Determination of Whole-Body Rotenone Residues in the Brown Tree Snake (*Boiga irregularis*)

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The brown tree snake (*Boiga irregularis*) is an introduced pest in Guam, responsible for extensive agricultural damage, the extinction of several bird species, and severe and frequent electrical power outages. Rotenone, a naturally occurring pesticide, has been investigated as a possible chemical control agent. An analytical method was developed to assess whole body rotenone residues ranging in concentration from 0.035 to 250 $\mu g\ g^{-1}$ in snakes. The method employed ethyl acetate extraction of 2 g samples of cryogenically frozen, pulverized snakes, followed by silica and Florisil solid-phase extraction cleanup. Extract analysis was performed using a high-performance liquid chromatography system employing a cyanopropyl analytical column. Tissues fortified to concentrations of 0.035, 4.82, and 250 $\mu g\ g^{-1}$ yielded analyte recoveries of 85.1, 85.6, and 83.5%, respectively. The linear response of rotenone standard solutions was assessed from 0.025 to 0.25 $\mu g\ mL^{-1}$ ($r^2 = 0.9968$) and from 0.250 to 125 $\mu g\ mL^{-1}$ ($r^2 = 0.9999$). The method was simple, rugged, and reliable.

Keywords: *Rotenone; brown tree snake; Boiga irregularis; high-performance liquid chromatography; solid-phase extraction*

INTRODUCTION

The brown tree snake (*Boiga irregularis*) is a nocturnal, arboreal, and mildly venomous reptile with an original distribution throughout Papua New Guinea, coastal northern and eastern Australia, and the islands of northwestern Melanesia (Kinghorn, 1964; McCoy, 1980). The accidental introduction and subsequent proliferation of the brown tree snake on the island of Guam following World War II has created a number of serious problems including (1) extensive agricultural losses resulting from predation on animals ranging from chicks to pigs and dogs, as well as other household pets (Fritts, 1988; Fritts and McCoid, 1991); (2) the near or complete extinction of several bird species (Savidge, 1987); and (3) interruptions of electrical service due to power line and equipment damage from short circuits resulting from snakes crawling along electrical lines (Fritts et al., 1987).

In addition to these problems, high snake population densities increase the likelihood of dispersal to, and similar consequences for, other Pacific islands via the high volume of civilian and military cargo from Guam. Individual snakes have been observed on several other islands, including Hawaii (Fritts, 1987, 1988).

In an effort to reduce snake populations on Guam, Wildlife Services (WS) personnel are investigating a number of control measures, including traps, lures, and a variety of toxicants. One chemical that was recently demonstrated to be highly toxic to brown tree snakes is rotenone (Brooks et al., 1998), a naturally occurring compound found in several Fabaceae plant genera. Rotenone is currently registered with the U.S. Environmental Protection Agency and is used extensively as both an insecticide and piscicide. To support the investigation into rotenone toxicity to brown tree snakes and to provide tissue residue data for the evaluation of secondary hazards to scavengers/predators, an analytical method for the determination of whole body rotenone residues ranging in concentration from 0.035 to 250 $\mu g\ g^{-1}$ was required.

Due to its comparative lack of volatility and thermal instability, the majority of analytical methods developed for rotenone have employed high-performance liquid chromatography (HPLC) as a separation technique. Most early methods involved formulation analysis (Bushway and Hanks, 1977; Bushway, 1983, 1984). Other techniques focused on rotenone determination in more complex tissue samples and the need for decreased limits of detection and extraction/sample cleanup procedures. Bowman et al. (1978) sampled (2.5−5 g) mouse fetuses and gastrointestinal (GI) tracts for ethyl ether extraction. Extracts were washed with hexane/acetonitrile, loaded (in benzene) onto an Na_2SO_4−silica gel−Na_2SO_4 column, and eluted in benzene/acetone. Rotenone recovery was 59.4% (±8.4%) for fetuses and 93.6% (±11.6%) for GI tracts. Rach and Gingerich (1986) examined radiolabled ^{14}C-rotenone uptake of various tissues in three fish species. Samples were lyophilized, extracted in acetone and methanol followed by water/ethyl ether extraction, and then reconstituted in 50:50 cyclohexane/methylene chloride. Gel permeation chromatography (GPC) was used to remove lipid contaminants, and the extract was analyzed by reverse-phase HPLC. GPC lipid removal was also employed by Dawson and Allen (1988), who determined rotenone content in fish, crayfish, mussels, and sediments. Samples (10 g) were blender ground over dry ice and then left overnight for CO_2 to sublime before transfer to a glass column for ethyl ether extraction. Extracts were redissolved in 50:50 cyclohexane/methylene chloride, loaded/eluted from a GPC column, and then transferred to a silica column

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for further ethyl ether cleanup. Samples were then analyzed using HPLC with a C_{18} column and methanol/water (70:30 or 60:40) mobile phase. Triplicate samples of crayfish, mussels, fish muscle, and fish offal were fortified to a concentration of 0.1 μg g^{-1} just prior to extraction, and recoveries for all tissues exceeded 83%.

Although useful, these methods can be time-consuming and require large solvent volumes and/or use of carcinogenic or chlorinated solvents (i.e., benzene, methylene chloride). This paper describes an analytical method that utilizes simple liquid extraction of small tissue samples (2 g) followed by solid-phase extraction (SPE) cleanup using silica and Florisil packings to assess rotenone residues in the brown tree snake ranging from 0.035 to 250 μg g^{-1}.

MATERIALS AND METHODS

Sample Preparation. Snakes ranging in mass from 30 to 250 g were collected by WS personnel on Guam and used to screen toxicants. Untreated snakes from these studies were later used for the validation of this method. Following post-experimental euthanization, snakes were weighed, coiled, wrapped in aluminum foil, frozen, and shipped to the WS/National Wildlife Research Center in Fort Collins, CO, where they were stored at −20 °C. For sample preparation, snakes were partially thawed and the aluminum foil was removed. Snakes were then weighed and transferred directly to a stainless steel cylinder for cryogenic pulverization as described by Sterner and Mauldin (1995). The pulverized tissue was then transferred to a polyethelene jar for frozen storage until used.

Sample Extraction. A flow chart summary of the extraction and cleanup procedures is shown in Figure 1. All glassware was pentane-washed prior to usage. Two grams of frozen, powdered tissue was weighed into a 50-mL screw-top glass test tube (tube) and allowed to thaw at room temperature. Four grams of sodium sulfate (anhydrous) was added to the tissue, which was then vigorously hand mixed. The tissue was allowed to stand for 15 min, with intermittent vortex mixing. Ethyl acetate (10 mL) was added to the tube, which was capped, vortex mixed, placed into a mechanical shaker (Equalpoise, model 6550, 2$^3/_8$ stroke, Eberbach, Ann Arbor, MI), and shaken at ≈175 strokes min^{-1} for 15 min. The tube was then centrifuged at ≈2000 rpm for 5 min. The supernatant was transferred to a 25-mL tube, which was placed in a 50 °C water bath, and the contents were partially evaporated under nitrogen (N_2). A second 10-mL aliquot of ethyl acetate was added to the original sample tube, which was then vortex mixed, shaken, and centrifuged as previously described. The supernatant was transferred to the collection tube and the volume further reduced with N_2. Ethyl acetate (5 mL) was again added to the tissue, which was then vortex mixed and centrifuged. The supernatant was transferred to the collection tube and evaporated to near dryness. *n*-Pentane (2 mL) was added to reconstitute the tissue extract, which was then vortex mixed, sonicated for 5 min, and mixed again.

Sample Cleanup. *Preparation.* Freshly unsealed silica SPE columns (1 g, 3 mL, Isolute, 460-0100-B, IST, Mid Glamorgan, U.K.) were inserted into Teflon stopcocks in a sample processing station (VacMaster-10, IST). Prewashed plastic syringes (10 mL, without plungers) were modified by removing the Luer cuff and used as additional solvent reservoirs by inserting one into each SPE column using a column adaptor. With the stopcock closed, *n*-pentane (5 mL) was added to the 10-mL reservoir. Because of solvent volatility, it was necessary to allow ≈0.5 mL of solvent from the 10-mL syringe reservoir into the SPE column reservoir prior to elution to maintain solvent flow continuity. This was accomplished by gently loosening the adaptor (by twisting or rocking to break the seal) and then allowing solvent to flow slowly into the SPE column reservoir. The stopcock was opened to allow ≈2 mL of pentane to flow through and wet the SPE column packing

With solvent flow stopped, the SPE column was allowed to stand for 5 min, with occasional gentle tapping to release air bubbles. Solvent flow was resumed and continued until all solvent had passed the upper frit of the SPE column. The stopcock was closed and the SPE column was never allowed to dry out. This solvent preload procedure was followed before each cleanup step.

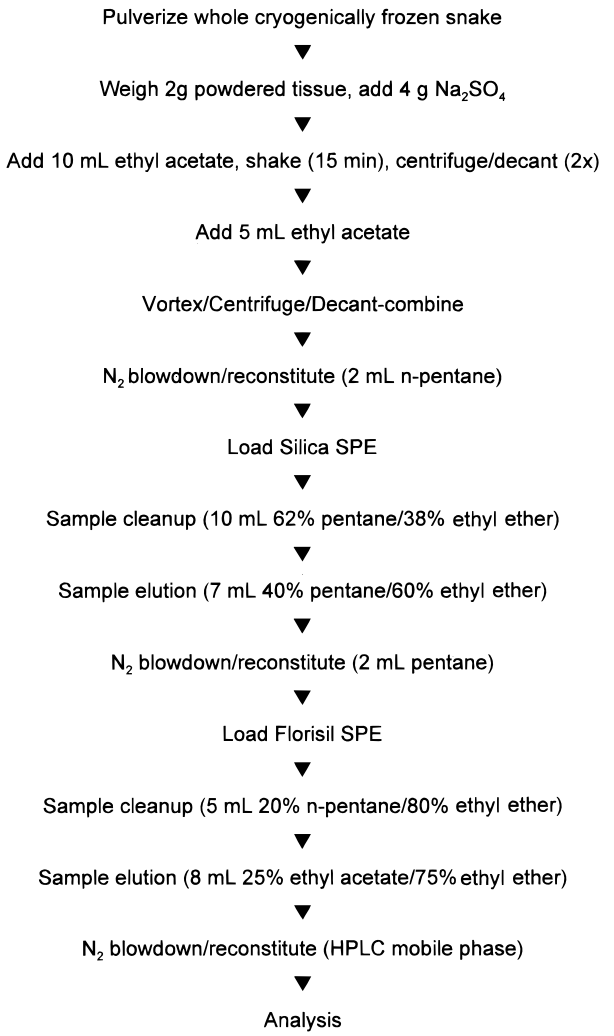

Figure 1. Flow chart summary of sample extraction and cleanup procedures.

Sample Cleanup/Elution, Silica SPE Column. With the stopcock closed to prevent solvent flow into the SPE column, the reconstituted extract was added to the 10-mL reservoir, followed by two 1-mL pentane rinses of the extract collection tube. The stopcock was opened and the sample extract loaded onto the SPE column at a rate of ≈1 mL min^{-1}. When the upper reservoir emptied, solvent flow was stopped and 0.5 mL of pentane was used to rinse the reservoir walls and allowed to flow into the SPE column reservoir and onto the column.

Cleanup solvent (10.0 mL, 62% *n*-pentane/38% ethyl ether) was added to the 10-mL reservoir and allowed to flow through the SPE column at a rate of ≈1 mL min^{-1}. The analyte was then eluted into a clean 25-mL tube with 7.0 mL of elution solvent (40% *n*-pentane/60% ethyl ether). The eluate was blown to dryness under N_2 as previously described. *n*-Pentane (2 mL) was then added, and the sample was vortex mixed, sonicated for 5 min, vortex mixed, and retained for further cleanup.

Sample Cleanup/Elution, Florisil SPE Column. Florisil SPE columns were prepared by inserting a 20-μm polyethylene frit (Isolute, 120-1033-B, Jones Chromatography, Lakewood, CO) to the bottom of an empty 3-mL empty reservoir (Isolute, 120-1002-B). One gram of Florisil (Alltech, PR, 60/100 mesh) stored at >100 °C was weighed and added to the reservoir in three ≈333-mg aliquots. Each aliquot was tamped down repeatedly

with a polypropylene plunger to pack. When all of the Florisil had been added and packed, a second frit was inserted into the reservoir to contain the Florisil. SPE columns were packed in advance and stored in a vacuum over desiccant prior to use.

Each Florisil SPE column was wetted as previously described. With the stopcock closed, the pentane-reconstituted sample extract was added to the 10-mL reservoir, followed by two 1-mL rinses of the extract collection tube. The sample extract was then loaded onto the Florisil column as previously described for the silica SPE column. Cleanup solvent (5.0 mL, 20% *n*-pentane/80% ethyl ether) was added to the 10-mL reservoir and eluted through the SPE column as described earlier. The analyte was then eluted with 8.0 mL of elution solvent (25% ethyl acetate/75% ethyl ether) into a volume-calibrated 10-mL centrifuge tube. The final eluate was blown to dryness (N_2) and reconstituted by first adding 350 μL of acetonitrile to the tube, which was vortex mixed, sonicated for 5 min, and vortex mixed again. Water was then added to bring the final sample volume to 1.0 mL, the solvent proportions in the HPLC mobile phase. The sample was then vortex mixed and filtered through a 0.45-μm Teflon filter into an amber LC vial for analysis.

Chromatographic Conditions. Whole-body snake extract samples were analyzed using a Hewlett-Packard (HP) 1090 liquid chromatograph (Hewlett-Packard Co., Sunnyvale, CA) equipped with an HP 1050 variable wavelength detector (295 nm): column, Keystone Cyano/B, 5 μm, 250 mm × 4.6 mm i.d. with cyanopropyl guard; oven, 40 °C; mobile phase, 35% acetonitrile/65% water (Milli-Q, Millipore, Molsheim, France); flow rate, 1 mL min^{-1}; injection volume, 0.1 mL; run length, 20 min.

Selectivity, Bias and Repeatability, and Method Limit of Detection (MLOD). Eight control brown tree snakes were pulverized, and two aliquots (2.0 g each) from each snake were weighed into separate 50-mL glass tubes. Each set of eight samples was fortified with 100 μL of either a 0.70 μg mL^{-1} or a 5.00 mg mL^{-1} rotenone standard solution in acetonitrile, yielding tissue concentrations of 0.035 and 250 μg g^{-1}. Tissues were then processed and analyzed as previously described. Final reconstituted extracts from the 250 μg g^{-1} fortification samples were diluted 1:5 with mobile phase to bring rotenone concentrations into validated standard curve range. During actual analysis of treated snakes from Guam, extracts yielding rotenone concentrations >62.5 μg g^{-1} were diluted 1:5 with mobile phase and reanalyzed, and nine additional control samples were prepared and fortified with 100 μL of a 96.4 μg mL^{-1} rotenone standard solution in acetonitrile, yielding a fortified tissue concentration of 4.82 μg g^{-1}. The MLOD was defined as the rotenone concentration required to generate a signal 3 times the baseline noise (measured peak-to-peak) in control snake extract chromatograms at the retention time of rotenone.

RESULTS AND DISCUSSION

Linearity. Two independent sets of calibration standards for each of two concentration ranges (0.040−0.250 and 0.250−125 μg mL^{-1}) were prepared and analyzed under the conditions described for this method. Linear regression analysis using the SAS PROC REG program (SAS, Inc., 1989) of the 0.250−125 μg mL^{-1} yielded an r^2 of 0.9999, a *y*-intercept of −26.77 (±17.4; HO: $y_{int} = 0$, $p = 0.1442$), and a slope of 156.68. Response factors (concentration/response) were essentially unchanged throughout the rotenone concentration range, with a mean value of 6.67×10^{-3} ($\pm 2.4 \times 10^{-4}$) and a coefficient of variation (CV) of 3.6%. A log versus log regression of the same data yielded a slope of 1.014. Taken together, these data indicated a highly linear and directly proportional relationship between rotenone concentration and detector response and justified the use of single-point (10.0 μg mL^{-1}) calibration during field sample analysis for samples of appropriate concentrations. Regression analysis of the 0.04−0.250 μg mL^{-1} range yielded an r^2 of 0.9968, a *y*-intercept of −0.0549 (HO: $y_{int} = 0$, $p = 0.833$), and a slope of 143.81. Response factors were essentially constant throughout the concentration range, with a mean value of 7.0×10^{-3} ($\pm 3.6 \times 10^{-4}$) and a CV of 5.1%. A log versus log regression gave a slope of 1.007, indicating a linear, proportional relationship between rotenone concentration and detector response. During field sample analysis, a five-point standard curve ranging from 0.04 to 0.250 μg mL^{-1} was used to quantify samples in that range.

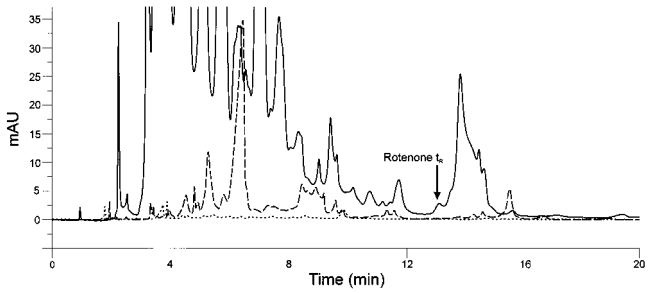

Figure 2. Comparison of chromatograms from control snake tissue extract with no SPE column cleanup (—), with silica SPE column cleanup only (− − −), and with silica and Florisil SPE column cleanup (- - -).

Selectivity, Bias and Repeatability, and MLOD. Mean rotenone recoveries for the 0.035, 4.82, and 250 μg g^{-1} fortification concentrations were 85.1% (s = 15.2%, $n = 9$), 85.6% ($s = 6.0\%$, $n = 9$), and 83.5% (s = 1.7%, $n = 8$), respectively. The MLOD was 0.012 ug g^{-1}.

General. In addition to drying tissue prior to extraction, the presence of Na_2SO_4 during extraction also served to grind the tissue, decreasing sample particle size and increasing surface area. Analyte recovery (from glass tube walls) was improved by adding the acetonitrile portion of the final reconstitution solvent first, followed by aggressive mixing and sonication prior to the addition of water.

Extraction of pulverized snake tissue yielded a yellow, oily residue upon final drying, and initial use of a silica SPE trapped much of the visible pigmented material and allowed most of the lipids to elute during the cleanup step. A small amount of lipid was visible in the dried post-silica residue, but subsequent cleanup and elution through the Florisil column removed all remaining visible pigment and allowed the remaining lipids to pass through before final analyte elution, leaving no visible residue in the collection tube. A comparison of chromatograms from control snake tissue extract (1) with no SPE column cleanup, (2) with silica SPE column cleanup only, and (3) with silica and Florisil SPE column cleanup is presented in Figure 2.

Initially, a chromatographic system using an octadecylsilane stationary phase with a methanol/water mobile phase was employed, but a significant peak coeluting with rotenone was consistently observed in sample extracts. The use of a cyanopropyl bonded phase optimized peak separation and shape. A comparison of chromatograms from tissue extracts of an unfortified control snake and the same snake fortified at 0.035 μg g^{-1} is shown in Figure 3.

Conclusions. The analytical method described in this paper is reliable and repeatable. Analyte recoveries at concentrations ranging from 0.035 to 250 μg g^{-1} exceeded 83%. The method is simple, requires less equip-

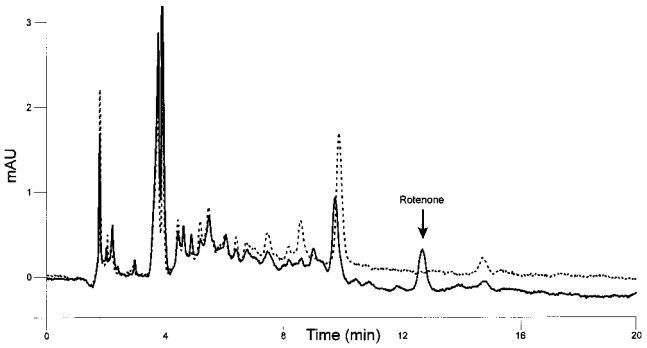

Figure 3. Comparison of chromatograms from tissue extracts of an unfortified control snake (- - -) and the same snake fortified at 0.035 μg g^{-1} (—).

ment and solvent, and utilizes less tissue (2 g) than comparable methods. With an MLOD of 0.012 μg g^{-1}, this method offers suitable sensitivity and precision for the determination of rotenone residues in the brown tree snake. Data resulting from the use of this method will enable researchers to identify toxicants with minimal associated environmental hazards when used to reduce the brown tree snake population on Guam.

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