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Gas Chromatographic Method for Residues of Baygon and Its Major Metabolite in Animal Tissues and Milk

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A gas chromatographic method for the determination of Baygon (*o*-isopropoxyphenyl *N*-methylcarbamate) and its major metabolite, *o*-hydroxyphenyl] *N*-methylcarbamate, in animal tissues and milk has been developed. The residues are extracted from animal tissues and milk by successive blending with acetonitrile and hexane. Baygon and the conjugated metabolite are separated from each other and cleaned up individually. The metabolite is released from its conjugate by acid hydrolysis before cleanup. Detection is by electron capture gas chromatography of the trichloroacetyl derivatives.

Baygon (*o*-isopropoxyphenyl *N*-methylcarbamate) is an insecticide being developed for agricultural use by Chemagro, Division of Baychem Corp., under license from Farbenfabriken-Bayer A.-G. It has been shown to be effective against insects affecting man and animals such as flies and mosquitoes. Since it is being developed for use on cereal grains and pasture crops, studies were required as to the fate of any residues in such crops when consumed by grazing animals. For this purpose, an analytical method for Baygon and its metabolites in animal tissues and milk was needed.

Metabolism studies on rats with radioactive Baygon (Everett and Gronberg, 1970) showed that rats which were treated orally eliminated 85% of the radioactivity within 16 hr. The major routes of metabolism were depropylation to *o*-hydroxyphenyl *N*-methylcarbamate (hereafter called *o*-OH Baygon) and hydrolysis to isopropoxy phenol. The structures of Baygon and *o*-OH Baygon are shown in Figure 1. Minor metabolites included ring-hydroxylated compounds and the N-hydroxymethylated compound. The metabolites are probably conjugated through both the nitrogen and one of the oxygens. The identified metabolites include those found in plants (Everett and Gronberg, 1968; Gronberg, 1970) and insects (Kuhr, 1970; Metcalf *et al.*, 1967) and those derived from microsomes (Oonnithan and Casida, 1968).

The residue analysis procedure is outlined in Figure 2. This procedure is similar to that for Baygon and metabolites in plant tissues (Stanley *et al.*, 1972). It differs slightly because of different characteristics of plant and animal tissues and because the metabolite must be released by a different technique from the conjugated forms present in the animal tissues (glucuronides) as compared with plant tissues (glycosides). The cleanup by column chromatography and determination by gas chromatography with an electron capture detector are essentially the same for plant and animal tissues.

Residues of Baygon and the conjugated metabolite are extracted from animal tissues or milk using a blender extraction. Baygon is then separated from the conjugated metabolite and cleaned up by column chromatography on Florisil. The metabolite is released from the conjugated form by acid hydrolysis and cleaned up by column chromatography on silica gel. Baygon and *o*-OH Baygon are both determined by gas chromatography after derivatization with trichloroacetyl chloride to obtain the desired sensitivity of detection with the electron capture detector.

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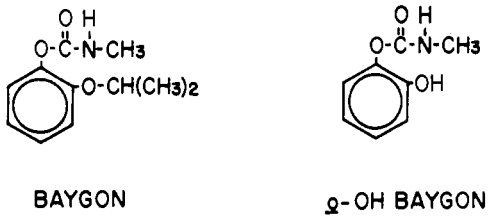

Figure 1. Chemical structures of Baygon and its major metabolite

ANALYTICAL METHOD

Apparatus. A Hewlett-Packard Model 5750B gas chromatograph equipped with an electron capture detector was used. 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° for 24 hr to remove moisture. It was then deactivated by adding 2.5% H_2O (2.5 ml of H_2O + 97.5 g of dried Florisil) and allowed to equilibrate for at least 24 hr in a tightly stoppered bottle before use. Phosphate buffer, pH 11, was prepared by dissolving 27.2 g of KH_2PO_4 and 10 g of NaOH in water and diluting to 1000 ml. Silica gel, Fisher S-679, was heated in an oven at 130° for 24 hr to remove moisture. It was then deactivated by adding 10% H_2O (10 ml of H_2O + 90 g of dried silica gel) and allowed to equilibrate for at least 24 hr in a tightly stoppered bottle before use. All chemicals were analytical reagent grade. Organic solvents were redistilled in glass stills prior to use. Trichloroacetyl chloride (Eastman No. 2032) was used for derivatization reactions.

Sample Preparation. Grind animal tissues in a Hobart food chopper in the presence of Dry Ice and place the samples in frozen storage overnight to allow the Dry Ice to sublime.

Sample Extraction. Extraction of Animal Tissues (Except Fat). Weigh a 20-g sample into a Waring Blendor jar. Add 200 ml of acetonitrile and about 5 g of Hyflo Super-Cel to the sample and blend at high speed for 5 min. Filter the sample through a 9-cm Whatman GF/A filter paper in a Büchner funnel. Return the filter cake to the blender jar and add 200 ml of hexane. Blend the sample at high speed for 3 min and filter as before. Wash the blender jar and filter cake with 100 ml of acetonitrile and then with 100 ml of hexane. Transfer the filtrate to a 1000-ml separatory funnel. Shake the separatory funnel for 30 sec and drain the acetonitrile into a second separatory funnel containing 250 ml of hexane. Shake the second separatory funnel for 30 sec and drain the acetonitrile into a 1000-ml flask. Add 50 ml of acetonitrile to the first separatory funnel and repeat the two-stage extraction described above and combine the acetonitrile phases. Evaporate the acetonitrile to dryness on a rotary vacuum evaporator.

Place 5 g of sodium chloride in the flask. Add 25 ml of 3 N H_2SO_4 to the flask and swirl the solution until the sodium chloride is dissolved. Transfer the solution to a 125-ml separatory funnel. Rinse the flask thoroughly with 75 ml of chloroform. Transfer the chloroform to the separatory funnel. Shake the separatory funnel for 30 sec and centrifuge. Drain the chloroform into a 300-ml round-bottomed flask, passing it through about 10 g of granular sodium sulfate supported in a funnel by a plug of glass wool. Extract the water layer twice more with 50-ml portions of chloroform, centrifuging each time and draining the chloroform through the sodium sulfate. Combine all chloroform phases and evaporate the solution to dryness. Reserve the chloroform residue for "Baygon—Florisil Column" below. Reserve the aqueous solution for "Metabolite—Hydrolysis" below.

Extraction of Fat. Extract the sample as described for animal tissues above, except blend the sample first with hexane and reblend the sample with acetonitrile.

Extraction of Milk. Weigh a 100-g sample into a Waring Blendor jar. Add 250 ml of acetone and about 5 g of Hyflo Super-Cel and blend at high speed for 3 min. Filter the sample through a 9-cm Whatman GF/A filter paper in a Büchner funnel. Transfer the filtrate to a 1000-ml separatory funnel. Rinse the filter flask with 250 ml of chloroform and add the rinse to the separatory funnel. Shake the separa-

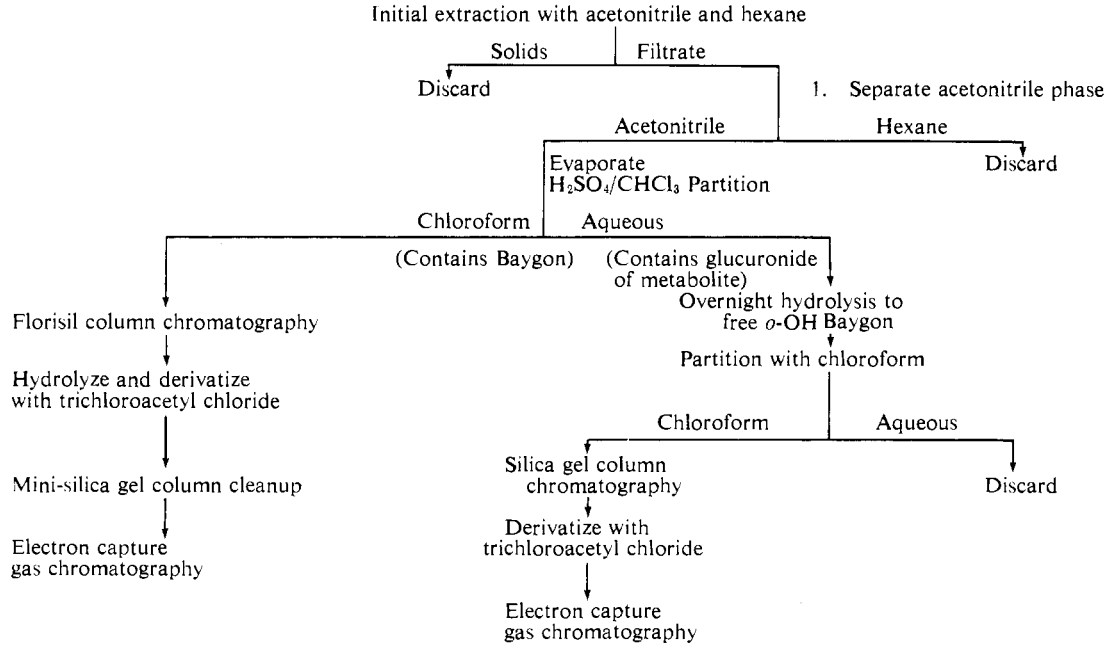

Figure 2. Outline of procedure

tory funnel for 30 sec and drain the chloroform–acetone layer into a 1000-ml round-bottomed flask. Repeat the partition twice with 100 ml of chloroform each time, combining all chloroform phases in the flask. Cool the aqueous phase in an ice bath and add 7 ml of concentrated H_2SO_4 slowly to make the solution 3 *N* in acid concentration. Reserve the solution for "Metabolite—Hydrolysis" below.

Evaporate the chloroform just to dryness. (Note: In the following steps use hexane and acetonitrile which have been equilibrated with each other.) Dissolve the residue in the flask in 50 ml of hexane and transfer to a 125-ml separatory funnel. Rinse the flask with 15 ml of acetonitrile and transfer the rinse to the separatory funnel. Shake the separatory funnel for 30 sec and drain the acetonitrile into a second separatory funnel containing 20 ml of hexane. Shake the second separatory funnel for 30 sec and drain the acetonitrile into a 125-ml round-bottomed flask. Add 10 ml of acetonitrile to the first separatory funnel and repeat the two-stage extraction described above. Combine the acetonitrile phases and evaporate just to dryness.

Baygon. Florisil Column. Tamp a glass wool plug into the bottom of a 20 × 400 mm chromatography tube. Cover the glass wool with a layer of Superbrite beads (Minnesota Mining and Manufacturing Company) and fill the tube with methylene chloride. Slowly sprinkle in 15 g of Florisil and allow the Florisil to settle. Top the column with 20–30 mm of granular sodium sulfate and drain the solvent down to the top of the column. Transfer the residue in the flask to the column with four washes of 5 to 10 ml of methylene chloride, elute the column with a total of 250 ml of methylene chloride at a rate of 2–4 drops per sec, and discard. Then elute the Baygon from the column with 250 ml of chloroform at the rate of 2–4 drops per sec. Transfer the chloroform to a 500-ml separatory funnel containing 50 ml of 0.001 *N* NaOH and shake the separatory funnel for 15 sec. Drain the chloroform into a 300-ml round-bottomed flask and evaporate to dryness. Eliminate all traces of chloroform by inverting the flask for a few minutes.

Bayon. Hydrolysis and Derivatization. Begin a 2-μg Baygon standard for meat tissues or a 1-μg standard for milk here. Add 1 ml of acetone to the flask to dissolve any residue on the walls. Add 15 ml of 0.1 *N* NaOH and place the flask in a 60° water bath unstoppered for 30 min. Swirl the flask occasionally. Cool the solution and transfer it to a 60-ml separatory funnel which contains 1 ml of 5 *N* H_2SO_4 and 10.0 ml of benzene. Rinse the flask with 15 ml of H_2O, adding the rinse to the separatory funnel. Shake the separatory funnel for 30 sec and drain off the aqueous layer. Decant 5 ml of the benzene layer through the top of the separatory funnel into a 13-ml glass-stoppered tube. Add 30 μl of trichloroacetyl chloride (4 drops from a Pasteur pipet) to the tube, mix the solution, and let it stand for at least 10 min. Add 2 ml of 1 *N* NaOH to the tube and shake the tube for 2 min.

Prepare a silica gel mini-column by placing a glass wool plug in a Pasteur pipet. Fill the tube to a height of 5 cm with silica gel, tapping the tube to settle the silica gel. Top the silica gel with 2 cm of granular sodium sulfate. Pass about 2 ml of the solution through the mini-column. Do not elute the column with additional solvent. Reserve this solution for gas chromatography.

Metabolite. Hydrolysis. Allow the acid solution to sit at room temperature for 16 to 24 hr. (For milk samples, dissolve 18 g of NaCl in the sample after the incubation period.) Transfer the solution to a 250-ml separatory funnel, add 75 ml of chloroform to the separatory funnel, and shake the separatory funnel for 30 sec. Drain the chloroform into a 500-ml flask. Repeat the partition twice with 75 ml of chloroform each time. Combine all chloroform phases in the flask, add 4 drops of keeper solution (1 g of mineral oil, U.S.P., in 100 ml of benzene) to the chloroform, and evaporate just to dryness using a room temperature water bath or with the flask just touching the water if the bath temperature is higher. Do all evaporations where free metabolite may be present in this manner.

Metabolite. Silica Gel Column. Tamp a plug of glass wool into the bottom of a chromatography tube, cover the glass wool with a layer of Superbrite beads, and fill the column with methylene chloride. Slowly add 5 g of silica gel and allow the silica gel to settle. Drain the methylene chloride to the top of the column, adding about 5 g of granular sodium sulfate to the top of the column while the methylene chloride is draining. Transfer the residue in the flask to the column with four washes of 5–10 ml of methylene chloride. Elute the column with 200 ml of methylene chloride (including the washes) at a rate of 2 to 4 drops per sec and discard. Elute the *o*-OH Baygon from the column with 300 ml of chloroform at the rate of 2–4 drops per sec and collect in a 500-ml round-bottomed flask. Add 4 drops of keeper solution to the chloroform and evaporate just to dryness. Invert the flask for a few minutes to remove the last traces of chloroform. Transfer the residue in the flask to a 15-ml glass-stoppered tube with 5.0 ml of benzene.

Metabolite. Derivatization. Begin a 4 μg *o*-OH Baygon standard for animal tissues or a 2 μg standard for milk samples here. Add 30 μl of trichloroacetyl chloride to the solution in the tube, mix the solution, and allow the tube to sit for 30 min. Add 10 ml of pH 11 buffer to the tube and shake for 2 min. Allow the tube to sit until the benzene turns cloudy and shake the tube for 10 sec. Repeat the shaking until the solution remains clear for 10 min before gas chromatography. This washing procedure may require up to 2 hr of elapsed time.

Gas Chromatographic Analysis. Inject 5 μl of the standard solution or the sample solution into the gas chromatograph maintained at the following conditions: standard column, 4 ft × 6 mm o.d. standard wall borosilicate glass column, packed with 3% OV-1 on 80–100 mesh Gas Chrom Q (packing supplied by Applied Science Laboratories); carrier gas, 95% argon, 5% methane, 45 ml/min at 50 psig, no make-up gas; pulse interval, 15 μsec; temperatures, Baygon, 150°, *o*-OH Baygon, 170°; injection port, 200°, detector, 200° (tritium) or 250° (Ni-63). The following conditions were used for the confirmatory column: 4 ft × 3 mm o.d. standard wall borosilicate glass column packed with 3% DC-200 + 1.5% OV-17 on 80–100 mesh Gas Chrom Q, prepared by the solution coating technique (Applied Science Laboratories, Inc., 1967); carrier gas, 95% argon, 5% methane, 40 ml/min at 40 psig, no make-up gas; pulse interval, 15 μsec; temperatures, Baygon, 140°, *o*-OH Baygon, 170°; injection port, 200°; detector, 200°. The retention time on the standard column for the trichloroacetyl derivative of Baygon is about 4 min and that for the derivative of *o*-OH Baygon is about 5 min. For the confirmatory column, the retention times are about 4.8 and 5.8 min, respectively.

Identify the Baygon or *o*-OH Baygon peak by retention time and measure the area on the recorder strip chart with a polar planimeter. Calculate the parts per million of residue in a sample by comparing the response obtained for the unknown to the response obtained for a known amount of the corresponding standard derivative.

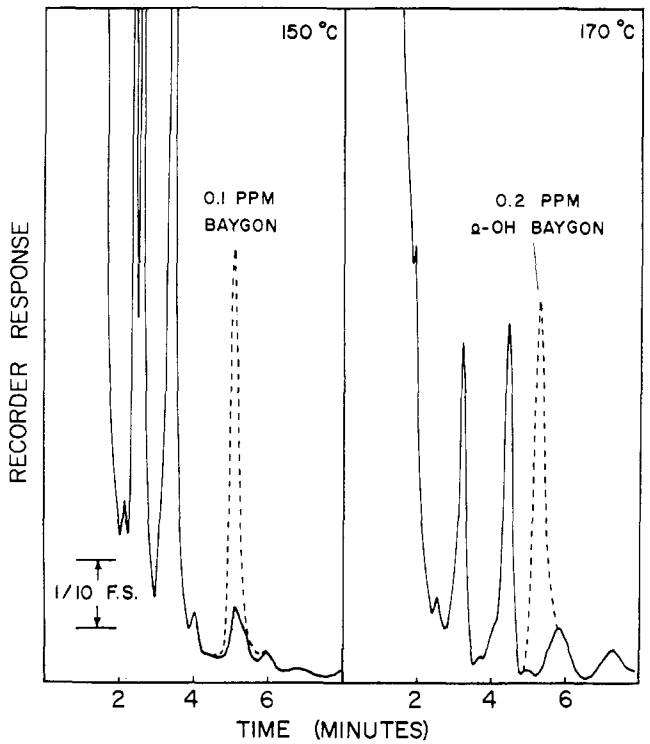

Figure 3. Gas chromatograms showing recovery of Baygon and its metabolite from bovine liver

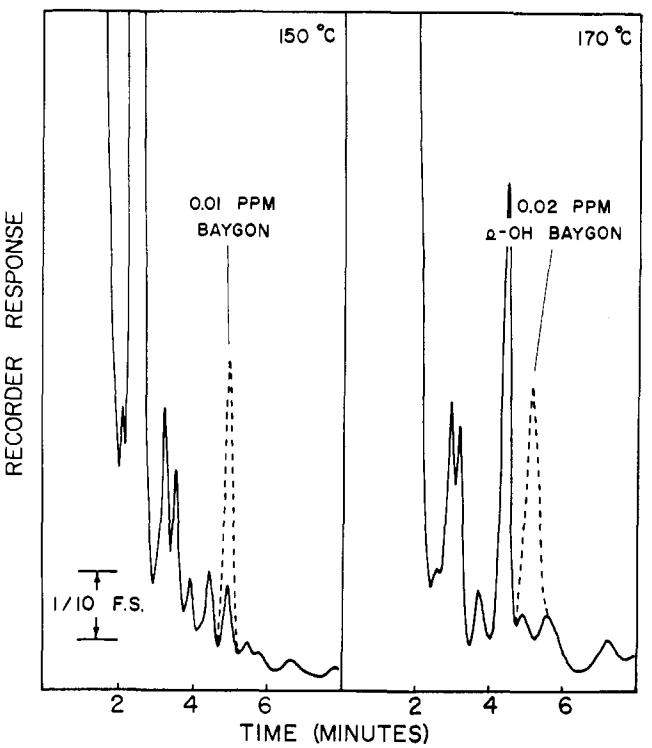

Figure 4. Gas chromatograms showing recovery of Baygon and its metabolite from bovine milk

DISCUSSION

The method described in this paper measures the residues of both Baygon and its major metabolite, *o*-OH Baygon, in animal tissues and milk. The procedure was not designed to detect isopropoxyphenol because of its low toxicity. The *o*-OH Baygon is linked as a glucuronide, which may be conjugated either through one of the oxygens or through the nitrogen. In metabolism studies, enzyme hydrolysis with β-glucuronidase released some metabolite from the conjugated state and further acid hydrolysis released more (Everett and Gronberg, 1970). Further work indicated that as much metabolite was released by acid hydrolysis alone as by enzyme hydrolysis followed by acid hydrolysis (Gronberg, 1971). Therefore, in the analytical method for meat tissues, only the acid hydrolysis was used.

In the analytical method after extraction from animal tissue, Baygon is separated from the conjugated metabolite and cleaned up by column chromatography on Florisil. Some interferences are removed by a methylene chloride wash before the Baygon is eluted from the column with chloroform. The *o*-OH Baygon is released from the conjugate by acid hydrolysis and cleaned up by column chromatography on silica gel.

Final determination is by gas chromatography with electron capture detection. To obtain sufficient sensitivity for the compounds at the residue level, it is necessary to derivatize them. Baygon is hydrolyzed to isopropoxyphenol, which is then reacted with trichloroacetyl chloride. The *o*-OH Baygon is reacted directly with trichloroacetyl chloride. The procedure will detect less than 0.02 ppm of Baygon or *o*-OH Baygon in animal tissues or less than 0.002 ppm of Baygon or *o*-OH Baygon in milk.

Recovery experiments were run on a number of animal tissues by adding known amounts of Baygon at the blending step or *o*-OH Baygon at the hydrolysis step. Representative values are shown in Table I for recoveries from the various tissues and milk. Typical chromatograms are shown for liver and milk analyses in Figures 3 and 4.

Table I. Recovery of Baygon and *o*-OH Baygon from Animal Tissues and Milk

	Baygon			*o*-OH Baygon		
Tissue	Level fortified, ppm	% Recovery	Control, ppm	Level fortified, ppm	% Recovery	Control, ppm
Brain	0.1	63	<0.02	0.2	60	<0.02
Fat	0.1	84	<0.02	0.2	68	<0.02
Heart	0.1	88	0.03	0.2	66	<0.02
Kidney	0.1	69	0.06	0.2	81	0.08
Liver	0.1	90	0.02	0.2	100	<0.02
Steak	0.1	63	<0.02	0.2	77	<0.02
Milk	0.01	65	0.002	0.02	80	0.005

In a feeding study, cattle were fed dairy ration containing 25, 75, or 250 ppm of Baygon for 28 consecutive days. The cattle were then slaughtered and the various tissues were examined for Baygon and *o*-OH Baygon. Detectable amounts of the two compounds were found only in kidney samples from the cattle fed 250 ppm of Baygon; 0.04 ppm of Baygon and 0.2 ppm of *o*-OH Baygon were found. Most other samples contained less than 0.04 ppm of either compound. Milk samples were also run. Milk from cattle fed the highest level showed less than 0.004 ppm of Baygon and from 0.02 to 0.05 ppm of *o*-OH Baygon, whereas nondetectable amounts were found at the lower feeding levels. These results indicate very low residues, if any at all, of Baygon or *o*-OH Baygon would be found in cattle tissues and milk.

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Corrections

FLUOROMETRIC ESTIMATION OF CHLOROPHYLLS, CHLOROPHYLLIDES, PHEOPHYTINS, AND PHEOPHORBIDES IN MIXTURES

In this article by Raymond C. White, Ivan D. Jones, Eleanor Gibbs, and Lillian S. Butler [*J. Agr. Food Chem.*, **20**(4), 773–778 (1972)], the following corrections should be made. On page 775, the sixth line of the first incomplete paragraph should read: "To 10 ml of the pigment solution was added 1 ml of 0.5 *M* oxalic acid in 80% acetone." In Table VI, on page 777, the symbol for pigment third line from the bottom should be "Py_b" and not "Py_bt." On page 778, the fourth citation in the Literature Cited section should be volume **70** of *J. Chromatogr.*, not volume **80**. The following statement should be added at the end of the first three lines on page 778. "Values expressed in Table VII should be multiplied by 0.025 to obtain correct Averages of Estimates of True Values. Values reported are expressed in terms of μmol/l. rather than μmol/25 ml."