPT HCl added (mg ± s)	CPT HCl measured (mg ± s)	RSD	%D
Day 1	11.0 ± 0.1	11.1 ± 0.6	5.6	–0.6	10.8 ± 0.5	9.9 ± 0.5	5.5	7.8
(N = 7)	31.5 ± 0.2	32.0 ± 0.5	1.5	–1.4	31.5 ± 1.3	29.7 ± 1.3	4.5	5.8
Day 2	10.4 ± 1.4	9.9 ± 1.1	11.2	4.8	9.6 ± 0.2	8.8 ± 0.1	1.7	9.0
(N = 3)	30.6 ± 1.9	30.9 ± 1.7	5.5	–0.9	28.8 ± 1.1	26.0 ± 1.3	5.0	9.6
Day 3	10.3 ± 0.7	10.3 ± 0.7	7.1	0.5	9.5 ± 0.2	8.5 ± 0.3	4.0	10.0
(N = 3)	29.8 ± 2.8	29.7 ± 2.9	9.9	0.3	29.1 ± 0.6	27.5 ± 1.9	6.9	5.5
Interday	10.6 ± 0.4	10.4 ± 0.6	5.6	1.5	9.4 ± 0.7	9.1 ± 0.8	8.3	3.1
(N = 3)	30.6 ± 0.9	30.8 ± 1.1	3.7	–0.7	29.8 ± 1.5	27.7 ± 1.9	6.7	6.9
Aged extracts	10.4 ± 1.4	10.0 ± 1.1	11.2	4.1	9.6 ± 0.2	8.8 ± 0.1	1.6	8.1
(N = 3)	30.6 ± 1.9	30.7 ± 1.6	5.2	–0.5	28.8 ± 1.1	26.3 ± 1.2	4.7	8.6

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