Liquid Chromatographic Determination of Rotenone in Fish, Crayfish, Mussels, and Sediments

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An analytical procedure is described for determining residues of rotenone in fish muscle, fish offal, crayfish, freshwater mussels, and bottom sediments. Tissue samples were extracted with ethyl ether and extracts were cleaned up by gel permeation chromatography and silica gel chromatography. Sediment samples were extracted with methanol, acidified, partitioned into hexane, and cleaned up on a silica gel column. Rotenone residues were quantitated by liquid chromatography, using ultraviolet (295 nm) detection. Recoveries from sediment samples fortified with rotenone at 0.3 μg/g were 80.8%, whereas recoveries from tissue samples fortified with 0.1 μg/g ranged from 87.7 to 96.8%. Samples fortified with 0.3 μg/g and stored at −10°C for 6 months before analysis had recoveries ranging from 83.2 to 90.5%. Limits of detection were 0.025 μg/g for sediments and 0.005 μg/g for tissue samples.

Rotenone, the active constituent of derris root, has long been used as an insecticide and piscicide. Its use for removing unwanted fish populations in the United States began in the 1930s. It is currently the most widely used fish toxicant in the United States (1).

Several procedures have been reported for the determination of rotenone, including colorimetry (2, 3), infrared spectrometry (4), thin-layer chromatography (5), gas chromatography (6), and liquid chromatography (LC) (7–11). However, none of these procedures provides the sensitivity and specificity required for the analysis of rotenone in fish exposed to piscicidal concentrations.

We describe a modification of the LC procedure reported by Bowman et al. (9) with sample cleanup and concentrating steps that can be used to determine residues of rotenone remaining in fish, crayfish, mussels, and bottom sediments after piscicide treatments.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Waters Associates, Inc., Model 510 dual pump system, Model 680 controller, Model 481 variable wavelength ultraviolet absorbance detector, Model 740 integrator, and optional autoinjector. Operating conditions: stationary phase, 150 × 3.9 mm Nova-Pak C_{18} (reverse phase), 4 μm; mobile phase, methanol–water (70 + 30, v/v) (or 60 + 40 for analysis of samples of fish offal); flow rate, 1.0 mL/min; chart speed, 0.5 cm/min; wavelength, 295 nm; and attenuation, 0.04 absorbance full scale.

(b) *Gel permeation chromatograph (GPC).*—Analytical Bio Chemistry Labs, Inc., Model 1001 Autoprep. Operating conditions: stationary phase, 400 × 28 mm column packed with SX-3 BioBeads; mobile phase, methylene chloride–cyclohexane (1 + 1); flow rate, 4 mL/min; and timing cycles, waste = 28 min, collect = 28 min, and wash = 5 min.

(c) *Extraction column.*—Insert pledget of glass wool and 5 cm Na_2SO_4 at lower end of glass column (500 × 22 mm) fitted with Teflon stopcock.

(d) *Chromatography column.*—Insert pledget of glass wool at lower end of glass column (400 × 11 mm) fitted with Teflon stopcock. Partially fill column with benzene and add 5 g Na_2SO_4. Slurry-pack column with 5 g 3% deactivated silica gel in benzene followed by 5 g Na_2SO_4.

(e) *Blender.*—Waring commercial model with stainless steel blender cup.

(f) *Mixer.*—Sorval Omni-mixer Model 17150 with 11 × 3.3 cm stainless steel mixer cup and screw-cap.

(g) *Roto-evaporator.*—Buchi Model R110.

(h) *Centrifuge.*—Beckman Model J2-21M.

Reagents

(a) *Solvents.*—LC grade methanol, water, methylene chloride, cyclohexane, acetone, and hexane were obtained from J. T. Baker Chemical Co. Distilled-in-glass ethyl ether and benzene were obtained from Burdick and Jackson Laboratories, Inc. (*Caution*: Benzene is a possible carcinogen.)

(b) *Sodium sulfate* (Na_2SO_4).—Anhydrous, 12-60 mesh (J. T. Baker Chemical Co.).

(c) *Silica gel.*—Mesh 40-140 (J. T. Baker Chemical Co.) (activated at 130°C for 24 h).

(d) *Rotenone.*—Purified grade from Aldrich Chemical Co.; prepare standard solutions fresh daily in methanol.

Extraction and Cleanup Procedure

(a) *Fish or crayfish tissues.*—Fish species used to evaluate method were channel catfish (*Ictalurus punctatus*), black bullheads (*Ictalurus melas*), largemouth bass (*Micropterus salmoides*), and bluegills (*Lepomis macrochirus*). Recoveries of rotenone from crayfish (*Orconectes* sp.) were also determined.

Homogenize each frozen sample of fish fillet, offal, or crayfish (whole body) in high-speed blender with enough dry ice to retain frozen state (12). Let carbon dioxide sublime overnight in freezer. Mix 10 g muscle tissue with 60 g sodium sulfate and let stand 15 min with occasional stirring (13). Use 70 g sodium sulfate with fish offal or crayfish to accommodate additional moisture content. Transfer mixture to extraction column and add 5 cm additional sodium sulfate to top of column. Extract column with 100 mL ethyl ether at flow rate of ≤ 1.5 mL/min, and rotary-evaporate ethyl ether to dryness at 30°C. Dissolve residue in 10 mL methylene chloride–cyclohexane (1 + 1) and load 5 mL on gel permeation chromatograph (GPC) equipped with column of SX-3 BioBeads. Elute GPC column with methylene chloride–cyclohexane (1 + 1) mobile phase at 4 mL/min. Discard first 28-min fraction (112 mL) of eluate from GPC and collect next 28-min fraction. Rotary-evaporate collected fraction to dryness at 30°C. Transfer residue to silica gel chromatography column, similar to that described by Bowman et al. (9), with 5 mL benzene. Rinse flask and elute silica gel column with 5 consecutive 5 mL portions of benzene. Do not let level of solvent drop below top of column bed. Discard benzene eluate and elute column with 70 mL benzene–acetone (97 + 3). Rotary-evaporate benzene–acetone fraction to dryness at 30°C. Rinse sides of flask with methanol and again rotary-evaporate to dryness at 30°C. Dissolve residue in 5 mL methanol and analyze by LC.

(b) *Freshwater mussels.*—Homogenize, extract, and clean up soft tissues from mussels (*Lampsilis* sp.) as was done for fish, except mix 100 g sodium sulfate with 10 g homogenate.

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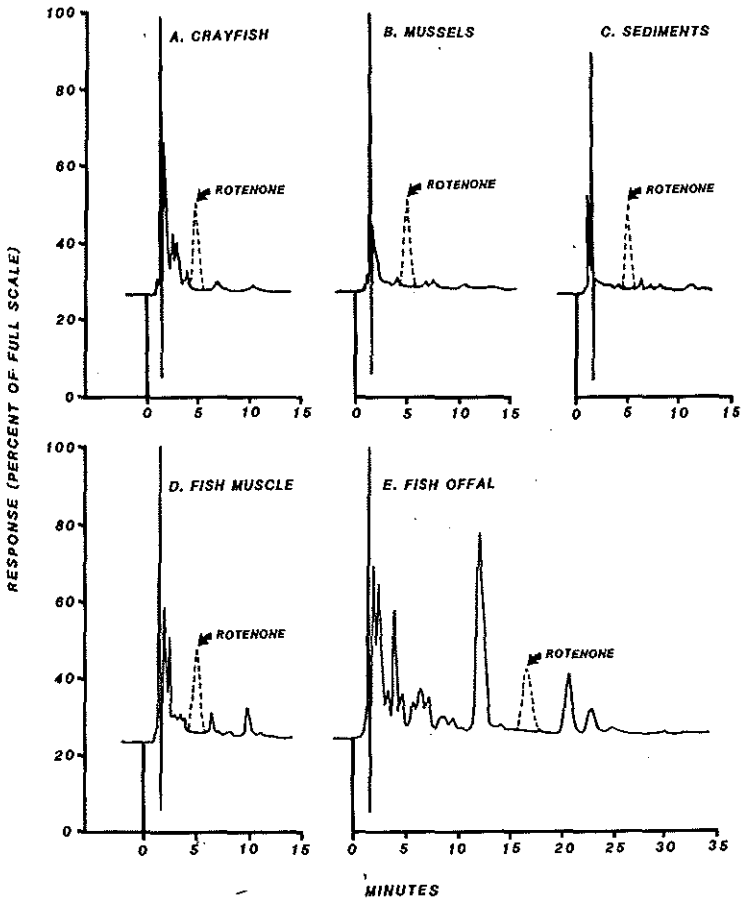

Figure 1. Typical LC chromatograms of cleaned up extracts of 5 types of samples; broken lines (superimposed) represent 0.3 μg/g of rotenone. Mobile phase was methanol–water (70 + 30, v/v) for all samples except fish offal (60 + 40, v/v); injection volume 25 μL.

(c) *Sediment.*—Place 10 g sediment in stainless steel Sorval mixing cup with 20 mL methanol and thoroughly mix in Sorval mixer. Centrifuge mixture in mixing cup for 5 min at 1000 × *g*; then decant the supernatant liquid to suction filter (Gelman type A/E glass fiber). Extract the sediment 3 more times with 10 mL volumes of methanol and combine extracts on suction filter. Rotary-evaporate combined filtrate at 30°C to about 25 mL and then transfer to 1 L separatory funnel containing 500 mL 0.1N HCl. Partition methanol-HCl mixture 3 times with 20 mL hexane. Rotary-evaporate combined hexane fraction to dryness at 30°C. Transfer to silica gel column with 5 mL benzene and continue with same procedure used for fish.

Table 1. Percent recovery (mean ± SE) of rotenone from control samples fortified just before analysis or fortified and stored in freezer (−10°C, 6 months) before analysis

	Fortified just before analysis		Fortified and stored before analysis	
Sample	Concn, μg/g	Mean recovery (SE), %[a]	Concn, μg/g	Mean recovery, %[b]
Sediment	0.3	80.8 (2.50)	0.3	85.0
Crayfish	0.1	88.7 (5.67)	0.3	83.5
Mussels	0.1	87.7 (2.33)	0.3	83.6
Fish muscle	0.1	94.5 (2.77)	0.3	90.5
Fish offal	0.1	96.8 (4.75)	0.3	83.2

[a] Average of triplicate assays.
[b] Average of duplicate assays.

Calculations

Inject a series of rotenone standards in range of 1–150 ng (not exceeding 100 μL) and plot peak height vs rotenone (ng). Inject samples and obtain ng rotenone from standard curve. Calculate rotenone concentrations (μg/g) as follows:

$$\text{Tissue concentration, } \mu g/g = (\text{rot.} \times \text{dil.})/(\text{inj.} \times \text{wt} \times 0.5).$$

$$\text{Sediment concentration, } \mu g/g = (\text{rot.} \times \text{dil.})/(\text{inj.} \times \text{wt})$$

where rot. = amount of rotenone (ng) from standard curve; dil. = final dilution volume (mL); inj. = injection volume (μL); wt = sample weight (g); and 0.5 = portion of sample lost in loading GPC.

Results and Discussion

Rotenone has an ultraviolet absorption maximum at 295 nm. A fixed wavelength detector (254 nm) can be used, but with a considerable loss of sensitivity and a potential loss of specificity. The performance of fixed wavelength detectors can be enhanced by the use of filters but a variable wavelength detector set at 295 nm provides optimal results.

The liquid chromatographic separation of rotenoids on a C_{18} reverse-phase column using 80 + 20 and 60 + 40 methanol–water mobile phases was shown by Bushway and Hanks (14). They showed separation of 6$\alpha\beta$,12$\alpha\beta$-rotenolone, tephrosin, rotenone, β-dihydrorotenone, deguelin, and dehydrorotenone using methanol–water (60 + 40) mobile phase and a C_{18} column system. Bowman et al. (9) showed the sepa-

ration of rotenone, 6αβ,12αβ-rotenolone, dehydrorotenone, and rotenononе mixtures using a C_{18} column system and a methanol–water (75 + 25) mobile phase. Our study involved only the quantitation of rotenone residues in fish, crayfish, mussels, and sediment. The separation of the rotenoids by reverse-phase chromatography on C_{18} columns and methanol–water mobile phase is well documented.

Because of interferences present in extracts of fish, crayfish, mussels, and sediment, cleanup by silica gel column chromatography and gel permeation chromatography or solvent partitioning was needed before LC analyses, especially at low residue concentrations. Gel permeation chromatography was used to clean up tissue samples by separating coextracted lipid material from rotenone. Since sediment samples contained no lipid interferences, solvent partitioning was used to clean up these samples.

The retention time for rotenone on the reverse-phase column with a mobile phase of methanol–water (70 + 30, v/v) was 5.0 min. Extracts of fish offal under these conditions contained peaks that interfered with the analysis of rotenone. A mobile phase of methanol–water (60 + 40, v/v) was used with fish offal samples to obtain baseline resolution of rotenone from matrix-related peaks with a retention time of 7 min. Typical LC chromatograms are shown in Figure 1 for a rotenone standard and cleaned up extracts of crayfish, mussels, bottom sediments, fish muscle tissue, and fish offal.

Triplicate samples of control tissue were fortified with rotenone at 0.1 μg/g and samples of control sediment were fortified at 0.3 μg/g and analyzed to evaluate consistency of the method. Mean recoveries ranged from 80.8% for bottom sediments to 96.8% for fish offal (Table 1).

Instrument responses were linear up to at least 140 ng of rotenone. The equation for a typical standard curve of peak height (mm) vs ng rotenone when a 10 mm flowcell is used is $Y = 0.2006 + 1.2779\ X$, ($r^2 = 0.999$). Limits of detection (signal : noise ratio of 3:1) for rotenone were determined to be 0.025 μg/g for bottom sediments and 0.005 μg/g for samples of fish muscle, fish offal, crayfish, and mussels.

Samples for analysis of rotenone residues can be frozen immediately after collection and analyzed within 6 months. Duplicate control samples of each matrix were spiked with 0.3 μg/g of rotenone and placed in a freezer (−10°C) for at least 6 months before analysis to evaluate the stability of rotenone during freezer storage. Mean recoveries ranged from 83.2% for fish offal to 90.5% for fish muscle (Table 1) and were not significantly different from recoveries from freshly spiked samples ($P > 0.05$).

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