Determination of Residues of Bay 9015 in Animal Tissues by Electron Capture Gas Chromatography

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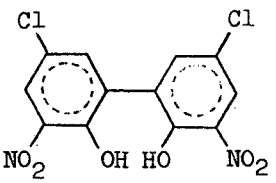
Bay 9015 (3,3'-dichloro-5,5'-dinitro-o,o'-biphenol) is an anti-fluke agent being developed by Chemagro Corporation under license. The pure material is a yellow crystalline powder which is insoluble in water. The structural formula is as follows:



The method described in this paper was developed for use in screening animal tissues for the purpose of obtaining an experimental permit. Although a previous method had been developed (1) for the analysis of Bay 9015, it was unsuitable for use as a residue determination because it lacked sensitivity and specificity.

In this procedure, the tissue sample is extracted and

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carried through a Skellysolve B-acetonitrile partition step to remove the excess oil. An acid charring step is included for further cleanup followed by conversion of the compound to the methyl ether with diazomethane. Interfering fatty acid esters are selectively hydrolyzed and removed in an aqueous extraction and the compound is measured gas chromatographically using electron capture detection.

Experimental

Special Reagents

Celite-sulfuric acid mixture - For each sample, mix 7 gms. Celite 545 with 6 ml. concentrated sulfuric acid in a mortar until homogeneous. Use 18 gms. of mixture per sample.

Bay 9015 standard solution - Weigh 0.05 gm. of Bay 9015 standard into a clean 100-ml. volumetric flask. Make to volume with reagent grade benzene and shake to mix. Transfer 1 ml. of the solution to a second clean 100-ml. volumetric flask. Make to volume with reagent grade benzene and shake to mix. This flask contains 5 γ/ml. of Bay 9015.

Celite 545 - (dried in a 110° oven overnight)

Diazomethane reagent - Dissolve 2.3 gms. of KOH in 2.3 ml. of distilled water in a 125-ml. Erlenmeyer flask. Cool the solution to room temperature and add 25 ml. of ethyl ether. Cool the flask in an ice bath. Working in a hood, add 1.5 gms. of N-methyl-N'-nitro-N-nitrosoguanidine in small portions over a period of a few minutes to the flask. Agitate the flask

thoroughly after each addition. Decant the ether solution from the aqueous slurry and store at ice bath temperature until ready for use.

N-methyl-N'-nitro-N-nitrosoguanidine - Aldrich Chemical Co.

Procedure

Preparation of Samples for Analysis - Grind the entire sample in a food chopper in the presence of an equal amount of dry ice. Place the sample material in frozen storage overnight to allow the dry ice to sublime.

Extraction of Fat Samples - Weigh 25 gm. of chopped sample into a Waring blendor jar. Add 200 ml. of Skellysolve B and blend for 3 minutes at high speed. Vacuum filter through Whatman No. 42 filter paper covered with a 1/8" layer of Super-Cel. Transfer the filtrate to a 500-ml. separatory funnel. (If the filtrate becomes cloudy, warm the solution before the transfer is made.) Reblend the filter cake with 200 ml. of acetonitrile for 2 minutes at high speed. Filter with suction, through No. 42 paper into the same filter flask and combine with the filtrate from the previous filtration step in the 500-ml. separatory funnel. Shake the funnel for 30 seconds. Draw off the lower, acetonitrile layer into a second 500-ml. separatory funnel containing 100 ml. of Skelly-solve B. Shake the second separatory funnel. Draw off the lower phase into a 1000-ml. round bottomed flask. Repeat the above extraction with two additional 200-ml. portions of

acetonitrile. Evaporate the combined acetonitrile extracts to dryness on a rotary vacuum evaporator (Swissco Instruments, Greenville, Ill.) at 40° C. (Proceed to Acid Charring Step)

Extraction of Meat Samples (other than fat) - Weigh 50 gm. of the chopped sample into a Waring blendor jar (use 25 gm. for brain tissue). Add 15 gm. of Super-Cel and 200 ml. of acetone. Blend for 3 minutes at high speed. Filter with vacuum through Whatman No. 42 filter paper. Reblend the filter cake with 200 ml. of chloroform for 3 minutes at high speed. Filter as above. Wash the blender and filter cake with 100 ml. of fresh chloroform. Transfer the filtrate to a 1000-ml. separatory funnel, rinsing the filter flask with two 10-ml. portions of chloroform. Shake the separatory funnel for 30 seconds. Allow the phases to separate, and drain the lower organic layer through a 32 cm. Whatman No. 12 fluted filter paper into a 1000-ml. round bottomed flask. Evaporate on a rotary vacuum evaporator at 40° C., just to dryness. Transfer the sample residue to a 250-ml. separatory funnel with 100 ml. of Skellysolve B. Rinse the flask with 100 ml. of acetonitrile and add to the separatory funnel. Shake the separatory funnel for 30 seconds. Allow the layers to separate, and draw off the lower phase into a second 250-ml. separatory funnel containing 50 ml. of Skellysolve B. Shake the second separatory funnel for 30 seconds. Allow the layers to separate and draw off the lower layer into a 500-ml. round bottomed flask. Repeat the above

extraction with another 100 ml. of fresh acetonitrile. Evaporate the combined acetonitrile extracts just to dryness on a rotary vacuum evaporator at 40° C.

Acid Charring - Dissolve the residue from the acetonitrile/Skellysolve B partition in 25 ml. of carbon tetrachloride. Add 18 gms. of the celite-sulfuric acid mixture (prepared just prior to use), and let sit at room temperature for 20 minutes with periodic swirling. Filter with light vacuum through a sintered glass filter (Allihn tube, 30 ml., medium porosity) onto which a 1/4" layer of dry celite has been lightly tamped. Wash the flask and filter with 50 ml. of CCl_4 in several portions. Transfer the filtrate quantitatively to a 250-ml. round bottomed flask. Add 5 drops of paraffin oil and evaporate just to dryness on a rotary vacuum evaporator at 40° C. Remove any last traces of solvent with a gentle stream of dry air.

Preparation of the Methyl Ether (Start a 5% Standard at this point) - Add 3 ml. of diazomethane reagent and let sit tightly stoppered for 10 minutes. Evaporate to dryness with a gentle stream of dry air.

Hydrolysis (Omit for the standard) - Add 15 ml. of 1.0 N KOH and place in a 60-70° C. water bath for 30 minutes. Swirl occasionally. Cool. Add 10 ml. H_2O and transfer to a 125-ml. separatory funnel. Rinse the flask with 25 ml. H_2O and add to the separatory funnel. Rinse the flask with 50 ml. of $CHCl_3$ and add to the separatory funnel. Shake the funnel for

30 seconds and drain the lower $CHCl_3$ layer into a 250-ml. round bottomed flask. Extract the aqueous layer with another 50 ml. of $CHCl_3$ and combine with the first extract in the 250-ml. round bottomed flask. Evaporate the combined extracts just to dryness on a rotary vacuum evaporator at 40° C. Remove any last traces of solvent with a stream of dry air. Dissolve the residue and the standard in 5 ml. of benzene.

Chromatographic Analysis - An F & M Model 700 gas chromatograph equipped with a pulsed type electron capture detector is used for this analysis. A 10 inch x 3 mm. i.d. borosilicate glass column packed with 5% D.C. 200 coated on 70-80 mesh DMCS treated Chromosorb G is maintained at the following operating conditions: oven - 195° C., inlet - 220° C., detector - 200° C. The carrier gas is 5% methane in Argon at a rate of 70 ml./ minute.

With the chromatograph stabilized at the above conditions, inject 5 microliters of the sample or standard solution. At the operating conditions employed, the retention time for Bay 9015 is 4.6 minutes.

Calculation of the ppm. of Bay 9015 in a sample is done by use of the following equation in which the response for an unknown is compared directly with the response for a known standard. Under the above conditions, 5 nanograms of standard Bay 9015 derivative injected corresponds to 0.1 ppm. in the unknown.

$$P.P.M. = \frac{\text{Sample Area}}{\text{Standard Area}} \times \frac{\text{Sample Attenuation}}{\text{Standard Attenuation}} \times \frac{\text{ng. Std. inj.}}{\text{Spl. wt. in gms.}} \times \frac{\text{Final vol.(ml.)}}{\mu\text{l. injected}}$$

Discussion

Analyses were conducted on a number of tissue samples which had been fortified with known amounts of Bay 9015. Recoveries were run on brain, fat, heart, kidney, liver, and steak tissues. Recoveries for eleven samples at the 0.1 ppm. level averaged 95%. No interference peaks from any of the tissue samples were noted at the retention time for the standard. Results are summarized in Table I.

Table I

Recovery of Bay 9015 From Animal Tissues

Sample	Added, P.P.M.	Recovery, %
Brain	0	-
"	0.1	83
"	0.1	82
Fat	0	-
"	0.1	75
Heart	0	-
"	0.1	105
"	0.1	105
Kidney	0	-
"	0.1	98
"	0.1	93
Liver	0	-
"	0.1	114
"	0.1	95
Steak	0	-
"	0.1	96
"	0.1	94

It was necessary to prepare a volatile derivative of Bay 9015 to be able to analyze it gas chromatographically. Initially, the acetate derivative was prepared using acetic anhydride. Although this gives a nice chromatographic peak it is unsatisfactory in the presence of tissue samples because of interferences from fatty acids which are acetylated along with the Bay 9015. The methyl ether derivative was finally chosen whereby a methyl group is substituted for the hydrogen on each of the hydroxyl groups. The esters of the long chain fatty acids are still formed but can be selectively hydrolyzed as the ether derivative of the compound is stable at the hydrolysis conditions employed.

Diazomethane is used to form the ether of Bay 9015. It is prepared immediately prior to use by alkaline hydrolysis of its precursor, N-methyl-N'-nitro-N-nitrosoguanidine (2). The ether of Bay 9015 thus formed is not stable over long periods of time therefore once a group of samples has been treated with diazomethane they should be carried through chromatographic analysis.

The method described has ample sensitivity for 0.1 ppm. analysis. A sample containing 0.1 ppm. of residue produces an area on the recorder strip chart of about 1 square inch. Somewhat greater sensitivity can be achieved by adjusting aliquot sizes and instrument attenuation.

Chromatographic response is linear over at least a five hundred-fold range up to 100 nanograms. Therefore, samples

containing residues in excess of 2 ppm. should be diluted and reinjected in order to have the response fall along the linear portion of the curve.

References

1. M. J. JOHNSON, Determination of Bay 9015 by Non-Aqueous Titration, Chemagro Corp. Report No. 15,354 (1965).

2. C. W. STANLEY, Anal. Chem. 14, 321 (1966).