Determination of Carbofuran and Its Toxic Metabolites in Animal Tissue by Gas Chromatography of Their *N*-Trifluoroacetyl Derivatives

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A glc procedure is described for the analysis of carbofuran and its toxic metabolites (3-hydroxycarbofuran and 3-ketocarbofuran) in animal samples. The residues are extracted in 0.25 *N* HCl, refluxed 1 hr at 100°, and partitioned into methylene chloride. Sample cleanup includes chromatography on activated Florisil columns and partition between 9:1 acetonitrile–water and petroleum ether. The carbamate residues are measured by glc with electron capture detection as *N*-trifluoroacetyl derivatives. Recoveries averaged 84.2% for carbofuran, 83.8% for 3-hydroxycarbofuran, and 72.8% for 3-ketocarbofuran; minimum sensitivities are approximately 0.5, 0.05, and 0.07 ppm, respectively.

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) (Figure 1, I) is a broad spectrum insecticide–nematocide with both contact and systemic activity. It is currently registered on a variety of food crops, including corn, sugar cane, and rice. In animals it reversibly inhibits the enzyme cholinesterase. Though a short residual toxicant, its oral toxicity in animals is relatively high (*e.g.*, rat LD_{50} 11 mg/kg) and several of its metabolites (*i.e.*, 3-hydroxycarbofuran and 3-ketocarbofuran) are also toxic. The metabolism of carbofuran has been studied extensively in both plants and animals (Metcalf *et al.*, 1968; Dorough, 1968a,b); oxidation at the 3 position of the benzofuranyl ring results in the formation of its principal toxic metabolite 3-hydroxycarbofuran (Figure 1, II). This metabolite can be further metabolized to 3-ketocarbofuran (Figure 1, III) or conjugated to a sugar as a water-soluble glycoside (Figure 1, IV); the latter is generally the predominate carbofuran residue in plants. 3-Hydroxycarbofuran glycoside is moderately persistent and can exhibit anticholinesterase activity after hydrolysis by a β-glucosidase. 3-Ketocarbofuran is hydrolytically unstable (Metcalf *et al.*, 1968) and is not found in significantly high levels in plants and animals; however, in soil and soil water runoff it may be an important residue (Caro *et al.*, 1973). Free and sugar-conjugated phenols are nontoxic hydrolysis products of carbofuran, 3-hydroxycarbofuran, and 3-ketocarbofuran.

Several analytical procedures employing gas–liquid chromatography (glc) have been developed for carbofuran and its toxic metabolites. Cook *et al.* (1969) employed a nitrogen-specific microcoulometric detector and a carefully deactivated gas chromatography column to prevent decomposition of the carbamate group. Butler and McDonough (1971) hydrolyzed carbofuran and its carbamate metabolites and derivatized the resulting phenols to trichloroacetyl compounds for determination with an electron capture detector. In both cases the carbamate residues were extracted from plant material, with different cleanup procedures employed. Extracted residues were prepared for glc after liquid chromatography on columns containing Florisil, Al_2O_3, and Nuchar-Attaclay (Butler and McDonough, 1971) or organic solvent partition and liquid chromatography with Nuchar-Attaclay–silica gel (Cook *et al.*, 1969).

The present procedure was developed for the analysis of carbofuran, 3-hydroxycarbofuran, and 3-ketocarbofuran in animal extracts. The cleanup procedure of Cook *et al.* (1969) was tested with animal extracts (*i.e.*, mullet) prior to development of this procedure. Samples obtained were not sufficiently clean for the routine analysis by glc. In this procedure residues are measured quantitatively by glc and electron capture detection as *N*-trifluoroacetyl derivatives following cleanup on activated Florisil columns and partition between 9:1 acetonitrile–water and petroleum ether. The *N*-trifluoroacetyl derivatives are highly sensitive to electron capture detection and can be resolved on the same gas chromatography columns (see text) currently employed in our laboratory for chlorinated hydrocarbon pesticide analysis. The initial step of the procedure is a dilute hot acid hydrolysis, first described by Cook *et al.* (1969), which releases 3-hydroxycarbofuran glycoside to its aglycone form for extraction with methylene chloride. The cleanup procedure gives rise to samples which are suitable for routine analysis by gas chromatography. Recoveries average 80% for fortified animal extracts and the procedure has a minimum sensitivity of approximately 0.5 ppm for carbofuran, 0.07 ppm for 3-ketocarbofuran, and 0.05 ppm for 3-hydroxycarbofuran.

EXPERIMENTAL SECTION

Apparatus. With the exception of the chromatographic tubes used in the cleanup procedure, all other glassware utilized ground-glass joints. Three-ball Snyder columns were used and the separatory funnels were fitted with Teflon stop cocks and glass stoppers.

The gas–liquid chromatograph employed was Varian Aerograph Model 2100, equipped with two 6-ft glass columns (2 mm i.d.) and tritium foil electron capture detectors. Column one was packed with 3% DC-200 on Gas-Chrom Q, 80–100 mesh. Column two contained a 1:2 mixture of 5% DC-200 and 5% QF-1 on Gas-Chrom Q, 80–100 mesh. Both columns were capable of resolving the *N*-trifluoroacetyl derivative of carbofuran, 3-hydroxycarbofuran, and 3-ketocarbofuran; however, because of more desirable retention time and peak symmetry, only column one was used for routine quantitative analysis of the carbamates. The columns were maintained at 165°, injection port at 220°, and detectors at 215°. Gas flow (zero-grade N_2) was 70 ml/min and the recorder was a dual pen Varian Model 20. Optimum oven temperature for column two is 195°.

Reagents. The solvents were either Mallinckrodt Nanograde or Fisher Pesticide Grade and were used without further purification. Sodium lauryl sulfate and anhydrous sodium sulfate were laboratory grade reagents obtained from Fisher Scientific Co. Florisil, 60–100 mesh, PR grade, was purchased from the Floridin Co., Pittsburgh, Pa. The Florisil was activated by heating for a minimum of 24 hr at 135° prior to use in the cleanup procedure. Trifluoroacetic anhydride for preparation of the derivatives

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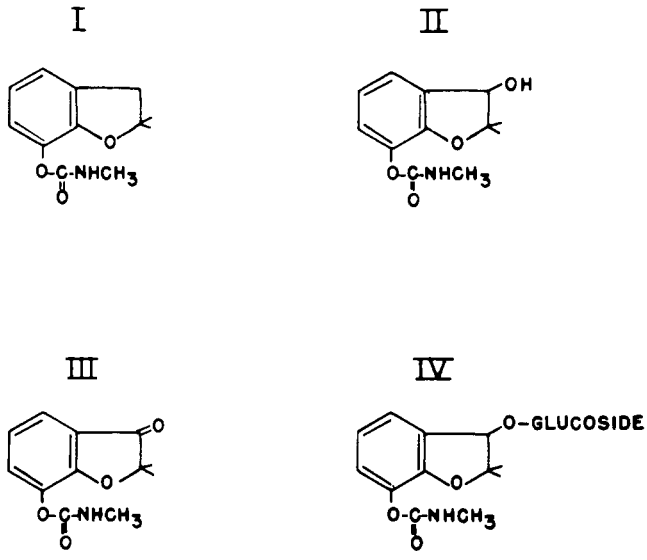

Figure 1. Carbofuran and its toxic metabolites: I, carbofuran; II, 3-hydroxycarbofuran; III, 3-ketocarbofuran; IV, conjugate of 3-hydroxycarbofuran.

was purchased from the Aldrich Chemical Co. Analytical standards of carbofuran, 3-hydroxycarbofuran, and 3-ketocarbofuran were generously supplied by the Niagara Chemical Division, FMC Corp., Middleport, N.Y.

Sample Preparation. A 20-g wet weight sample (*e.g.*, whole animal or tissue) was homogenized in a blender containing 200 ml of 0.25 *N* HCl. The homogenate was transferred to a 300-ml round-bottomed flask containing several boiling chips. The flask was fitted with an Allihn condenser and the mixture refluxed for 1 hr in an electric heating mantle.

Extraction of Residues. The homogenate was cooled to room temperature and transferred to a 1000-ml separatory funnel to which was added 20 ml of ethyl acetate and 1 g of sodium lauryl sulfate to prevent formation of an emulsion. The ethyl acetate and sodium lauryl sulfate were thoroughly mixed with the acid hydrolysate and 100 ml of methylene chloride was added with vigorous mixing for 2 min. Additional sodium lauryl sulfate was added if an emulsion formed and failed to break within several minutes. The methylene chloride was collected in a 500-ml flask following passage through anhydrous sodium sulfate contained in a funnel. The residue was extracted with two additional 100-ml volumes of methylene chloride and the anhydrous sodium sulfate rinsed with 50 ml of fresh methylene chloride. Boiling sand was added to the flask, a Snyder column affixed, and the solution evaporated to approximately 5 ml on a steam table.

Cleanup. *First Activated Florisil Column Cleanup.* A 400 × 20 mm chromatographic tube was prepared with 12.5 cm of activated Florisil topped with a 2.5 cm layer of anhydrous sodium sulfate. This column was attached to a vacuum flask. The concentrated methylene chloride extract was added in 2-4-ml aliquots, permitting enough time between additions for the complete evaporation of the solvent and collection of residue on the top 2.5-5 cm of the Florisil column. The flask was rinsed with three 5-ml volumes of methylene chloride. The column was disconnected from the vacuum flask and eluted with 200 ml of petroleum ether and the eluate discarded. A second elution was effected with 300 ml of 35% ethyl acetate in hexane (v/v). The eluate was collected in a 500-ml flask, boiling sand was added, and the solvent was evaporated to approximately 5 ml on a steam table using a Snyder column. Approximately 50 ml of petroleum ether was added through the Snyder column and the volume reduced to approximately 5 ml.

Petroleum Ether Partition of 9:1 Acetonitrile-Water. The above sample was transferred to a 250-ml separatory funnel and the flask rinsed with two 10-ml volumes of petroleum ether followed by two 50-ml volumes of 9:1 acetonitrile-water. Each rinse was transferred to the separatory funnel. The separatory funnel was shaken vigorously for 2 min and the phases allowed to separate. The lower 9:1 acetonitrile-water phase was drained into a second 250-ml separatory funnel and the petroleum ether phase discarded. The first separatory funnel was rinsed with fresh petroleum ether and this rinse was also discarded. The 9:1 acetonitrile-water phase was partitioned with two 20-ml

Table I. Chromatographic Data for *N*-Trifluoroacetyl Derivatives of Carbofuran, 3-Hydroxycarbofuran, and 3-Ketocarbofuran Relative to Heptachlor

	Response,[a] ng	Retention,[b] min
Carbofuran	2.9	1.43
3-Hydroxycarbofuran	0.3	1.69
3-Ketocarbofuran	1.3	1.98

[a] Nanograms required to produce a peak height equivalent to 0.1 ng of heptachlor (64 mm) on a 6-ft column packed with Gas-Chrom Q, 80–100, coated with 3% DC-200: sensitivity 16, 10^{-10} A/mV; column, 165°; detector, 215°; injector, 220°; gas flow (N_2), 70 ml/min. [b] Paper speed, 30 in./hr.

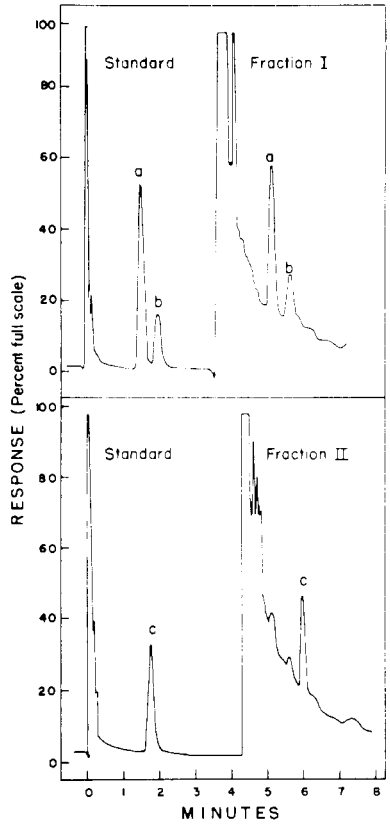

Figure 2. Recovery of carbofuran (2.5 ppm), 3-hydroxycarbofuran (0.12 ppm), and 3-ketocarbofuran (0.28 ppm) from fortified mullet (20 g): (a) carbofuran; (b) 3-ketocarbofuran; (c) 3-hydroxycarbofuran. The standards represent 2.5, 0.12, and 0.28 ng, respectively. An equivalent of approximately 4 mg of mullet was injected.

Table II. Recovery of Carbofuran, 3-Hydroxycarbofuran, and 3-Ketocarbofuran from Fortified Animal Homogenates

Species	Carbofuran		3-Hydroxycarbofuran		3-Ketocarbofuran	
	Added, ppm	Recovery, %	Added, ppm	Recovery, %	Added, ppm	Recovery, %
Oyster	2.5	83	0.12	80	0.28	34
	2.5	84	0.12	87	0.28	74
	25.7	100	8.2	84	8.2	60
	25.7	71	8.2	83	8.2	72
Shrimp	2.5	81	0.12	84	0.28	102
	25.7	99	8.2	82	8.2	78
Fish (Menhaden)	25.7	91	8.2	80	3.2	72
	25.7	71	8.2	85	8.2	77
Fish (Mullet)	2.5	63	0.12	70	0.28	77
	2.5	75	0.12	76	0.28	71
	12.5	74	1.3	89	2.8	67
	12.5	88	1.3	92	2.8	62
	12.5	87	1.3	91	2.8	70
	25.7	89	8.2	85	8.2	75
	25.7	100	8.2	83	8.2	70
Skate liver	25.7	87	8.2	93	8.2	84
	25.7	84	8.2	78	8.2	89
	25.7	87	8.2	71	8.2	88
Bird (red-winged blackbird)	2.5	79	0.12	94	0.28	68
	25.7	93	8.2	87	8.2	72
	25.7	83	8.2	86	8.2	68
Average		84.2		83.8		72.8

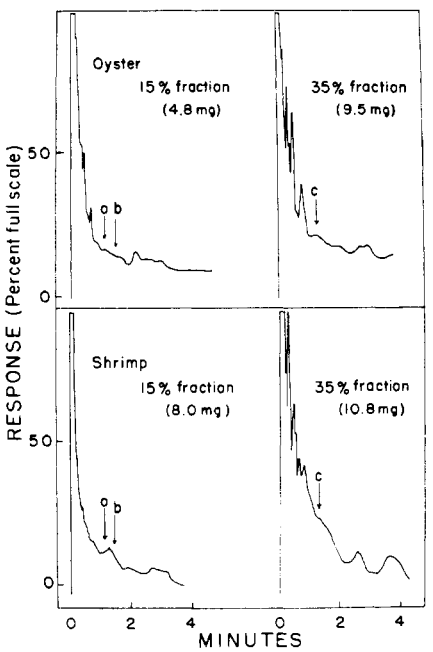

Figure 3. Chromatographic scan of unfortified samples. Numbers in parentheses represent the gram-sample injected. Arrows show the points at which the *N*-trifluoroacetyl derivatives of carbofuran (a), 3-ketocarbofuran (b), and 3-hydroxycarbofuran (c) appear.

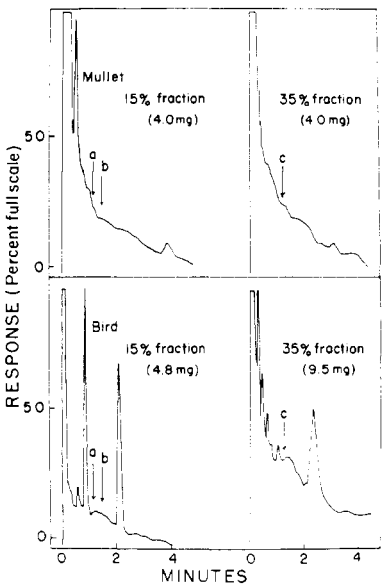

Figure 4. Chromatographic scan of unfortified samples. Numbers in parentheses represent the gram-sample injected. Arrows show the points at which the *N*-trifluoroacetyl derivatives of carbofuran (a), 3-ketocarbofuran (b), and 3-hydroxycarbofuran (c) appear.

volumes of petroleum ether, discarding the petroleum ether hypophase each time. The resulting 9:1 acetonitrile–water phase was transferred to a crystallizing dish and evaporated to dryness on a slide warming tray (45°).

Second Activated Florisil Column Cleanup. A second 400 × 20 mm chromatographic tube was prepared with 12.5 cm of activated Florisil. The column was topped with 1.25 cm of anhydrous sodium sulfate and prewetted with approximately 40 ml of hexane. The 9:1 acetonitrile–water residue was redissolved in a small volume of ethyl acetate (less than 1 ml), diluted with 5 ml of hexane, and transferred to the column. The crystallizing dish was rinsed with three 10-ml volumes of hexane containing 6% ethyl acetate and 1% acetonitrile (v/v) and each rinse was quantitatively added to the column. The column was then eluted with 200 ml of the same hexane–ethyl acetate–acetonitrile mixture and the eluate was discarded. This elution removed the sodium lauryl sulfate carried through the extraction process from the initial step. The column was then eluted with 300 ml of hexane containing 15% ethyl acetate and 1% acetonitrile (v/v) to obtain the carbofuran–3-ketocarbofuran fraction. A second elution with

300 ml of hexane containing 35% ethyl acetate (v/v) removed 3-hydroxycarbofuran from the column. Each carbamate fraction was collected in a separate 500-ml flask. Sand was added to the flask, a Snyder column affixed, and the solvent evaporated to approximately 50 ml on a steam table. The samples were quantitatively transferred to crystallizing dishes with three 5-ml ethyl acetate rinses of each flask. The solvent was then evaporated on a slide warming tray (45°).

Derivatization. The residue was redissolved in 1–4 ml of ethyl acetate and 0.2 ml transferred to a 2-dram vial. Trifluoroacetic anhydride (0.1 ml) was added and the vial sealed with a Teflon-lined cap. The vial was wrapped in aluminum foil to protect from light and the shielded vial placed on a slide warming tray (45°) for a minimum of 16 hr for complete derivation. At the completion of the reaction, the reaction mixture was diluted with 2 ml of hexane and washed with three 4-ml volumes of water to destroy the unreacted trifluoroacetic anhydride. The organic phase was dried over anhydrous sodium sulfate and brought to a convenient volume in a 25- or 50-ml graduate cylinder with hexane. Aliquots of 5.0 μl of this hexane solution were examined by gas–liquid chromatography.

Gas Chromatography. Fresh standards were prepared with each group of samples analyzed. Stock solutions of carbofuran (102 μg/ml), 3-hydroxycarbofuran (25 μg/ml), and 3-ketocarbofuran (5.0 μg/ml) were kept at 5° in ethyl acetate. It was convenient to have carbofuran and 3-ketocarbofuran in a single standard solution. With the setting of our gas chromatograph (attenuation, 8–16; range, 10^{-10} A/mV) 0.2 ml of the standards after derivatization required dilution to approximately 25 ml for full scale response. Quantitation was by peak height.

RESULTS AND DISCUSSION

N-Trifluoroacetyl derivatives of *N*-methylcarbamate insecticides have been prepared for glc determination by Seiber (1972) and Lau and Marxmiller (1970); our procedure is basically that of Seiber (1972) with minor modifications. The derivatives of carbofuran, 3-hydroxycarbofuran, and 3-ketocarbofuran are stable under the glc conditions employed and standards show no decrease in peak height when maintained at room temperature during the course of a working day. Reference standards, nevertheless, were prepared along with each group of samples as a check of the reagents and conditions for derivative synthesis. Retention times and peak response relative to heptachlor are given in Table I for column one. The retention times on our present columns are not sufficiently different for the complete resolution of peaks. Overlapping peaks are avoided, however, since the residues are collected in two fractions in the final step of the cleanup procedure (Figure 2). Sensitivity to electron capture detection is at the nanogram level; the higher sensitivity of 3-hydroxycarbofuran is apparently due to trifluoroacetylation of the hydroxy and carbamate groups (Seiber, 1972).

The recoveries given in Table II were determined using whole animal (oyster, shrimp, fish, bird) and tissue homogenates (skate liver) fortified with 2.5–25.7 ppm of carbofuran, 0.12–8.2 ppm of 3-hydroxycarbofuran, and 0.23–0.82 ppm of 3-ketocarbofuran prior to acid reflux. Recoveries averaged 84.2% for carbofuran, 83.8% for 3-hydroxycarbofuran, and 72.8% for 3-ketocarbofuran. Background interference (Figures 3 and 4) limits the sensitivity of this procedure to approximately 0.5 ppm of carbofuran, 0.07 ppm of 3-ketocarbofuran, and 0.05 ppm of 3-hydroxycarbofuran for the commodities tested (oyster, shrimp, mullet, Red-winged blackbird). This was checked by injecting extracts of untreated samples concentrated to an appropriate volume for sensitivity determination. It should be noted that in animal tissue carbofuran is rapidly converted to 3-hydroxycarbofuran (Metcalf *et al.*, 1968; Dorough, 1968b), the residue measured with the greatest sensitivity by this procedure.

Low recovery in this procedure can result from evaporative loss of residues in the final step of the cleanup, when samples (*i.e.*, the 15% and 35% ethyl acetate elution fractions) are evaporated to dryness in crystallizing dishes. This was determined by adding a nanogram quantity of carbofuran (102 ng), 3-ketocarbofuran (11.5 ng), and 3-hydroxycarbofuran (5 ng) to the 15 and 35% elution fractions of an unfortified shrimp sample. After solvent evaporation, the residues were air dried for 2 days at 23°. Recoveries were 51.7, 40, and 53.3%, respectively. Therefore, residues should be redissolved in ethyl acetate for derivative synthesis as soon as the last of the eluting solvent is evaporated.

Four trifluoroacetyl derivatives of other *N*-methylcarbamate insecticides were examined for potential interference with carbofuran residue determination. On the basis of retention time, carbofuran (1.43 min), 3-hydroxycarbofuran (1.69 min), and 3-ketocarbofuran (1.98 min) can be distinguished from aminocarb (1.89 min), baygon (1.02 min), carbaryl (2.87 min), and methiocarb (3.60 min).

The extraction method used in this procedure is basically that of Cook *et al.* (1969), with the exception that ethyl acetate is added to the aqueous animal homogenate prior to extraction with methylene chloride. The addition of ethyl acetate was found to significantly improve the methylene chloride extraction step.

Sodium lauryl sulfate was added as a detergent to prevent the formation of a thick, persistant emulsion when extracting lipid containing samples with methylene chloride. Generally, a gram was sufficient for each 20 g wet weight of tissue to be extracted; however, as much as 2.5 g has been used with no subsequent interference in the cleanup or detection of these carbamates.

The elution pattern of the carbamate residues should be carefully examined with each new lot of Florisil, since adsorption properties may vary and occasionally this variation is significant. Adjustments to overcome these variations can be made in the quantity of Florisil used or in the concentration of ethyl acetate in the eluting solvent mixtures.

Contaminating substances not removed in the cleanup may eventually cause deterioration of the packing in the glc columns. To avoid this, the Silane-treated glass wool plugs in the injection ports of our columns were changed at approximately 3-week intervals, and, at the same time, the top 1–2 cm of column packing was removed and replaced with fresh packing.

LITERATURE CITED

Butler, L. I., McDonough, L. M., *J. Ass. Offic. Anal. Chem.* **54**, 1357 (1971).
Caro, J. H., Freeman, H. P., Glotfelty, D. E., Turner, B. C., Edwards, W. M., *J. Agr. Food Chem.* **21**, 1010 (1973).
Cook, R. F., Stanovick, R. P., Cassil, C. C., *J. Agr. Food Chem.* 17, 277 (1969).
Dorough, H. W., *Bull. Environ. Contam. Toxicol.* **3**, 164 (1968a).
Dorough, H. W., *J. Agr. Food Chem.* **16**, 319 (1968b).
Lau, S. C., Marxmiller, R. L., *J. Agr. Food Chem.* **18**, 413 (1970).
Metcalf, R. L., Fukuto, T. R., Collins, C., Borck, K., abd El-Aziz, S., Munoz, R., Cassil, C. C., *J. Agr. Food Chem.* **16**, 300 (1968).
Seiber, J. N., *J. Agr. Food Chem.* **20**, 443 (1972).

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