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Agricultural Analytical Chemistry
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DETERMINATION OF FLURIDONE AND ITS MAJOR METABOLITE
IN FISH BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

AM-AA-CA-R063-AA-755

PRINCIPLE

Fish samples are extracted with methanol, and the extracts are subjected to acidic hydrolysis to release conjugated forms of fluridone^{1/} and its major metabolite (compound 125670)^{2/}. Purification of the sample extract is accomplished by liquid-liquid partition and Florisil Sep-Pak[®] cartridge chromatography. Both compounds are separated and measured simultaneously by high-performance liquid chromatography with UV detection at 313 nm.

CHEMICALS AND REAGENTS

A. Solvents

1. Acetone, reagent-grade, redistilled
2. Dichloromethane, reagent-grade, redistilled
3. Ethyl acetate, reagent-grade, redistilled
4. Hexane, reagent-grade, redistilled
5. Methanol, reagent-grade
6. Methanol, HPLC-grade
7. Water, HPLC-grade

B. Solutions

1. HPLC mobile phase—methanol (HPLC-grade):water (HPLC-grade), 65:35, v/v, filtered and degassed
2. Sodium chloride, aqueous, saturated
3. Hydrochloric acid, 2N

^{1/} 1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1H)-pyridinone

^{2/} 1-methyl-3-(4-hydroxyphenyl)-5-[3-(trifluoromethyl)phenyl]-4(1H)-pyridinone

C. Reagents

1. Sodium sulfate, anhydrous, methanol-washed
2. Boiling chips, carborundum
3. Florisil Sep-Pak cartridges

EQUIPMENT

1. Rotary vacuum evaporator (Rinco or equivalent) with water bath heated to approximately 35-45°C.
2. Magnetic stirrer and stirring bar
3. Gyrotory shaker (New Brunswick Model G33 or equivalent) or Omni mixer (Sorvall or equivalent)
4. Hot plate with temperature control
5. Condensing tubes, air-cooled, 18.5 cm x 2.2 cm i.d. or equivalent
6. Syringe, glass, 10-50 ml, with Luer tip
7. Folded filter paper (Schleicher and Schuell No. 588 or equivalent)
8. A high-performance liquid chromatograph consisting of the following components (or equivalent models):

Waters Model 6000A solvent delivery system
Waters Model 440 absorbance detector (fixed wavelength, 313 nm)
Waters Model 710B Intelligent Sample Processor
Houston Instruments Omni Scribe strip chart recorder, 1-10 mV

PROCEDURE

A. Preparation of Standard Solutions

1. Standard Solution A (fluridone plus compound 125670, 1.0 mg/ml each)—Dissolve 100 mg each of fluridone and compound 125670 analytical standards in methanol (HPLC-grade) in a 100-ml volumetric flask and dilute to volume.
2. Standard Solution B (fluridone plus compound 125670, 25 mcg/ml each)—Transfer a 1.0-ml aliquot of Standard Solution A to a 40-ml volumetric flask and dilute to volume with methanol (HPLC-grade).
3. Standard Solution C (fluridone plus compound 125670, 12.5 mcg/ml each)—Transfer a 10.0-ml aliquot of Standard Solution B to a 20-ml volumetric flask and dilute to volume with methanol (HPLC-grade).

4. Standard Solution D (fluridone plus compound 125670, 0.25 mcg/ml each)—Transfer a 1.0-ml aliquot of Standard Solution B to a 100-ml volumetric flask and dilute to volume with HPLC mobile phase (methanol:water, 65:35).

B. Fortification of Recovery Samples

With each set of samples, prepare recovery samples in duplicate by fortifying 25-g aliquots of untreated control fish tissue with 1.0 ml of Standard Solution C. If insufficient control sample is available, prepare duplicate system recoveries by fortifying system blanks (100 ml of methanol) with 1.0 ml of Standard Solution C. Also analyze an untreated control (if available) and a system blank with each set of samples.

C. Extraction of Fish Tissue

1. Weigh 25 g of chopped and finely ground fish tissue into a pint jar. If less than 25 g of tissue is available, weigh the entire sample into the jar and record the weight.
2. Add a volume of methanol equivalent to 100 ml with allowance for the moisture content of the fish tissue (normally assumed to be about 80 percent).
3. Blend the sample for 5 minutes on an Omni Mixer, or shake finely ground samples on a rotating platform table for 30 minutes at 300 rpm.
4. Transfer the methanol extract to a graduated cylinder by pouring the supernatant liquid through a funnel containing folded filter paper.
5. Transfer a 10.0-ml aliquot of the extract to a 250-ml evaporating flask and add 10 ml of methanol to minimize foaming during evaporation. Evaporate the methanol using a Rinco rotary vacuum evaporator and a 35-45°C water bath. A small amount of water and oil will remain in the flask after the methanol has evaporated.

D. Hydrolysis of Conjugated Residues

1. Add 15 ml of 2N HCl to each flask and swirl the flask briefly. Add a few boiling chips and an air-cooled condensing tube and place the flasks on a hot plate preheated to approximately 150-160°C. Heat the contents for one hour.
2. Allow the solution to cool for 5-10 minutes and rinse the condensing tube by pouring 20 ml of saturated sodium chloride solution through the top of the tube. Remove the tube and transfer the solution to a 250-ml separatory funnel. Rinse the flask with 40 ml of hexane and transfer the rinse to the separatory funnel.

E. Liquid - Liquid Partition

1. Shake the separatory funnel for approximately 20 seconds. Allow the phases to separate and drain the aqueous (lower) phase into a beaker. Discard the hexane (upper) phase.
2. Return the aqueous phase to the separatory funnel. For inedible fish tissue extracts, repeat the hexane wash with a second 40 ml of hexane. (Do not repeat the hexane wash for edible tissue extracts.)
3. Return the aqueous phase to the separatory funnel, add 40 ml of ethyl acetate, and shake the separatory funnel for approximately 20 seconds. Allow the phases to separate and drain the aqueous (lower) phase into a beaker. Drain the ethyl acetate (upper) phase through a funnel containing sodium sulfate into a 250-ml evaporating flask.
4. Return the aqueous phase to the separatory funnel and repeat the extraction twice with two additional 40-ml aliquots of ethyl acetate. Rinse the sodium sulfate with 15 ml of ethyl acetate.

F. Florisil Sep-Pak Purification

1. Evaporate the ethyl acetate extract from step E-4 to dryness using a rotary vacuum evaporator and a 35-45°C water bath, and dissolve the residue in 5 ml of dichloromethane.
2. Attach the long end of a Florisil Sep-Pak cartridge to the Luer tip of a glass syringe and rinse the cartridge with 10 ml of acetone, followed by 10 ml of dichloromethane.
3. Pump the sample extract from step F-1 through the cartridge. Rinse the flask with two separate 5-ml aliquots of dichloromethane and pump each rinse separately through the cartridge. Rinse down the sides of the syringe with 5 ml of dichloromethane and pump the rinse through the cartridge. Discard all of the eluate collected thus far.
4. Pump 10 ml of acetone through the cartridge and collect the eluate in a 125-ml evaporating flask.
5. Evaporate the acetone eluate to dryness using a rotary vacuum evaporator and a 35-45°C water bath.
6. Dissolve the residue in 5.0 ml of HPLC mobile phase (methanol:water, 65:35).
7. Filter the solution through folded filter paper into an HPLC sample vial. Cap the vial.

G. HPLC Measurement

1. Measure the HPLC peak height responses for fluridone and compound 125670 using the instrumentation listed in the Equipment section and the following analytical parameters.

NOTE: The parameters listed below may be modified as needed to compensate for daily variations in instrument performance. The parameters used for the analysis should be recorded.

Column— μ Bondapak C₁₈ (or equivalent) with a Co-Pell ODS (or equivalent) guard column

Mobile phase—methanol:water (65:35)

Flow rate—1.0 ml/min

Injection volume—100 microliters

Attenuation—0.02 AUFS

UV wavelength—313 nm

Chart speed—0.167 cm/min

Direct standard—Standard Solution D

2. During the sample analysis, periodically determine the HPLC peak height for Standard Solution D. Use the average peak height for calculating the results in Section H.

H. Calculations

Perform the following calculations for both fluridone and compound 125670:

1. Percent Recovery =
$$\frac{PH_{rec}}{PH_{std}} \times C \times V \times AF \times 100\%$$

 mcg fortified

where: PH_{rec} = net peak height (cm) of recovery sample

PH_{std} = average peak height (cm) of standard

C = concentration (mcg/ml) of standard

V = final volume (ml), including dilutions

AF = aliquot factor (normally 10)

2. parts per million =
$$\frac{PH_{sa}}{PH_{std}} \times C \times V \times AF \times 100\%$$

 W x % Recovery

where: PH_{sa} = peak height (cm) of sample

W = weight (g) of sample extracted

3. Total residue (ppm) = fluridone (ppm) + compound 125670 (ppm)

DISCUSSION

Recoveries obtained by analyzing six replicates of untreated control fish tissues fortified with various levels of fluridone and its metabolite (compound 125670) are summarized in Table I. Overall, recoveries averaged 110 percent for fluridone and 92 percent for the metabolite. Chromatograms demonstrating recoveries of both compounds from edible and inedible fish tissue are contained in Figure 1 and 2, respectively.

A total of 10 system (reagent) blank samples assayed along with control and recovery samples contained no detectable background peaks at the HPLC retention times of either compound. In the system blanks, the baseline width was 0.1 cm or less. The least detectable quantity (three times baseline width) has been approximately 4 ng of fluridone and 2 ng of compound 125670.

In eight different fish species, the background interference for fluridone was nondetectable (ND) in control edible tissue and ranged from ND-0.02 ppm in control inedible tissue. Likewise, the background level for compound 125670 ranged from ND-0.02 ppm and ND-0.03 ppm in control edible and inedible tissue, respectively.

For edible fish tissue, the residue method has exhibited a detection limit (three times background or baseline width) of approximately 0.10 ppm of fluridone and 0.05 ppm of compound 125670. For inedible tissue, the method has exhibited a detection limit of approximately 0.10 ppm for both compounds. The higher detection limit for the metabolite in inedible tissue compared to edible tissue was due to lowered recoveries (probably as a result of requiring a second hexane wash for purification of sample extracts) and a slightly higher background level (up to 0.03 ppm) in control fish.

S. D. West

Ref. Notebooks 88W and 4P3

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I.D. 1564a

TABLE I

RECOVERIES OF FLURIDONE AND ITS MAJOR METABOLITE
(COMPOUND 125670) FROM CONTROL FISH
FORTIFIED WITH VARIOUS LEVELS OF BOTH COMPOUNDS

Level of Fortification (ppm)	N	Percent Recovery ($\bar{X} \pm s.d.$)			
		Edible Tissue		Inedible Tissue	
		Fluridone	125670	Fluridone	125670
0.50	6	94±11	103±10	108±14	83±20
0.20	6	99±6	98±5	100±11	60±8
0.10	6	141±26	116±25	118±12	86±6
0.05	6	ND ^{1/}	96±7	ND	ND

^{1/} Nondetectable (less than three times background in control or blank samples)

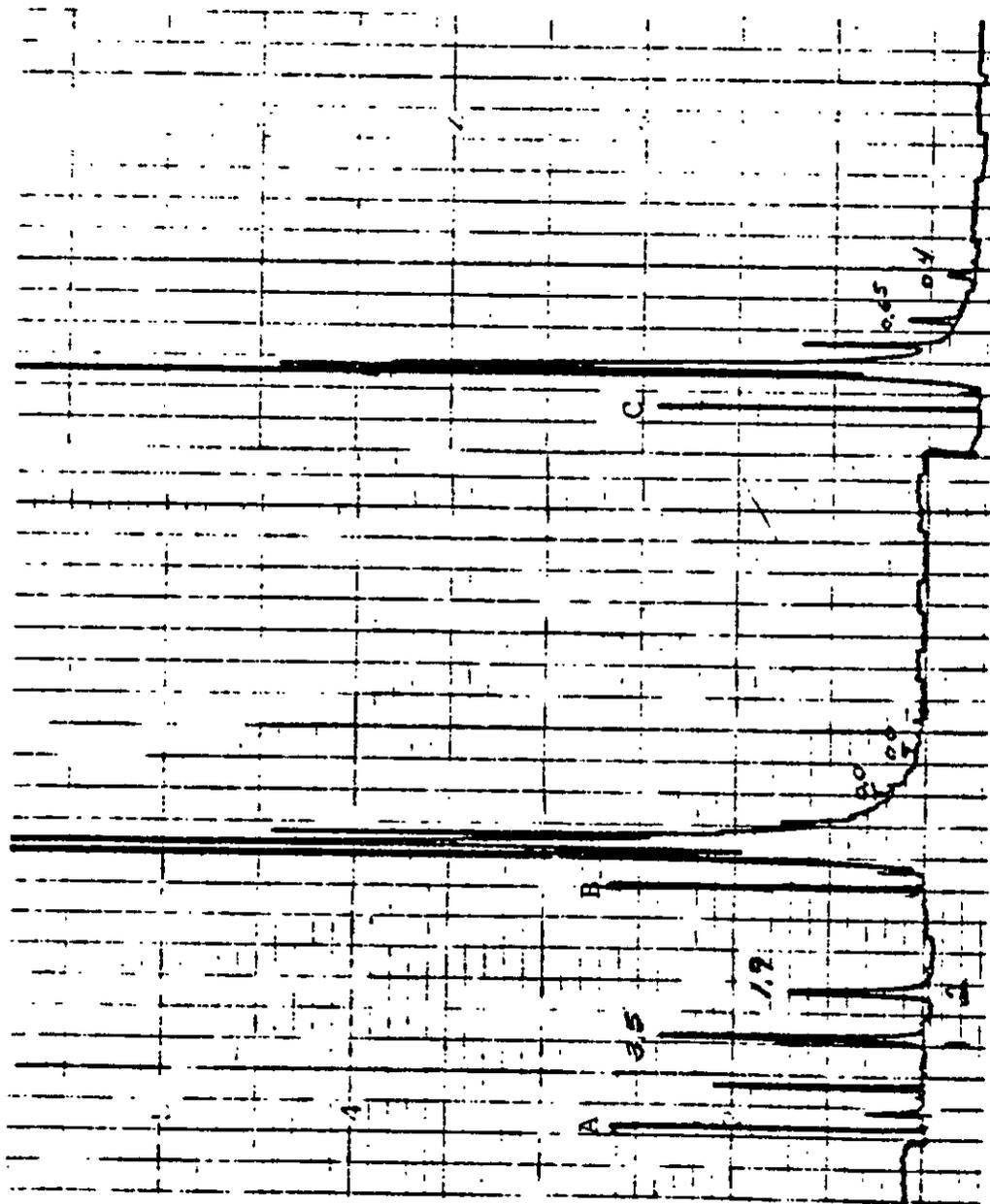


Figure 1. Chromatograms demonstrating the recovery of fluridone and compound 125670 from edible fish tissue:
(A) compound 125670 standard (1) and fluridone standard (2), 25 ng each; (B) control fish tissue containing no detectable residue of either compound; (C) control fish tissue fortified with 0.10 ppm of compound 125670 (93% recovery) and 0.10 ppm of fluridone (103% recovery).

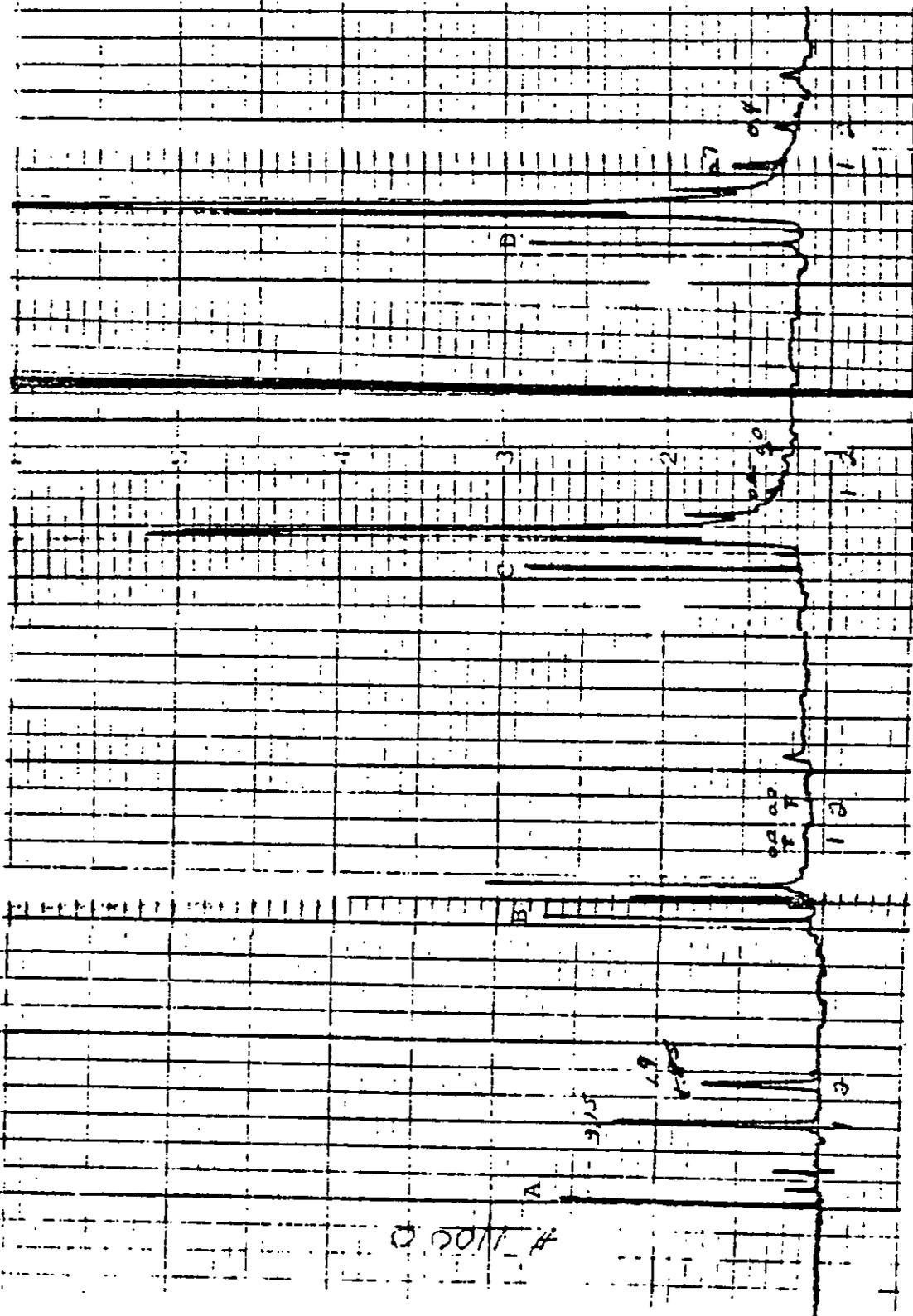


Figure 2. Chromatograms demonstrating the recovery of compound 125670 and fluridone from inedible fish tissue: (A) compound 125670 standard (1) and fluridone standard (2), 25 ng each; (B) system (reagent) blank; (C) control fish tissue containing no detectable residue of either compound; (D) control fish tissue fortified with 0.10 ppm of compound 125670 (80% net recovery) and 0.10 ppm of fluridone (105% recovery).