FIELD METHOD FOR ANALYZING BIRDS FOR AVICIDE 3-CHLORO-*P*-TOLUIDINE HYDROCHLORIDE

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Abstract: We developed a fast and simple method to detect presence or absence of DRC-1339 (CPTH: 3-Chloro-*p*-toluidine Hydrochloride) in birds that fed on DRC-1339 bait sites. We compared the effectiveness of the colorimetric method to the previously published analytical method using birds collected from DRC-1339 bait sites in Louisiana and Texas. We also conducted tests with caged red-winged blackbirds (*Agelaius phoeniceus*) to determine if time from consumption of DRC-1339-treated bait to death and time from death to colorimetric analysis affected test results. The colorimetric assay was effective in detecting the presence or absence of DRC-1339 in birds collected from bait sites. In the tests with caged birds, the method resulted in the detection of four grains of treated rice consumed up to 120 minutes post consumption, but failed to detect 1 grain of treated rice consumed at 120 minutes. Frozen samples of 4 treated consumed rice grains could be detected up to 90 days post collection.

Key words: 3-Chloro-*p*-toluidine Hydrochloride, CPTH, detection, colorimetric method, rice, stability

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INTRODUCTION

The avicide 3-chloro-4-methyl benzamine Hydrochloride [3-chloro-ρ-toluidine HCl] (CPTH) or DRC-1339 is currently used for operational baiting programs around the country for a number of avian species. It is used in Louisiana under a state-sponsored Emergency Use Permit through the Environmental Protection Agency (EPA) and in Texas under the Compound DRC-1339 Concentrate - Staging Area label (EPA Registration No. 56228-30). Although an analytical method for quantification of CPTH has been developed (Hurlbut et al. 1998, Stahl et al. 2002), a faster and simpler qualitative method is required to detect presence or absence of the chemical during field studies. We developed a modified colorimetric method for field use and compared its reliability to the colorimetric method of Stahl et al. (2002) for detecting DRC-1339 residues in birds collected from bait sites in Louisiana and Texas. We also conducted tests with captive red-winged blackbirds (*Agelaius phoeniceus*) to

determine if time from consumption of DRC-1339-treated bait to death and the duration of sample storage affected detectable residues.

MATERIALS AND METHODS

Colorimetric Method

Reagents: Solvents include 2N hydrochloric acid (166 ml conc HCL per liter solution), 0.10% sodium nitrite (w/v), urea, 3N Sodium hydroxide (120 g NaOH per liter solution) with 2.5 g 2-napthol, tetrahydrofuran (THF), and anhydrous magnesium sulfate.

Standard Preparation and Quantification: DRC-1339 (from Pocatello Supply Depot, SCN-128) was recrystallized in acetonitrile/ethanol and assayed at essentially 100%. A standard curve was prepared at concentrations of 5, 25, 50, and 125 µg CPTH.

Initial Method

We conducted lab analysis on our field samples between April 1 and May 16, 2003 using the initial colorimetric method. Field samples consisted of birds collected from DRC-1339 bait sites that were not used for the residue analysis and at least one pretreatment bird of each species was analyzed as a control. Field birds were shipped to the lab on ice, and the gizzard and esophagus (to the base of the mouth) were removed. The contents of the gizzard and esophagus were scraped from the tissue and placed into a labeled plastic bag. The esophagus and gizzard were also placed in the bag. The mouth of the bird was checked for particles of rice that we might have missed. If a particle was present in the mouth, it was placed into the plastic bag. The number of particles of rice eaten was enumerated.

The reaction conditions basically followed those found in a qualitative organic text (Kemp 1979). The contents and organs were rinsed with 4 ml of 2N HCl in the plastic bag. The contents were massaged into one corner of the bag and left to sit for a minimum of 5 minutes to achieve extraction and cooled in an ice bath. Samples were filtered, and the filtrate was transferred to a test tube containing 2 mls of the napthol-sodium hydroxide solution. Prior to transfer, temperature was monitored until it reached 10º C, at which point 10 µl of sodium nitrite (10% w/v) was added, swirled, and returned to the ice bath. After approximately one minute, a few grains (No. 9 sieve) of urea were added to the tube, which was returned to the ice bath. Every 2-4 minutes, each tube was lifted out and checked for bubbling from the urea pellets. When the bubbling ceased, approximately 5-10 minutes, 2 ml of the HCL/DRC-1339 was transferred to the napthol-sodium hydroxide solution and the mixture was shaken. The development of an orange color indicated the presence of DRC-1339. The test tube was then centrifuged to force the particles containing the orange color to the bottom of the test tube. A qualitative assessment was then made on whether the mass at the bottom of the test tube was positive for the presence of DRC-1339 (i.e. was orange in color).

Modified Method

We conducted the lab analysis of our field samples between February 18 and April 30, 2004 using a modified colorimetric method. Field samples consisted of birds collected from DRC-1339 bait sites and at least one pretreatment bird of each species was analyzed as a control. Contents and the gizzard and esophagus of birds were processed as in the initial method.

Some modifications and improvements were made to the initial colorimetric method to allow for quantification of the CPTH in the gastro

intestinal tract. These modifications include the addition of 2.5 ml of tetrahydrofuran to the napthol/sodium hydroxide tube to act as a solvent extracting the diazo compound from reaction solution for spectrophotometric analysis at 472 nm.

DRC-1339 Residue Analysis: Residue analyses were conducted on 40 birds between April 3 and May 12, 2003. The entire gastrointestinal tract (intestines, gizzard and esophagus) was removed from each bird, placed in a plastic bag, and stored in a freezer until analyzed. Two matrices were analyzed using the method described by Stahl et al. (2002); esophagus/gizzard contents and gastrointestinal tract tissues (esophagus, gizzard, and intestines) combined, and breast muscle tissue. Samples from each bird were sub-sampled, and 2 replicates for each matrix were assayed. The CPTH concentration for each matrix, from each bird, was calculated as the average of the two values.

Cage Study

Up to 60 captive red-winged blackbirds per cage were housed in an outdoor aviary (3.0 × 6.1 × 3.0 m) with free access to a maintenance diet consisting of a combination of millet, milo, safflower, and sunflower. Water was available *ad libitum*. Birds were quarantined for at least 14 days prior to testing. While in quarantine and prior to testing birds were visually inspected for signs of illness or discomfort, and weighed, and banded with individually numbered bands. Environmental conditions were ambient and uncontrolled.

Blackbirds were placed one bird per cage (61 × 46 × 33 cm test cage; 2 × 4 = 8 cages/rack; 97 × 61 × 406 cm rack) in an outdoor building and provided with brown rice and water *ad libitum*. Birds were weighed before being placed in cages and randomly assigned to treatments based on weight (i.e., the heaviest birds assigned one to each group, the next heaviest birds assigned one to each group, until all the birds were assigned to treatment groups). Each group was randomly assigned to a treatment. Birds were gavaged with their respective treatments, after which they were allowed free access to rice *ad libitum* until they were scheduled to be euthanized.

Gavage: For gavaging each bird was held by one individual while another person administered the dose by gently prying the mouth open while a single rice grain was placed in the mouth. A 5-mm-diameter glass tube with a rounded end was used to gently slide the grain of rice lengthwise down the esophagus approximately 1 cm past the esophagus opening (just past the point where the bird can work the grain back out of the mouth). The esophagus was gently massaged externally to move the rice grain lengthwise down the esophagus. This process was repeated on each bird to administer up to four grains.

Phase 1 - Dose and Time to Death: Sixty-five birds were gavaged with 1 or 4 grains of brown rice treated with DRC-1339 at a 2% by weight concentration. Thirteen birds from each treatment group were euthanized at 0 (within 2 minutes), 10, 30, 60, and 120 minutes post dosing, and spectroscopic analysis was performed immediately following euthanization and processing. After euthanization, the birds were taken to the lab and processed using the same method as for our field samples. To provide a comparison between the spectroscopic assay and analytical method, 3 birds at each time interval were randomly selected and analyzed via the analytical method. The entire gastrointestinal tract (intestines, gizzard and esophagus) was removed from the bird, placed in a plastic bag, stored in a freezer, and analyzed the day following treatment.

Phase 2 - Time to Analysis: Fifty birds were gavaged with a 2% DRC-1339

dose of 4 grains of brown rice and euthanized within 5 minutes of dosing. After euthanization, the birds were taken to the lab and processed using the same method as for our field samples. The contents of 10 birds from each treatment group was analyzed using the colorimetric method at 0, 1, 30, 60, and 90 days following euthanization to determine loss of DRC-1339 during freezer storage at -21°C.

During phase 1 - one group of 65 birds were dosed with 1 grain of DRC-1339-treated rice and 13 birds each were euthanized at 0, 10, 30, 60, and 120 minutes; or phase 2 - one group of 50 birds were dosed with 4 grains of DRC-1339-treated rice and 10 birds each were analyzed at 0, 1, 30, 60 and 90 days.

Statistical Analysis: We conducted F-tests to compare sample variances between methods. We ran t-tests to compare the means of the analytical groups for each dose and time period to the means of the colorimetric groups for each dose and time period. We ran an ANOVA test to compare means of the time to analysis data.

RESULTS

Colorimetric Method

During the first year of analysis 407 total birds were tested using the first method. Of these, 247 birds ate less than 25 rice grains and 160 birds ate more than 25 rice grains. One treated grain to 25 untreated grains is the maximum ratio of treated to untreated allowed under Compound DRC-1339 Concentrate - Staging Area label (EPA Reg. No. 56228-30). We detected 31% positive from the birds that ate less than 25 rice grains, and 78% positive from the birds that ate more than 25 rice grains. During the second year of analysis 210 total birds were tested using the second method. Of these, 163 birds ate less than 25 grains of rice and 47 birds ate more than 25 grains of rice. We detected 32% positive from the birds that ate less than 25 rice grains, and 72% positive from the birds that ate more than 25 rice grains (Figure 1). We were able to detect concentrations ranging from 0.725 to 90.7 µg of DRC-1339 among these birds.

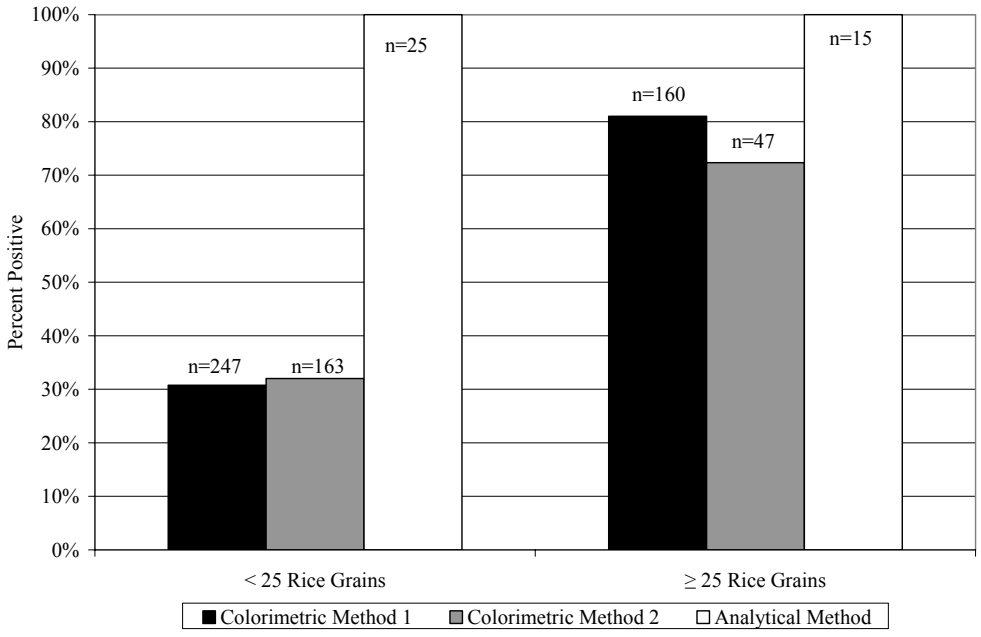

Figure 1. Colorimetric method percent positive results from the two field groups over two years.

DRC-1339 Residue Analysis

Results of the residue analysis on 40 birds collected from field sites showed that 25 ate less than 25 rice grains, and 15 ate more than 25 rice grains. Residue analysis detected 100% positive from the birds that ate less than 25 rice grains, and 100% positive from the birds that ate more than 25 rice grains (Figure 1). Concentrations from 0.067 to 70.45 µg of DRC-1339 were detected.

Cage Testing

Phase 1 – Dose and Time to Death: Results of the colorimetric analysis of 50 red-winged blackbirds dosed with a single grain of rice and then euthanized at 0, 10, 30, 60, and 120 minute time intervals showed average µg of DRC-1339 that were not statistically significantly different (α = 0.05) from results of the analytical method except at 60 and 120 minutes when the colorimetric method was unable to detect µg of DRC-1339 (Figure 2). Results of the colorimetric analysis of 50 red-winged blackbirds dosed with 4 grains of rice and then euthanized at 0, 10, 30, 60, and 120 minute time intervals showed average µg of DRC-1339 that were not statistically significantly different (α = 0.05) from results from the analytical method (Figure 3) except at the 10 and 120 minute group. In the four grain treatment group DRC-1339 was detected at an average of 1.78 µg after 120 minutes.

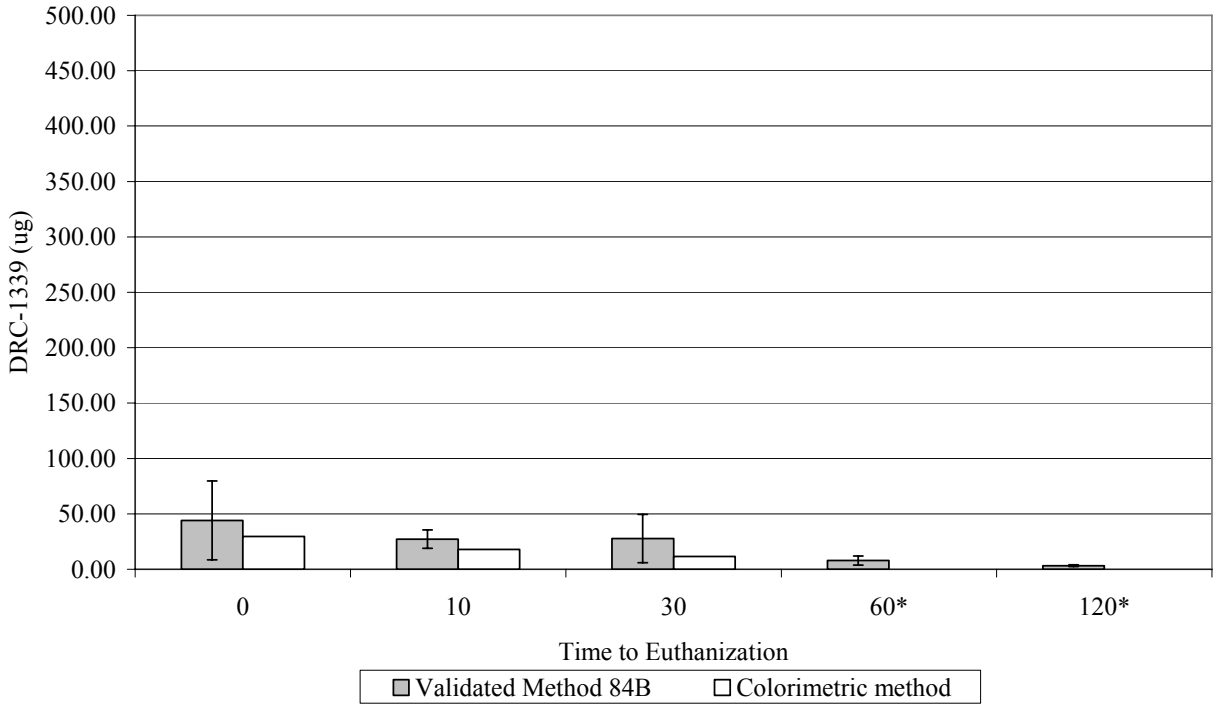

Figure 2. Single grain treatment ug DRC-1339 comparison between validated NWRC method 84B and Colorimetric method. Time categories marked with '*' indicate significance at the α = 0.05 level.

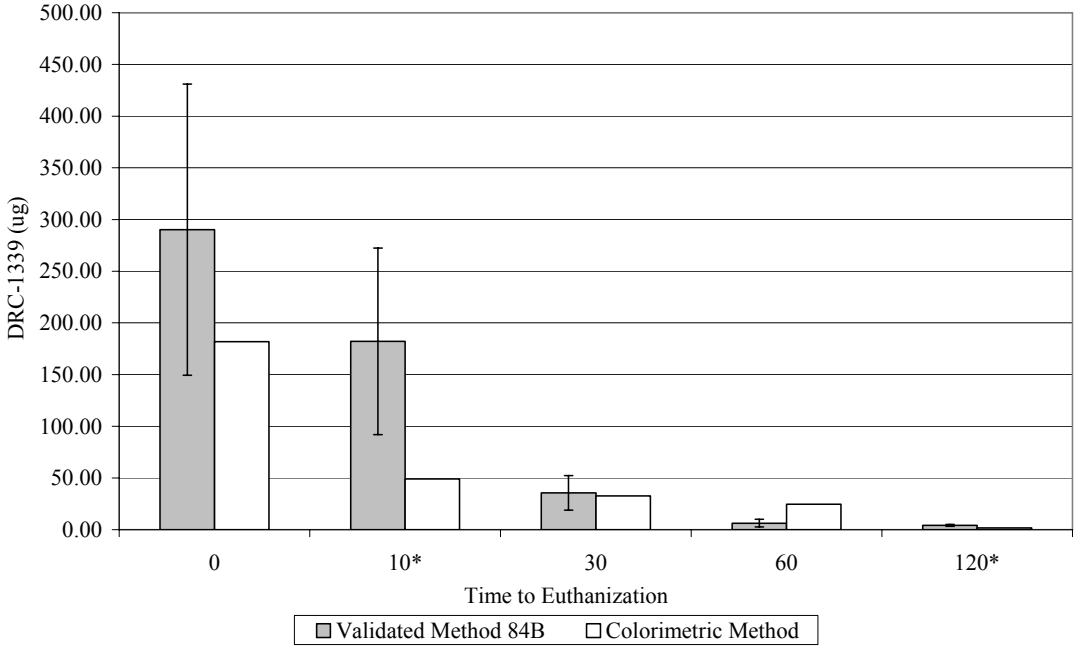

Figure 3. Four grain treatment comparison of validated NWRC method 84B to Colorimetric method time categories marked with '*' indicate significance at the $\alpha = 0.05$ level.

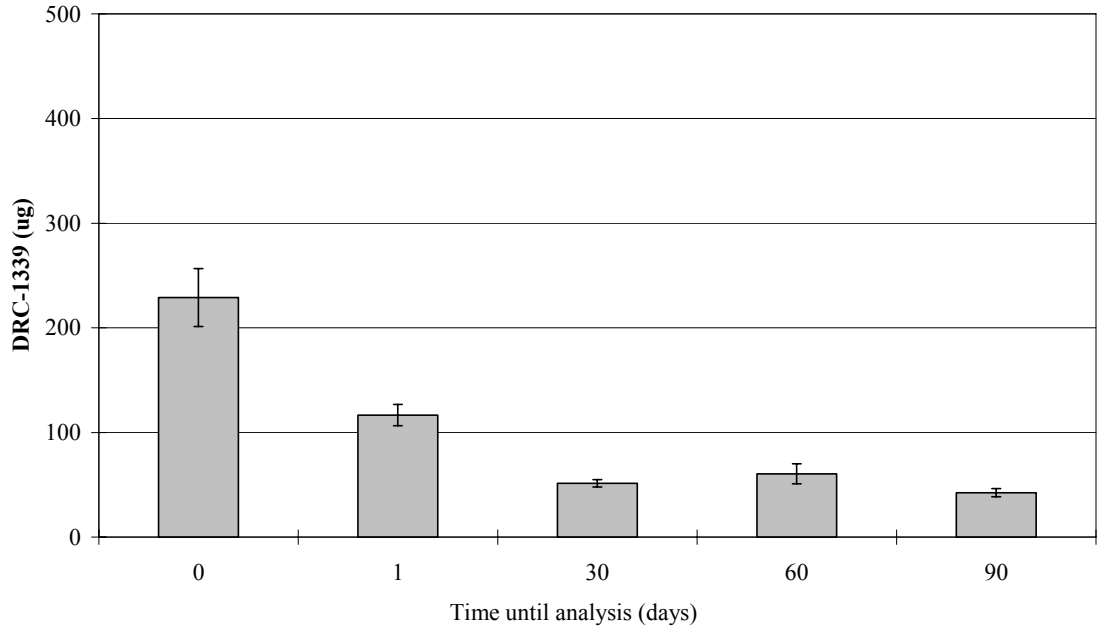

Figure 4. Average µg of DRC-1339 detected after gavaging 4 grains of DRC-1339 treated rice and analyzed at 0, 1, 30, 60, and 90 days from euthanization.

Phase 2 – Time to Analysis: Duration of storage impacted recoveries shows the combined averaged results of these analyses (Figure 4). Zero day analysis returned the highest observable levels of DRC-1339 with an average of 228.97 µg. Results from the 1, 30, 60, and 90 day analyses had averaged values of 116.67 µg, 51.50 µg, 60.57 µg, and 42.48 µg, respectively. The averages of the 0, 1, 30, 60, and 90 day analyses were statistically significantly different from each other at the $\alpha = 0.05$ level.

DISCUSSION

DRC-1339 is a primary aromatic amine, a functional group not commonly seen in biological systems. Primary aromatic amines react with nitrous acid to form an azo intermediate (reaction 1), which react with an activated aromatic (naphthol) (reaction 2) to form a highly colored diazocompound (Figure 5). The reaction (labeled reaction 1) works best in acid, and reaction 2 occurs in base. The compound is a pink-orange color generally not found in stomach contents of granivorous birds. Thus, this method gives few false positives. Fortuitously, the dizacompound associates with colloidal starches and is concentrated to a small "button" at the bottom of a tube after centrifugation making qualitative assessment easy (method one).

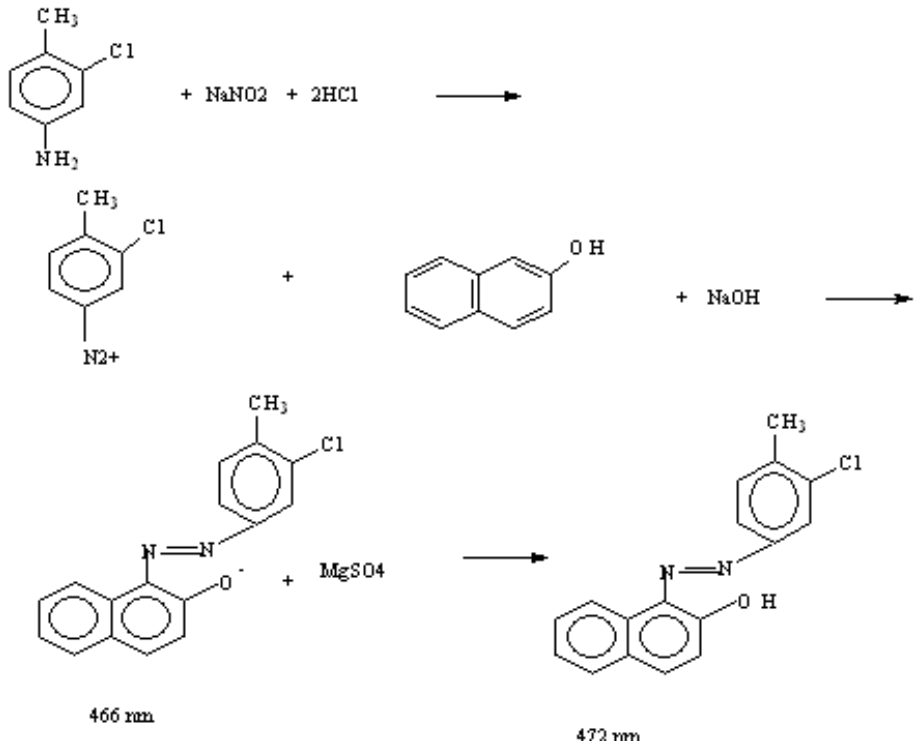

Figure 5. DRC-1339 Diazo/ coupling reaction

The reaction sequence to generate the colored adduct is amazingly robust, as evidenced by the R^2 of the calibration curve (0.9991) (Figure 6). Small variations in timings, multiple persons doing pippetings (six liquid transfers are performed), differences in temperatures of the ice baths, did not make observable differences as evidenced by very repeatable DRC-1339 standards that were done with each set of birds analyzed.

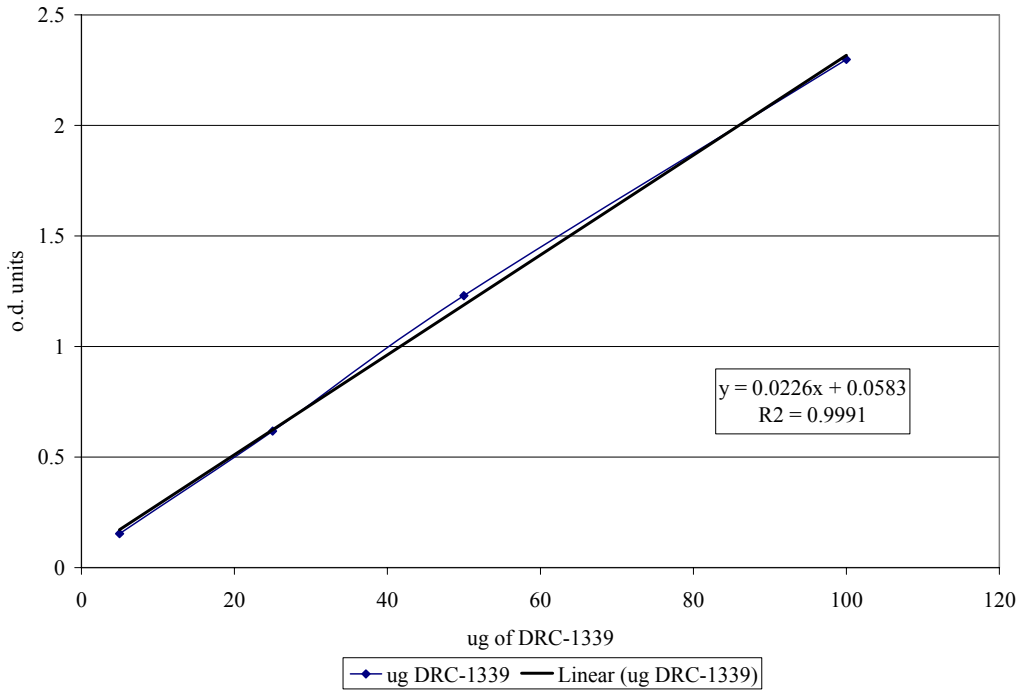

Figure 6. Standardization of Colorimetric Reaction

To establish a more quantitative nature for the diazotization reaction, we added the diazo product to the basic naphthol in the presence of tetrahydrofuran (THF) as an extraction solvent (method two). Simply rocking the reaction tube end to end ten times and subsequent centrifugation for about 10 minutes at 3,000 rpm gave good phase separation and allowed the nearly total separation of the THF and at least to the eye the total extraction of all color. The addition of anhydrous magnesium sulfate removed water from the THF and acidified the product changing the original reddish-orange to a golden tan color. The THF volume was normalized to 1.3 mL in small tubes with a factory pre-applied white mark. The absorbance at 472 nm is linear to about 125 µg (Figure 6) with a method level of detection (mlod) of 0.6 µg and a method limit of quantification (mloq) of 1.8 µg and an R^2 of 0.9991.

Analysis from the two sets of treated seeds (2 treated plus 50 untreated) gave a recovery of 95 ± 4%.

The colorimetric method revealed the presence of DRC-1339 in most field samples containing greater than 25 rice grains (Figure 1), but was not as sensitive as the analytical method for samples containing less than 25 rice grains. Of the analytical samples containing less than 25 rice grains, 10 of 25 were at the analytical method MLOD of 0.025 µg/g and the 100% positives may actually be realistically 68% positives. Of the analytical samples containing greater than 25 rice grains 2 of 15 were at the MLOD and 87% positive is a more likely result.

Our cage testing yielded complimentary results to our field trials. DRC-1339 was detected in birds that ate only a single grain of rice only if the birds were collected within 30 minutes of consumption. For birds that ate multiple grains, DRC-1339 was detected up to 120 minutes after consumption of treated rice. The four grain group did show a significant result (colorimetric analysis of 4 grains was significantly different from the analytical

method) after 10 minutes. The processing time (time to remove the gizzard, esophagus and contents) of the birds in the 10-minute group was longer than for any other treatment group. During this time more of the DRC-1339 probably was absorbed by the body, which caused the observed levels of DRC-1339 to be lower than with the analytical method. The gastrointestinal juices in a bird might have caused more rapid degradation of DRC-1339 than samples not exposed to gastrointestinal juices. Data from a liquid gavage study indicated that uptake of DRC-1339 was rapid in birds, resulting in maximum absorption within 15 minutes (Goldade et al. 2004). Another factor that could contribute to the detection limits of our method is that we extracted only the gizzard and esophagus and their contents, whereas the analytical method uses the entire gastrointestinal tract.

In our time-to-analysis experiment, samples processed from birds that ate four treated grains and subsequently stored in a freezer at -21°C were detectable for up to 90 days post collection. Storage stability was established for the analytical method, and samples maintained for longer than 90 days had little if any detectable CPTH (Stahl et al. 2002).

We expected to be able to determine number of hot grains consumed by the colorimetric analysis. However, the reported concentration of DRC-1339 did not correspond to the number of hot grains gavaged. When we compared known dosages (either 1 or 4 grains) with the amount of DRC-1339 detected, we did not see results corresponding to the number of hot grains gavaged because the compound either degraded or was absorbed by the body too quickly (Figures 2 and 3).

We processed up to 40 samples a day with our modified colorimetric method; the main limiting factors were available manpower for processing the birds and our ability to keep track of the number of test tubes. We could process only 24 samples with the analytical method of Stahl et al. 2002. The colorimetric assay for detecting DRC-1339 in birds can be used to analyze a large numbers of birds by one technician with only moderate laboratory skills, with the only restriction that collection be achieved within a relatively short time following ingestion of the treated bait.

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