Determination of Residues of Maretin in Animal Tissues and Milk by Photofluorometry and Gas Chromatography

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An adaptation of an earlier photofluorometric procedure is described which gives control values for animal tissues which are reduced by at least a factor of ten. Milk analyses are now possible with the new procedure because of lower control values. The procedure involves initial extraction and solvent partitioning to remove fats and oils followed by hydrolysis of the Maretin (*N*-hydroxynaphthalimide diethyl phosphate) to naphthostyril. The extract is then further cleaned up on a Florisil column and the fluorescence of the resulting solution measured at 460 mμ. Sensitivity is good to at least the 0.1 ppm level for meat tissues and to 0.01 ppm for milk. A confirmatory procedure has been devised which employs a completely different means of detection. For this, the naphthostyril which was used for fluorescence measurement is brominated and analyzed by gas chromatography employing electron capture detection. An interference study was also conducted which includes all pesticides currently registered on meat and milk.

Maretin (*N*-hydroxynaphthalimide diethyl phosphate), also known as BAY 9002 and Rametin, is an anthelmintic which is being developed for use as a bolus administration, feed additive. A sensitive method is therefore necessary for determining residues of this compound in animal tissues and milk.

Anderson *et al.* (1966) developed a photofluorometric procedure for determining residues of Maretin. The procedure was based on conversion of Maretin to the alkaline hydrolysis product, naphthostyril, which is stongly fluorescent at 460 mμ. It is this same product which Giang (1961) found as the alkaline hydrolysis product of BAY 22408, the sulfur analog of Maretin (Figure 1).

This earlier method for Maretin depended upon alumina adsorption column cleanup of the tissue extracts prior to hydrolysis and fluorometric measurement. The procedure was highly reliable but control values often ran as high as 0.1 to 0.2 ppm, especially with liver and kidney samples. Milk analyses were not even attempted because of the high controls.

The procedure described in this paper is an adaptation of this earlier method. Various column chromatography systems were tried, without success, in attempts to improve the cleanup of the tissue extracts prior to hydrolysis and measurement. However, if Maretin was first hydrolyzed to naphthostyril and then passed through a Florisil column, the fluorescent interferences were successfully removed. This system involves discarding the first 65 ml of column eluate, which contains most of the interfering substances, followed by collecting the next 100 ml of eluate which contains the hydrolyzed Maretin. Thus, it is the hydrolysis product naphthostyril which is cleaned up by column chromatography instead of Maretin.

Recently it has become desirable to have a separate procedure for use as a confirmatory method for positive identification of pesticide residues. In this case, a separate specific procedure was devised whereby the extract left after fluorometric measurement was brominated and analyzed by electron capture gas chromatography for confirmation purposes.

ANALYTICAL METHOD

Apparatus. An Aminco Bowman No. 4-8100 Spectrophotofluorometer equipped with a type IP28 photomultiplier tube was used for the fluorometric analysis. A Hewlett-Packard Model 5750 gas chromatograph equipped with a high temperature ^{63}Ni electron capture detector operated in the pulsed mode was used to detect the brominated derivative. Explosion proof blender motors were used to minimize the fire hazard from volatile organic solvents.

Reagents. Florisil (PR grade, 60–100 mesh) was heated in an oven at 130°C for 24 hr to remove moisture. It was then deactivated by adding 2.5% water (2.5 ml of H_2O + 97.5 g of dried Florisil) and allowed to equilibrate for 24 hr in a tightly stoppered bottle before use. Maretin standard (99.5% pure) was diluted to 25 μg per ml in benzene.

Sample Extraction. Extraction of Animal Fat. Grind the sample in a food chopper in the presence of Dry Ice and place in frozen storage overnight to allow the Dry Ice to sublime. Weigh a 50-g portion of finely chopped fat into a 1-qt blender jar. Add 250 ml of Skellysolve B and blend for 2 min at high speed. Filter with vacuum through Whatman No. 42 filter paper covered with a 1/8-in. layer of Super-Cel in a Size 2A Büchner funnel. Return the filter cake to the blender and blend with 200 ml of acetonitrile for 2 min. Filter as before into the same filter flask containing the Skellysolve B extract. Rinse the blender with 50 ml of fresh Skellysolve B and use this to wash the filter cake. Transfer the combined filtrates to a 500-ml separatory funnel. Rinse the filter flask with a few milliliters of fresh acetonitrile and add to the separatory funnel. Shake the separatory funnel for 30 sec. Allow the phases to separate and drain the lower phase into a 500-ml round-bottomed flask. Repeat the extraction with 100 ml of fresh acetonitrile. Evaporate the combined extracts just to dryness on a rotary vacuum evaporator at 30°C. Proceed to "Hydrolysis."

Extraction of Animal Tissues (Except Fat). Grind the sample in a food chopper in the presence of Dry Ice. Place the sample in frozen storage overnight to allow the Dry Ice to sublime. Weigh a 50-g portion of finely chopped tissue into a 1-qt blender jar. Add 200 ml of acetone and 5 g of Super-Cel and blend for 3 min at high speed. Filter through Whatman No. 42 filter paper covered with a 1/8-in. layer of Super-Cel. Return the filter cake to the blender and blend with 200 ml of chloroform for 3 min. Filter as before into the same filter flask containing the acetone extract.

Rinse the blender jar with 100 ml of fresh chloroform and use this to wash the filter cake. Transfer the combined filtrates to a 500-ml separatory funnel and shake to extract.

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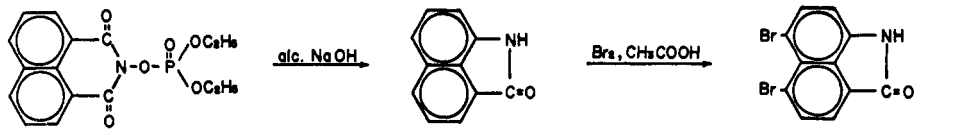

MARETIN NAPHTHOSTYRIL BROMINATED NAPHTHOSTYRIL

Figure 1. Structure of Maretin and derivatives used in the residue analysis procedure

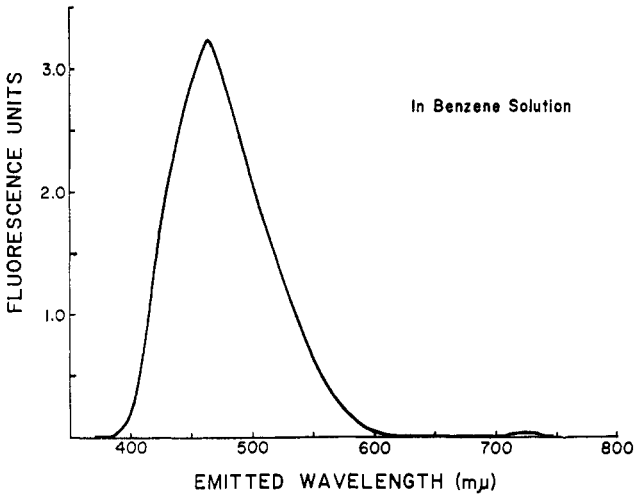

Figure 2. Fluorescence spectrum of naphthostyril in benzene solution using an activating wavelength of 360 mμ

Allow several minutes for the phases to completely separate; then drain the lower phase through 32-cm Whatman No. 2V fluted filter paper containing 5–10 g of Super-Cel into a 1000-ml round-bottomed flask. Rinse the filter paper with 25 ml of fresh chloroform. Evaporate the filtrate just to dryness on a rotary vacuum evaporator at 30°C. Dissolve the residue in 200 ml of Skellysolve B and transfer to a 500-ml separatory funnel. Rinse the flask with 200 ml of acetonitrile and add to the separatory funnel. Shake the separatory funnel for 30 sec; allow the phases to separate and drain the lower phase into a 500-ml round-bottomed flask. Repeat the extraction with 100 ml of fresh acetonitrile. Evaporate the combined extracts just to dryness on a rotary vacuum evaporator at 30°C. Proceed to "Hydrolysis."

Extraction of Milk. Mix the milk sample thoroughly to disperse the cream. Weigh 200 g of milk into a 1-qt blender jar. Add 400 ml of acetone, 10 g of Super-Cel, and blend for 3 min. Filter with vacuum through Whatman No. 541 filter paper covered with a $^1/_4$-in. layer of Super-Cel in a Size 3 Büchner funnel. Rinse the blender jar with 50 ml of acetone and use the acetone to wash the filter cake. Transfer the filtrate to a 1000-ml separatory funnel. Rinse the filter flask with 250 ml of chloroform and add to the separatory funnel containing the acetone filtrate. Shake the separatory funnel for 30 sec. Allow the phases to separate and drain the lower phase through 32-cm Whatman No. 2V fluted filter paper into a 1000-ml round-bottomed flask. Repeat the extraction using 100 ml of fresh chloroform. Evaporate the solvent using a rotary vacuum evaporator at 30°C. Dissolve the residue from the previous steps in 50 ml of Skellysolve B and transfer to a 125-ml separatory funnel. Rinse the flask with 50 ml of acetonitrile and add to the separatory funnel. Shake the separatory funnel for 30 sec. Allow the phases to separate and drain the lower phase into a 250-ml round-bottomed flask. Repeat the extraction with 25 ml of fresh acetonitrile. Evaporate the combined acetonitrile extracts just to dryness on a rotary vacuum evaporator at 30°C. Proceed to "Hydrolysis."

Hydrolysis. To the residue left after initial extraction add 10 ml of 0.5 *M* methanolic NaOH and swirl to dissolve the residue. Allow to stand 10 min at room temperature with occasional swirling. Add 50 ml of distilled water, 3 ml of 2 *N* HCl, and swirl to mix. Transfer to a 125-ml separatory funnel. Rinse the flask with 25 ml of chloroform and transfer to the separatory funnel containing the aqueous phase. Shake the funnel for 30 sec and allow the phases to separate. Drain the lower chloroform phase through 10 g of granular sodium sulfate supported in a powder funnel with a loose plug of glass wool. Collect the filtrate in a 100-ml round-bottomed flask. Repeat the extraction with a fresh 25-ml portion of chloroform. Rinse the sodium sulfate with about 5 ml of fresh chloroform. Evaporate the chloroform extract just to dryness on a rotary vacuum evaporator at 30°C.

Florisil Column Cleanup. Tamp a plug of glass wool into the bottom of a 20 × 400 mm glass column with integral 300-ml reservoir. Add about 1 in. of Superbrite glass beads. Fill the column up to the reservoir with benzene. Slowly add 10 g of Florisil (2.5% water deactivated), and allow this to settle. Top the column with 5 g of granular sodium sulfate. Drain the benzene down to the level of the sodium sulfate. Transfer the sample residue to the column using several 3 to 5 ml rinses of the flask with benzene. Rinse down the sides of the column with benzene and drain the level of benzene down to the level of the sodium sulfate. Discard the eluted benzene. Place a 100-ml graduated cylinder under the column. Add 165 ml of 4% acetonitrile in benzene (v/v). Allow the solvent to percolate through the column at a rate of 1 to 2 drops per second and discard the first 65 ml. Change receivers and collect the next 100 ml in a 125-ml round-bottomed flask. Evaporate the eluate on a rotary vacuum evaporator at 30°C.

Preparation of Increment Solution. Add 2 ml (50 μg) of Maretin standard solution to a 100-ml round-bottomed flask. Treat according to the "Hydrolysis" procedure above. (Do not pass the hydrolyzed increment through the Florisil column.) Dissolve the hydrolyzed Maretin (naphthostyril) in 30 ml of benzene. Swirl to mix and stopper.

Increment Addition and Fluorescence Measurement. To the dry sample residue left after the Florisil column, add 3 ml of benzene. Mix thoroughly. Pipet 1-ml portions of this solution into each of two centrifuge tubes labeled A and B. Add 1 ml of benzene to tube "A" and 1 ml of increment solution to tube "B." Mix thoroughly. Transfer 1 ml of aliquots A and B to spectrophotofluorometer cuvettes and measure the fluorescence. Use slit arrangement of No. 3 of the Aminco-Bowman instrument for all measurements. Determine the fluorescence using an activating wavelength of 360 mμ and a measuring wavelength of 460 mμ. Arbitrarily standardize the instrument at a convenient fluorescence value using 1 ml of the prepared increment solution (50 μg/30 ml). This is done so that readings taken on successive days will be comparable in absolute fluorescence units.

Calculation Procedure. Calculations are carried out by comparing the fluorescence obtained for aliquot A to the

fluorescence for aliquot B plus the internal standard increment, taking into account dilutions and sample size. The general equation is as follows:

$$\text{ppm} = \frac{(\text{fluorescence due to aliquot A}) \times (\mu\text{g of hydrolyzed Maretin added to aliquot B})}{(\text{fluorescence due to the increment, B-A}) \times (\text{sample wt in grams} \times 1/3)}$$

EXAMPLE: If the fluorescent values obtained were 0.50 units for aliquot A and 1.00 units for aliquot B, the ppm in a 50-g sample would be

$$\frac{(0.50) \times (1.67)}{(1.00-0.50) \times (50 \times 1/3)} = 0.1 \text{ ppm.}$$

GAS CHROMATOGRAPHIC CONFIRMATORY PROCEDURE

Transfer and Bromination of Extracts. Rinse the transfer pipet, the contents of cuvette A, and the remaining one-third of the sample in the round-bottomed flask into the centrifuge tube marked A. Use acetone for a rinse solvent. Place 2 ml of the increment solution into a clean 13-ml centrifuge tube for use as a standard. Evaporate the solvent in the tubes just to dryness using a rotary tube Evapo-Mix. Add 0.5 ml of 6% bromine in glacial acetic acid. Rotate the tube so that the bromine reagent contacts all sides of the tube. Stopper the tube and place it in a 60°C water bath for 30 min so that the water level is just above the level of the liquid in the tube. Evaporate the bromine reagent using a rotary tube Evapo-Mix with the water bath at 45°C (10–15 min evaporation time.) Remove any last traces of acetic acid with a gentle stream of dry air. Dissolve the residue in 5 ml of benzene. (Use 2 ml for milk samples.)

Gas Chromatographic Analysis. Using a microliter syringe, inject an appropriate aliquot of the sample or standard solution into the gas chromatograph maintained at the following conditions: Column: 3-ft × 1/8-in. o.d. (approx. 1 mm i.d.) borosilicate glass column packed with 3% FFAP (Varian Aerograph) solution coated (Applied Science Laboratories, 1967) on 80–100 mesh Gas Chrom Q. Carrier Gas: 5% methane in argon, 60 ml per min. Temperatures: column, 240°C; injection port, 260°C; detector, 275°C. Range, 10; Attenuation, 8. Pulse interval, 15 μsec. Recorder chart speed, 0.25-in. per min.

Identify the brominated naphthostyril peak by its retention time and measure the area produced on the recorder strip chart with a polar planimeter. At the operating conditions employed, the retention time is approximately 9.6 min.

Calculation of the parts per million of Maretin in a sample is done by use of the following equation, in which the response for an unknown is compared to the response for a known amount of hydrolyzed, brominated Maretin standard.

$$\text{ppm} = \frac{\text{sample area}}{\text{standard area}} \times \frac{\text{ng std injected}}{\text{sample wt in grams} \times 2/3} \times \frac{\text{final vol (ml)}}{\text{microliters injected}}$$

DISCUSSION

The above procedure is an adaptation of an earlier photofluorometric procedure which was less desirable because of high animal tissue control values. Using the former method (Anderson *et al.*, 1966) reagent blanks were negligible in the absence of tissue extracts indicating the fluorescence method to be satisfactory if cleanup of sample extracts could be improved. Chromatography of the hydrolyzed Maretin on Florisil successfully removes interferences from all animal tissue extracts and milk. This adaptation, employing a discard of the initial 65 ml of column eluate, is the basis for the revised fluorometric procedure thus developed.

Table I. Recovery of Maretin from Various Tissues and Milk

		Fluorometric Analysis		glc analysis	
Tissue	ppm added	Net ppm found[a]	% recovered	Net ppm found[a]	% recovered
Brain	0.0	0.003			
	0.0	0.005			
	0.1	0.078	78	0.078	78
	0.05	0.038[b] ± 0.001	75	0.041	82
Fat	0.0	0.002			
	0.0	0.005			
	0.10	0.088	88	0.075	75
	0.05	0.050[b] ± 0.013	100	0.038	77
Heart	0.0	0.002			
	0.0	0.008			
	0.1	0.090	90	0.081	81
	0.05	0.042[b] ± 0.006	84	0.039	78
Kidney	0.0	0.004			
	0.0	0.017			
	0.1	0.084	84	0.084	84
	0.05	0.043[b] ± 0.007	86	0.046	92
Liver	0.0	0.005			
	0.0	0.034			
	0.1	0.089	89	0.078	78
	0.05	0.049[b] ± 0.011	98	0.046	92
Steak	0.0	0.003			
	0.0	0.016			
	0.1	0.075	75	0.078	71
	0.05	0.046[b] ± 0.012	92	0.033	65
Milk	0.0	0.002			
	0.0	0.002			
	0.005	0.004[c] ± 0.008	80	0.004	80

[a] Individual analysis except as noted. [b] Mean value of four determinations and standard deviation. [c] Mean value of six determinations and standard deviation.

The Florisil was activated by heating overnight to drive off water to a constant level before deactivating with 2.5% (v/w) water. In this way, reproducible 100–500-g batches of Florisil can be prepared from large stocks which are stored at ambient conditions in fiber containers and subject to changing humidity conditions.

Hydrolysis of Maretin using methanolic NaOH was necessary because the presence of water caused incomplete conversion to naphthostyril. Using methanolic NaOH, conversion of Maretin to naphthostyril is quantitative and practically instantaneous. Ten-minute reaction at room temperature ensures complete conversion even in the presence of tissue extracts.

Both Maretin and naphthostyril have been observed to be quite stable in the presence of a variety of organic solvents and tissue extracts. No problems were encountered due to volatility losses during evaporations. Samples may be left at any point in the analysis scheme without affecting the residues.

The fluorescence of naphthostyril was checked in various solvents, with benzene showing the maximum response for naphthostyril and minimum reagent blank response. The fluorescence maximum for naphthostyril in benzene solution was obtained at 460 mμ, using an activating wavelength of 360 mμ (Figure 2).

Response for naphthostyril in the photofluorometer was found to be linear up to a final concentration of at least 20 μg per milliliter in the final solution. Samples containing residues in excess of this concentration should be diluted and read

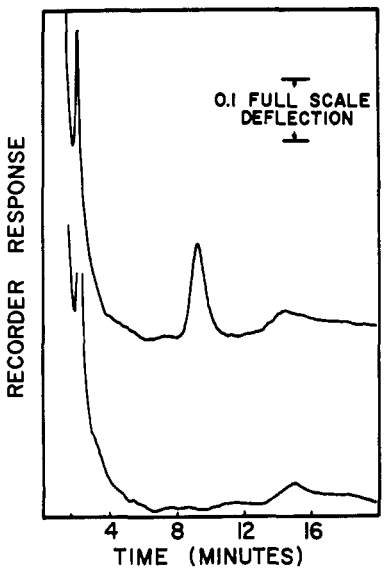

Figure 3. Gas chromatograms of control and 0.05 ppm recovery of Maretin from bovine fat tissue

again to ensure that the response falls within the linear portion of the response curve.

The fluorometric procedure was checked by adding known amounts of Maretin to the various tissues and milk at the blending step. Recoveries were run at the 0.1- and 0.05-ppm level for meat tissues and at the 0.005-ppm level for milk. Recoveries were generally in the 75–110% range. Representative values including controls are shown in Table I.

Naphthostyril can be analyzed directly by gas chromatography, but it does not contain any atom or functional groups which are specifically sensed by the various selective detectors available for use. Bromination converts the compound to a derivative to which the electron capture detector is quite sensitive. Mass spectrometry studies show that bromination adds two bromine atoms (mol wt 327), most probably in the para position on each of the two rings (Figure 1). Chromatography of this derivative on FFAP liquid phase yields a symmetrical peak with sensitivity down to the subnanogram range. The brominated derivative is quite stable and does not decompose even on standing for several weeks at room temperature in the presence of sample extracts.

Controls and fortified samples of each of the various tissues analyzed by the fluorometric procedure were further analyzed by the gas chromatographic confirmatory procedure. This procedure involved brominating the $^2/_3$ of the sample to which no increment was added and analyzing by electron capture gas chromatography. Resolution was obtained between the brominated naphthostyril peak and any peaks due to tissue extractives. Control values, recoveries, and sensitivity all compare favorably with the photofluorometric procedure, as shown in Table I. Representative control and recovery chromatograms are shown in Figure 3.

In order to check the specificity of the fluorometric method, an interference study was conducted. All of the 55 compounds registered for use on meat and milk as of May 1, 1970, were tested at or above their tolerances as listed in the Federal Register. Compounds with a zero tolerance were checked at 0.1- and 0.01-ppm for meat and milk, respectively. No interferences with the fluorometric method were noted above 0.05 ppm for meat or above 0.005 ppm for milk.

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Gas Chromatographic Method for Analysis of Chlorpyrifos and Endosulfan Insecticides in Topically Treated Houseflies

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Chlorpyrifos (Dursban) and endosulfan (Thiodan) can be determined in houseflies at the 10-pg level. Gas chromatographic methods and a high temperature Ni^{63} detector were used after *n*-hexane extraction without cleanup. Cuticle penetration was estimated along with rates of *in vivo* metabolism of these insecticides in the housefly. Rates of 50% disappearance of chlorpyrifos and endosulfan from the cuticle were 36 and 83 min, respectively. The 50% disappearance rates from the whole body were 67 and 176 min, respectively. Thoracic cholinesterase activity of flies surviving the LD_{50} dose treatment with chlorpyrifos declined to a plateau of 70% of the nontreated controls after 3 hr and remained unchanged for a 12-hr period. The enzyme activity of flies at "knock-down" averaged 36% regardless of knock-down time. Endosulfan did not inhibit thoracic cholinesterase.

Analysis of insecticides by gas chromatographic methods (glc) is generally limited to residue problems. There is a theoretical importance to the development of highly sensitive analytical methods for very low levels of insecticides in target insects. They are significant in the study of efficiency in the application of insecticides under field conditions. This is an area of increasing ecological importance. In the substantial absence of analytical methods for direct determination of dose–mortality relationships, the lethal dose is determined by application of measured amounts of insecticide (in microliter volumes) on individual insects. There is no known correlation between mortality from laboratory LD data and the methods of delivery which occur in typical field applications of insecticides.

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