pK_1 = 4.19, pK_2 = 5.59 (Simms 1928), permits a tentative assignment of pK_1 to the hydrazide moiety and pK_2 to the carboxyl group.

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Gas Chromatographic Method for Residues of Baygon and Metabolites in Plant Tissues

Charles W. Stanley, John S. Thornton,* and David B. Katague[1]

A gas chromatographic method for the determination of Baygon (*o*-isopropoxyphenyl *N*-methylcarbamate) and its metabolites in plants has been developed. The residues are removed from the plant sample by successive blending with acetone and chloroform. Baygon and the water-soluble conjugates are separated from each other and cleaned up separately. The conjugated metabolites are released by enzyme hydrolysis before the 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 on cereal grains and pasture crops by Chemagro, Division of Baychem Corp., under license from Farbenfabriken-Bayer A.-G. It is presently used in pest control operations.

Metabolism studies on green beans (Everett and Gronberg, 1968; Kuhr and Casida, 1967) and corn (Everett and Gronberg, 1968; Gronberg, 1970) show that Baygon is partially converted to the carbamate-containing moieties, *o*-hydroxyphenyl *N*-methylcarbamate (hereafter called *o*-OH Baygon) and *o*-isopropoxyphenyl *N*-hydroxymethylcarbamate (hereafter called N-CH_2OH Baygon), and to a minor degree, other compounds of less toxicity, such as isopropoxyphenol. Structures of Baygon and the major metabolites are given in Figure 1. In plants, intact Baygon appears to be present in free form but the metabolites are primarily in the form of conjugated *O*-glycosides. The sugar portions of the glycosides may include mono-, di-, and trisaccharides (Kuhr and Casida, 1967).

The primary concern in the development of a suitable residue method was to account for the parent compound and for those toxic metabolites which metabolism studies showed might be formed from the parent compound. Since metabolism studies indicated that *o*-OH Baygon and N-CH_2OH Baygon were formed in plants, an analytical method was developed to account for residues of Baygon and these two metabolites.

The procedure is outlined in Figure 2. Residues of Baygon and conjugated metabolites are extracted from the plant tissues. Baygon is then separated from the conjugated metabolites and cleaned up by hexane–acetonitrile partitioning and column chromatography on Florisil. The metabolites are released from the conjugated form by enzyme hydrolysis and cleaned up by column chromatography on silica gel. Baygon and the metabolites are determined by gas chromatography after derivatization to obtain the desired sensitivity of detection with the electron capture detector.

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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. β-Glucosidase enzyme, Emulsin from Almonds (Catalog No. G8625, Sigma Chemical Co.), was used as the enzyme. Phosphate buffer, pH 5, was prepared by adding 15 ml of 2/15 *M* $NaHPO_4 \cdot 7H_2O$ to 1000 ml of 2/15 *M* KH_2PO_4. 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-279, 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. Trichloracetyl chloride (Eastman No. 2032) was used for derivatization reactions.

Sample Preparation. Grind the plant 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. Weigh a 20-g sample into a Waring Blendor jar. Add 5 ml of H_2O and 200 ml of acetone and

Figure 1. Chemical structures of Baygon and its major metabolites

blend the sample for 5 min at high speed. Filter the sample through a 9-cm Whatman GF/A filter paper covered with about 6 mm of Hyflo Super-Cel in a Büchner funnel. Return the filter cake to the blender jar and add 200 ml of chloroform. Blend the sample at high speed for 3 min and filter again. Wash the blender jar and the filter cake with 100 ml of acetone and then 100 ml of chloroform.

Evaporate the filtrate to dryness on a rotary vacuum evaporator in a 38° water bath. Transfer the residue in the flask to a 125-ml separatory funnel with 50 ml of chloroform and 25 ml of pH 5 phosphate buffer. Shake the separatory funnel for 30 sec and centrifuge for 5 min. Drain the chloroform into a flask and extract the buffer solution twice more with 50-ml portions of chloroform, combining all chloroform phases in the flask. Reserve the chloroform for "Baygon—Hexane–Acetonitrile Partition" below. Add 40 ml of benzene to the buffer solution and shake the separatory funnel for 30 sec. Centrifuge the separatory funnel for 5 min and drain the buffer into a 125-ml flask. Reserve the solution for "Metabolites—Enzyme Hydrolysis" below.

Baygon. Hexane–Acetonitrile Partition. Evaporate the chloroform to dryness on a rotary vacuum evaporator. Transfer the residue in the flask to a 250-ml separatory funnel using 150 ml of hexane and 40 ml of acetonitrile successively. Shake the separatory funnel for 30 sec and drain the acetonitrile layer into another separatory funnel containing 80 ml of hexane. Shake the second separatory funnel for 30 sec and drain the acetonitrile into a round-bottomed flask. Add 40 ml of fresh acetonitrile to the first separatory funnel and repeat the two-stage extraction as described above. Evaporate the combined acetonitrile phases just to dryness.

Baygon. Florisil Column. Tamp a glass wool plug into the bottom of a 20 × 400 mm chromatography tube with integral reservoir. Cover the glass wool with a layer of Superbrite beads (Minnesota Mining and Manufacturing Company). Fill the tube to the reservoir with chloroform. Slowly sprinkle in 15 g of Florisil and allow the Florisil to settle. Top the column with 20–30 mm of granular anhydrous 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 5–10 ml of chloroform and elute the column with a total volume of 250 ml of chloroform, including the washes at a rate of 2 to 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 flask and evaporate just to dryness. Eliminate all traces of chloroform by inverting the flask for a few minutes.

Baygon. Hydrolysis and Derivatization. Begin a 2-μg Baygon standard 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 containing 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.

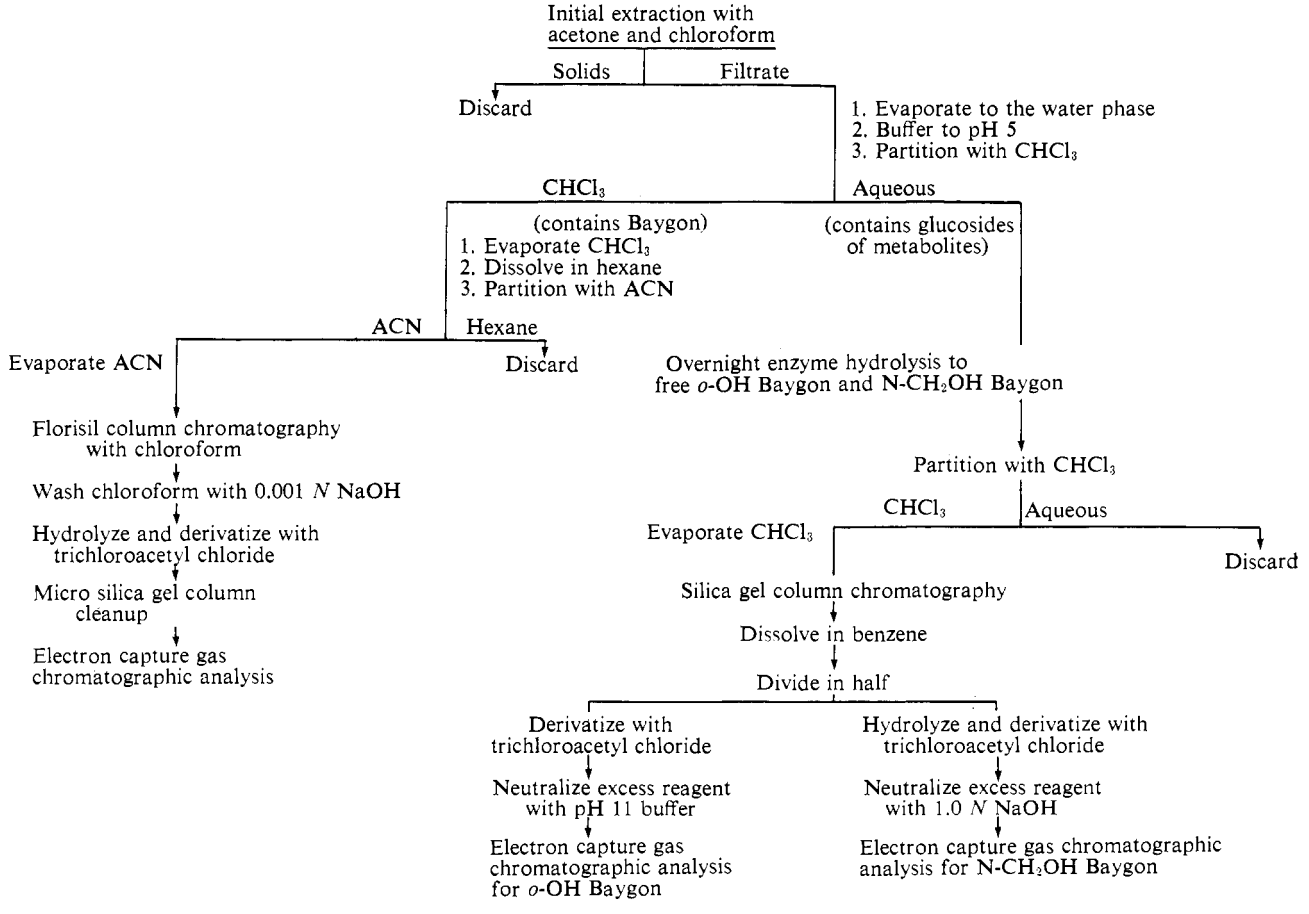

Figure 2. Outline of procedure

Table I. Recovery Data for Baygon and Its Metabolites from Crop Samples

Crop	Baygon			o-OH Baygon			N-CH_2OH Baygon		
	Level fortified, ppm	% recovery	Control, ppm	Level fortified, ppm	% recovery	Control, ppm	Level fortified, ppm	% recovery	Control, ppm
Alfalfa (green)	0.1	99	<0.01	0.5	98	<0.10	0.1	94	<0.01
Alfalfa hay	1.0	71	0.05	1.0	85	<0.10	1.0	108	0.03
Alfalfa hull	0.1	117	0.03	0.5	63	<0.10	0.1	81	<0.01
Corn cob	0.1	81	<0.01	0.5[a]	69	<0.10	0.1	100	<0.01
Corn cob	...	...	...	0.1[a]	61	<0.10	...	...	...
Dry corn cob	0.1	115	0.02	0.5	85	<0.10	0.1	124	<0.01
Corn fodder	0.1	96	0.03	0.5[a]	64	<0.10	0.1	76	<0.01
Corn fodder	...	...	...	0.1[a]	73	<0.10	...	...	...
Corn kernel	0.1	79	<0.01	0.5[a]	65	<0.10	0.1	84	<0.01
Corn kernel	...	...	...	0.1[a]	41	<0.10	...	...	...
Oat forage	0.1	69	0.02	0.5	72	<0.10	0.1	95	<0.01
Oat grain	0.1	87	0.04	0.5[a]	66	<0.10	0.1	73	<0.01
Oat grain	...	...	...	0.1[a]	64	<0.10	...	...	...
Oat straw	0.1	75	0.06	0.5	102	<0.10	0.1	77	<0.01
Pasture grass (green)	0.1	100	0.02	0.5	87	<0.10	0.1	82	<0.01
Pasture grass (dry)	0.1	89	0.03	0.5	112	<0.10	0.1	109	0.03

[a] Fortified in the blender jar with o-OH Baygon glucoside.

Shake the separatory funnel for 30 sec and discard 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 minicolumn 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 benzene solution through the minicolumn. Do not elute the column with additional solvent. Reserve this solution for gas chromatography.

Metabolites. Enzyme Hydrolysis. Place the buffer solution from the Baygon–metabolite separation on a rotary vacuum evaporator for about 10 min or until volatile organic solvents are removed. Add 100 mg of β-glucosidase enzyme to the flask, stopper the flask, and incubate the flask for 16 hr at 38°. Transfer the solution to a 250-ml separatory funnel and extract three times with 75-ml portions of chloroform. Combine the chloroform phases in a 500-ml flask, add 4 drops of keeper solution (1 g of mineral oil, USP, 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 metabolites may be present in this manner.

Metabolites. Silica Gel Column. Tamp a glass wool plug into the bottom of a chromatography tube and cover the glass wool with a layer of Superbrite beads. 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 metabolites from the column with 300 ml of chloroform at a rate of 2 to 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. Dissolve the residue in the flask in 10.0 ml of benzene. (Note: Begin a standard containing 10 μg of o-OH Baygon and 2 μg of N-CH_2OH Baygon in 10.0 ml of benzene here.) Transfer 5.0 ml of the benzene to a 15-ml glass-stoppered tube for o-OH Baygon analysis. Transfer the remaining solution quantitatively to a 125-ml flask for N-CH_2OH Baygon analysis.

Metabolite Derivatization. o-OH Baygon. 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.

Metabolite Derivatization. N-CH_2OH Baygon. Add 2 drops of keeper solution to the benzene and evaporate the solution to dryness. Continue as in "Baygon—Hydrolysis and Derivatization" above, beginning with "Add 1 ml of acetone."

Gas Chromatographic Analysis. Inject 5 μl of the standard solution or the sample solution into the gas chromatograph maintained at the following conditions: 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 or N-CH_2OH 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, 1967); carrier gas, 95% argon, 5% methane, 40 ml/min, at psig, no make-up gas; pulse interval, 15 μsec; temperatures,

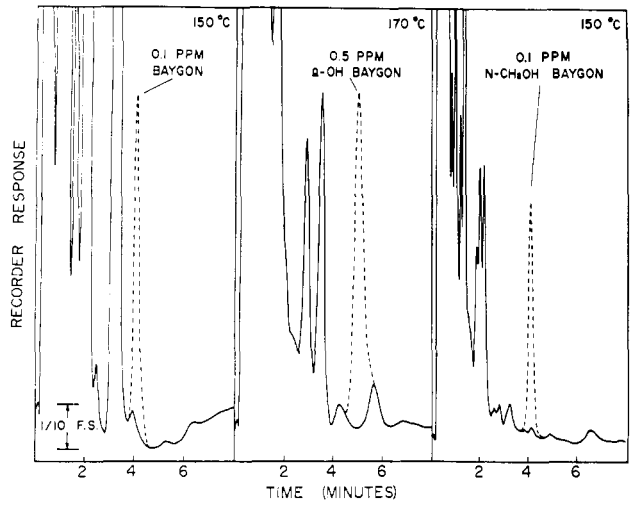

Figure 3. Gas chromatograms showing recovery of Baygon and metabolites from alfalfa

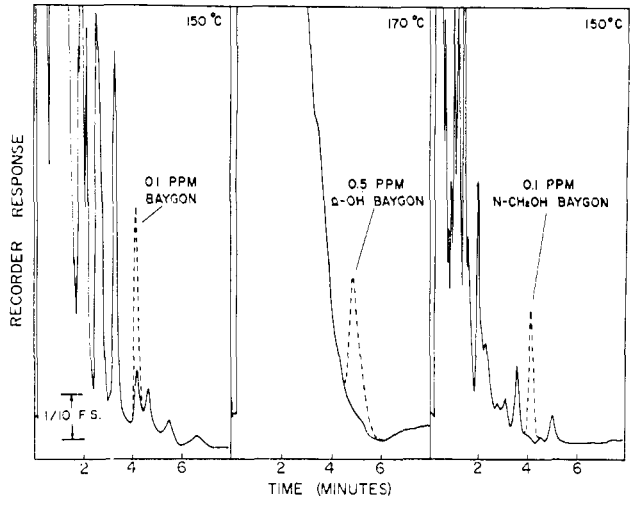

Figure 4. Gas chromatograms showing recovery of Baygon and metabolites from oat grain

Baygon or N-CH_2OH Baygon, 140°, *o*-OH Baygon, 170°; injection port, 200°; detector, 200°. The retention time on the standard column for the trichloroacetyl derivative of Baygon or N-CH_2OH 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, N-CH_2OH Baygon, or *o*-OH Baygon peak by retention time and measure the area on the recorder strip chart with a polar planimeter. Calculate 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.

DISCUSSION

The procedure described in this paper measures not only Baygon residues but also the major metabolites, *o*-OH Baygon and N-CH_2OH Baygon, which are present in plant tissue extracts as conjugated *O*-glycosides. Initial plant extracts are partitioned between immiscible solvent pairs to separate Baygon from the conjugated metabolites. Baygon is then cleaned up separately and analyzed using the procedure of Katague and Thornton (1969). This procedure gave adequate cleanup for all crops except oat grain, which required a wash with methylene chloride in the Florisil column step.

Table II. Levels of Baygon and Metabolites in Pasture Grass after Spraying with Baygon

Days after application	Net residues, ppm: Baygon	*o*-OH Baygon	N-CH_2OH Baygon	Total
Control	0.03	<0.05	0.01	0.04
0	73.10	<0.05	0.03	73.13
3	0.74	0.73	0.69	2.16
7	0.22	0.57	0.70	1.49
14	0.08	0.13	0.28	0.42
21	0.05	0.15	0.12	0.32
28	0.07	0.11	0.18	0.36

Foliar spray prepared with 70% wettable powder. Application rate: 16 oz/acre.

After separating the Baygon, the extract is hydrolyzed with enzyme to release the free metabolites. Enzyme hydrolysis releases about 90% of the water-soluble metabolites (Gronberg, 1970). Acid hydrolysis was tried and gave essentially no release and basic hydrolysis yielded methylamine from *o*-OH Baygon. While a method is available for methylamine in plant tissues (Tilden and Van Middlelem, 1970), crop samples give interferences which mask the response for the metabolites at the 0.1-ppm level. Following release of the metabolites from the conjugates, they are cleaned up on a silica gel column.

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 and N-CH_2OH Baygon are hydrolyzed to isopropoxyphenol, which is then derivatized with trichloroacetyl chloride. The *o*-OH Baygon is derivatized directly with trichloroacetyl chloride. The procedure will detect less than 0.02 ppm of Baygon or N-CH_2OH Baygon and less than 0.05 ppm of *o*-OH Baygon.

Recovery experiments were run on a number of crop tissues by adding known amounts of Baygon at the initial blending step. Recovery of the metabolites was checked in crop samples by adding known amounts of *o*-OH Baygon glucoside at the initial blending step. In addition, recovery of the free metabolites was checked by adding the compounds at the time of hydrolysis. Recovery data for Baygon and the two metabolites from several crops are shown in Table I. Typical chromatograms are shown in Figure 3 for the recovery of Baygon and metabolites in alfalfa and Figure 4 shows typical chromatograms for the recovery of Baygon and metabolites in oat grain.

To determine the specificity of the method in the presence of other pest control chemicals, an interference study was conducted. All chemicals for which Food and Drug Administration tolerances have been established for cereal grains were investigated. These compounds were carried through the procedure at the maximum registered level for a 50-g sample and no interferences were found. A number of field-treated samples were checked to determine the levels to be expected under conditions of usage and how quickly the residues would disappear after application. The crop samples included alfalfa, barley, corn, oats, pasture grass, rye, and wheat. Table II shows the levels found in one experiment on pasture grass. As would be expected, the Baygon level was highest immediately after spraying; it then decreased and the metabolite levels increased and then declined. At the end of 14 days, Baygon had decreased to very low levels and only traces of the metabolites were present.

ACKNOWLEDGMENT

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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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