Evaluation of Extraction Procedures for the Removal of ^{14}C-Carbofuran and Its Toxic Metabolites from Cabbage Leaves

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The soil of potted cabbage plants was treated with carbonyl-^{14}C-carbofuran and leaves were sampled 7, 14, 35, and 56 days after treatment. Cabbage leaves sampled 35 days after treatment were subjected to five extraction procedures to determine their relative efficiency for the removal of ^{14}C-carbofuran and its toxicologically significant metabolites. Three of these procedures (acid digestion, two successive 5-min blendings with methanol and 24-hr Soxhlet extraction using methanol) were found to be approximately 90% efficient. The least effective extraction procedure was two 5-min blendings with ethyl acetate. Thin-layer chromatographic–cholinesterase inhibition of the five extractions indicated that 85–90% of the ^{14}C in extracts of leaves sampled 35 days after treatment was associated with 3-hydroxycarbofuran. The extraction of ^{14}C from cabbage leaves appeared less efficient as the posttreatment sampling period increased from 7 to 56 days. For all sampling dates, at least 80% of the ^{14}C appeared to be associated with 3-hydroxycarbofuran.

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) is a broad-spectrum pesticide used for insect control on a wide variety of crops. This carbamate is effective as a contact toxicant and, when applied to the soil, is root absorbed and translocated to aerial portions of growing plants. This investigation was conducted to evaluate the effectiveness of several published extraction procedures for the removal of metabolized carbofuran from plant tissues. Most previous extraction efficiency studies have been directed primarily toward chlorinated hydrocarbons and, to a lesser extent, to organophosphate-type pesticides. Insufficient attention has been extended to specific extraction studies involving methylcarbamates, a relatively new class of compounds with insecticidal and nematocidal properties.

An effective means of evaluating extraction efficiencies for the quantitative removal of pesticide residues metabolized into biological tissues is to utilize radiolabeled compounds. However, few investigators have used labeled pesticides to determine extraction efficiencies. Klein *et al.* (1959) used radiolabeled methoxychlor to evaluate the removal of methoxychlor residues from the surface of spinach leaves by various extraction procedures. Wheeler *et al.* (1967) investigated the quantitative extraction of labeled dieldrin which had been root absorbed into aerial portions of various crops and noted that complete extraction of these internal residues required more exhaustive procedures, such as 24-hr Soxhlet extraction with chloroform–methanol. Bowman *et al.* (1968) concluded that 24-hr Soxhlet extraction using 10% methanol in chloroform was the most effective of the nine (9) extraction procedures evaluated for removal of weathered organophosphorus insecticides and their primary metabolites from field-treated crops. Watts (1971) evaluated two blending techniques as well as an exhaustive Soxhlet extraction using 10% methanol in chloroform for their relative efficiencies in the removal of ^{14}C-labeled and unlabeled residues of several organophosphates and one methylcarbamate from laboratory-grown bean plants and field-treated kale. This investigator observed that all three extraction procedures tested were equally effective for the removal of ^{14}C-carbaryl residues on bean leaves following 2 days "simulated weathering" in a controlled environment.

Previous studies of ^{14}C-labeled carbofuran indicated that its metabolic pathway involved oxidation followed by conjugation at the 3 position of the benzofuran ring and/or hydrolysis followed by conjugation at the 7 position of the benzofuran ring (Ashworth and Sheets, 1972; Cook *et al.*, 1969; Knaak *et al.*, 1970). In a study using field corn, Cook *et al.* (1969) reported that carbofuran, 3-hydroxycarbofuran, and 3-hydroxycarbofuran glycoside were the only carbamate residues found and stated that no accumulation of 3-ketocarbofuran was evident as a result of oxidation of 3-hydroxycarbofuran. It had been previously demonstrated that the conjugated forms of the carbamate residues could be quantitatively hydrolyzed to the aglycone forms by using hot acid digestion (Cook *et al.*, 1969). Acid hydrolysis converts the water-soluble conjugates to the organoextractable aglycone forms without destruction of the compound. Conventional extraction procedures using organic solvents are not considered to be effective in removing the conjugated carbamate residues.

The metabolism of ring-^{14}C- and carbonyl-^{14}C-carbofuran was investigated in alfalfa and bean plants and it was determined that the major metabolites in alfalfa were the glycosides of 3-hydroxycarbofuran (Knaak *et al.*, 1970). Previous radiotracer studies involving weathered corn foliage indicate that 80–90% of the original ^{14}C-carbofuran residues has been metabolized to 3-hydroxycarbofuran or its glycoside (Cook *et al.*, 1969). Ashworth and Sheets (1972) investigated the metabolism of carbofuran in tobacco using ring-labeled ^{14}C-carbofuran and reported that 3-hydroxycarbofuran and its glycoside were the major metabolites in root-uptake studies.

Toxicologically significant carbofuran metabolites extracted from plants exhibit cholinesterase inhibition. Therefore, of equal interest to extraction efficiency was the association of extracted ^{14}C activity with toxic components resulting from carbofuran metabolism. Since the phenolic hydrolysis products do not inhibit cholinesterase, they are not considered to be toxicologically important metabolites and were not considered in these investigations.

In this study five previously used extraction procedures were evaluated. Of initial interest was the acid digestion procedure recommended by Cook *et al.* (1969) for the removal of carbofuran and its carbamate metabolite residues from field-treated crops. The acetonitrile and ethyl acetate blending procedures were included since both have been used by other investigators and appeared to be relatively rapid. The methanol blending procedure for carbofuran and its metabolites (Knaak *et al.*, 1970) was used as a possible alternative procedure having possibilities of being both rapid and efficient in the removal of

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Table I. Percent[a] Extraction Efficiency of ^{14}C[b] from Cabbage Leaves by Various Extraction Procedures

Extraction fractions	Acid digestion	Soxhlet (methanol)	Methanol[c]	Aceto-nitrile[c]	Ethyl acetate[c]
First extraction	90.2	88.8	71.2	65.4	32.7
Second extraction			17.6	13.3	19.3
Residual tissues	9.8	11.2	11.2	21.3	48.0

[a]Calculated from comparing individual fractions to fraction total. Determined from combustion and scintillation counting. Each value represents the average of at least two replications. [b]^{14}C-Carbofuran added to soil 35 days prior to leaf sampling. [c]Extraction by blending for 5 min each time.

Table II. Percent[a] ^{14}C Associated with Components[b] in Extracts from Cabbage Leaves

Tlc-ChE components	Acid digestion	Soxhlet (methanol)	Methanol[c]	Aceto-nitrile[c]	Ethyl acetate[c]
Origin (ChE and non-ChE)	3.7	2.7	2.1	2.1	1.4
Carbofuran	2.0	2.2	1.8	2.6	4.5
3-Hydroxy-carbofuran	89.2	90.0	91.6	92.7	87.2
3-Keto-carbofuran	0.6	0.3	0.6	0.6	0.9
Total other ChE	2.9	1.3	0.8	0.6	0.5
Total non-ChE	1.7	3.5	3.2	1.6	5.7

[a]Calculated by dividing the cpm of individual tlc zones resulting from a duplicate aliquot applied to the plate by the cpm of the same volume of methylene chloride extract pipetted directly into a scintillation vial. Each value represents the average of at least two replications. [b]^{14}C-Carbofuran added to soil 35 days prior to leaf sampling. [c]Extraction by blending for 5 min each time.

metabolized carbamate pesticide residues. Finally, the 24-hr Soxhlet extraction using methanol was included as an exhaustive procedure to use for comparison with the other extraction methods.

Using carbonyl-^{14}C-carbofuran, these investigations primarily evaluated the following: (1) efficiency of five extraction procedures for the removal of metabolized ^{14}C from plant tissue and the percentage of ^{14}C associated with carbamate-containing components found in each type of extract; (2) extraction efficiency for the removal of ^{14}C from plants after varying periods of metabolism; (3) concentration of ^{14}C associated with carbamate-containing components following varying periods of metabolism.

MATERIALS AND METHODS

Application and Sampling. Cabbage seedlings (Round Dutch variety) were transplanted to 7 × 7 in. clay pots and placed outdoors in mid-January. On February 25, 1971, ^{14}C-carbofuran dissolved in 1:1 acetone–water was introduced into five 2-in.-deep prepunched pencil holes in the potted soil. Each pot received a total of 4.78 mg of carbonyl-^{14}C-carbofuran.

Samples were collected 7 and 14 days after soil treatment by randomly removing ten outer cabbage leaves. All the remaining leaves from eight of the ten treated cabbage plants were removed 35 days after the pesticide had been introduced into the soil. Fifty-six days after treatment, the remaining two treated and two untreated cabbage plants were stripped of their leaves. All the sample leaves were chopped finely, placed in plastic bags, and stored at −10° until analyzed.

^{14}C-Carbofuran. ^{14}C-Carbonyl-labeled carbofuran with a specific activity of 2.74 mCi/mmol was supplied by Niagara Chemical Division, FMC Corporation. Purity was verified by thin-layer chromatography and found to be satisfactory.

Extraction Procedures. Five-gram samples of finely chopped cabbage leaves from plants harvested 35 days after treatment were subjected to the following extraction procedures to evaluate their relative effectiveness in removing ^{14}C-carbofuran and its carbamate-containing metabolites.

Acid Digestion. The procedure used was similar to that presented by Cook *et al.* (1969).

Soxhlet 24-Hr. The sample was wrapped in filter paper and placed in an extraction thimble. The thimble was then placed into a Soxhlet extractor containing 250 ml of methanol and was refluxed for 24 hr. The extractor was rinsed with methanol and the final extract volume was adjusted to 300 ml.

Methanol, Acetonitrile, and Ethyl Acetate Blending. The sample was extracted in a Lourdes blender for 5 min with 25 ml of the appropriate solvent. The macerate was filtered through filter paper in a Buchner funnel and the recovered solvent was designated as the first extraction. The filter cake was reblended for 5 min with an additional 25 ml of solvent, filtered, and the extract was designated as the second extraction.

^{14}C Analysis. Following extraction, the extracted plant tissue was dried at 45–50° and weighed. Combustion was conducted as described by Watts (1971) in an oxygen atmosphere, and the resulting gases were trapped using a gas scrubbing apparatus containing a trapping-scintillation solution. The samples were composed of 25 μl of plant extract or 100 mg of dried previously extracted plant tissue. Both extracts and extracted plant tissue were combusted to reduce color and chemical quenching. Two replications of 16, 160, and 1600 ng of ^{14}C-carbofuran were applied to 100 mg of dried untreated cabbage leaves and carried through the described combustion procedure. Recoveries throughout this range were found to be over 95%. It was noted that vapors from the phenylethylamine used in the scintillation trapping solution caused bronchial irritation, necessitating the use of an efficient fume hood when handling this compound.

After combustion, an aliquot of trapping-scintillation solution was counted in a Packard Tri-Carb 3375 liquid scintillation spectrometer. The trapping solution was composed of 55% v/v toluene, 20% v/v methanol, 20% v/v phenylethylamine, 5% v/v Triton X-100, 0.5% w/v PPO, and 0.01% w/v POPOP. This trapping solution served not only to remove $^{14}CO_2$ from the combustion gases but also acted as the scintillation solution for determining the cpm associated with each sample. Using external standardization, samples were corrected for oxygen quenching when necessary. Percentage efficiencies were determined by dividing the counts per minute (cpm) for the individual extracts and tissue by the total cpm found in both components.

From the four organic solvent extractions, an aliquot equivalent to 0.5 g of crop was evaporated to dryness, refluxed for 1 hr with 0.25 *N* HCl, and partitioned into methylene chloride prior to thin-layer chromatography using cholinesterase inhibition (tlc-ChE). The acid-digested extracts were partitioned directly into methylene chloride. An aliquot of methylene chloride was applied to a precoated silica gel GF plate and developed in 2:1 ethyl ether–benzene. After solvent development the plate was air-dried and sprayed with a 25% solution of human blood plasma in distilled water. The plate was humidified for at least 30 min to facilitate cholinesterase inhibition. Following removal from the tank, the plates were sprayed with 1% indoxyl acetate in dioxane. Under long-wave uv,

Table III. Extraction[a] of ^{14}C from Cabbage Leaves Following Varying Periods of Metabolism

Extraction fractions	^{14}C-Carbofuran application interval,[b] days			
	7	14	35	56
Cabbage extract, %[c]	96.2	97.0	90.2	74.3
Residual tissues, %	3.8	3.0	9.8	25.7
Cabbage extract, ppm[d]	72.5	65.7	42.6	9.9
Residual tissues, ppm	2.9	2.0	5.5	3.4

[a]By acid digestion procedure. Each value represents the average of at least two replications. [b]Time interval between leaf sampling and application of labeled pesticide to potted soil. [c]Percent efficiency by comparing cpm of individual fractions to cpm fraction total. [d]Comparison of cpm of individual fractions to cpm/nanogram of ^{14}C-carbofuran.

spots appeared dark blue on a light blue background. Zonal plate scrapings were individually placed in scintillation counting vials. After the addition of scintillation solution (same as used for combustion), the samples were counted and color quench corrections were made using external standardization.

RESULTS AND DISCUSSION

Table I illustrates the relative efficiency of five extraction procedures for the removal of labeled carbon from cabbage plants, 35 days after ^{14}C-carbofuran application to the soil. Approximately 90% of the ^{14}C was removed from the plant tissue by acid digestion or methanol extraction *via* 24-hr Soxhlet or two 5-min blendings. Two 5-min periods of methanol blending were found to remove as much of the ^{14}C-carbon-associated compounds as by 24-hr Soxhlet refluxing with methanol. The methanol double-blending procedure looks promising, since it removed nearly 90% of the labeled ^{14}C and was more rapid than either the acid digestion or the 24-hr Soxhlet extraction. Double-blending with acetonitrile removed approximately 80% of the labeled compounds and therefore should not be ruled out of consideration. The only procedure that is not recommended as a result of these investigations would be ethyl acetate blending. However, these various solvent extracts would subsequently require acid hydrolysis in order to release the conjugated forms of the metabolites. Other considerations such as the ease of pesticide partitioning into another solvent during cleanup or the ease of concentration of the solvent should also be evaluated.

The percentage of labeled carbon in the various components of the five extractions as separated by tlc–ChE are shown in Table II. Results obtained indicate that approximately 90% of the total ^{14}C activity in leaf extracts partitioned into methylene chloride was associated with 3-hydroxycarbofuran, 35 days after applications of the ^{14}C-carbofuran to the soil. Only an insignificant percentage of the ^{14}C activity appears to remain associated with carbofuran.

Initially, it was found that carbofuran and its toxic metabolites could not be successfully separated by tlc–ChE when in aqueous acid media. Therefore, carbofuran and its metabolites were partitioned from the aqueous phase into methylene chloride as described by Cook *et al.* (1969). It was determined that approximately 85% of the ^{14}C activity was partitioned into the organic solvent using this procedure. Another complication was that sodium lauryl sulfate (added to break emulsion during partitioning) tended to produce spurious cholinesterase inhibitions which interfered with thin-layer chromatography. By eliminating the sodium lauryl sulfate, most of the spurious cholinesterase inhibitions were eliminated.

Percent efficiencies and corresponding concentrations of ^{14}C-associated compounds removed by acid digestion of cabbage leaves 1, 2, 5, and 8 weeks following ^{14}C-carbofuran application to the soil are illustrated in Table III. It would appear from the percentage efficiency data that the acid digestion extraction becomes less efficient after a period of metabolism and weathering. However, following conversion of the percentage data to concentrations equivalent to parts per million of carbofuran, the ^{14}C activity attributed to unextracted plant tissue remained relatively constant and the extractable ^{14}C decreased significantly after 2 weeks of metabolism. Consequently, consideration of only the percentage changes in the extract and tissues would tend to be misleading, unless the concentration data were properly evaluated.

Table IV shows the percentage and concentrations of ^{14}C associated with carbofuran and its metabolites at varying posttreatment sampling periods following acid digestion extraction. One week after the application of ^{14}C-carbofuran to the soil, less than 10% of the ^{14}C in the leaves remained associated with the parent compound. At this sampling, approximately 80% of the ^{14}C activity was associated with 3-hydroxycarbofuran. As the posttreatment time interval increased, the ^{14}C associated with carbofuran continued to decrease until it was less than 1% of the total activity in the 56-day posttreatment sampling. The percentage of ^{14}C associated with 3-hydroxycarbofuran increased to approximately 90% at 2 weeks posttreatment but gradually decreased to about 75% after 8 weeks of metabolism. The percentage data in Table IV indicate that the ^{14}C activity associated with the origin increased as the posttreatment period progressed. Concentrations of carbofuran and its primary metabolites should be compared against the percentage data. It is readily apparent that the concentration of the originally applied ^{14}C-carbofuran is a minor part to the total residue, regardless of the time that had lapsed since application to the soil. The

Table IV. Percent[a] and Concentration[b] of ^{14}C Associated with Components Extracted[c] from Cabbage Leaves Following Varying Periods of Metabolism

Tlc–ChE components	^{14}C-Carbofuran application interval,[d] days							
	7		14		35		56	
	%	ppm	%	ppm	%	ppm	%	ppm
Origin[e]	1.8	1.19	2.0	1.22	3.7	1.25	11.8	0.71
Carbofuran	8.8	4.30	5.6	3.25	2.0	0.73	0.5	0.04
3-Hydroxycarbofuran[f]	87.0	55.28	89.8	56.81	89.2	34.20	80.4	5.23
3-Ketocarbofuran[f]	0.5	0.29	0.7	0.36	0.6	0.12	0.3	0.02
Remaining ChE	1.0		0.7		2.9		3.9	
Remaining non-ChE	0.8		1.4		1.7		3.3	

[a] Calculated by dividing the cpm of individual tlc zones resulting from a duplicate aliquot applied to the plate by the cpm of the same volume of methylene chloride extract pipetted directly into a scintillation vial. Each value represents the average of at least two replications. [b] Concentrations calculated by comparing cpm of individual tlc sections to cpm/nanogram of ^{14}C-carbofuran, 3-hydroxycarbofuran, or 3-ketocarbofuran. (Origin calculated as carbofuran.) [c] By acid digestion procedure. [d] Time interval between leaf sampling and application of labeled pesticide to potted soil. [e] Calculated as carbofuran. [f]ppm corrected for molecular weight difference from carbofuran.

primary metabolite found at all sampling periods was 3-hydroxycarbofuran with very low concentrations of 3-ketocarbofuran being noted. The concentrations of ^{14}C activity associated with the origin and calculated as carbofuran remained relatively constant in the 1-, 2-, and 5-week samplings but decreased in the 8-week sampling.

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Use of Selected Plastics in Controlled Release Granular Formulations of Aldicarb and Dimethoate

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Granular formulations of aldicarb prepared with cellulose acetate, polyamide, polyester, polyvinyl chloride, polyurethane, and urea-formaldehyde and granular formulations of dimethoate prepared with cellulose acetate, polyvinyl chloride, and petroleum charcoal released the insecticides more slowly into soil and water than did the standard corn cob formulations of the two insecticides. Greenhouse bioassays indicated that most plastic formulations of aldicarb were more effective than the standard corn cob formulation in extending the period of systemic insecticidal activity of aldicarb against adult boll weevils, *Anthonomus grandis* Boheman. Greenhouse bioassays with cotton aphids, *Aphis gossypii* Glover, and radioassays of plants grown in soil treated with selected granular formulations indicated that certain plastic and charcoal formulations of dimethoate extended the uptake from soil and the biological activity.

Solid formulations of insecticides prepared with plastic materials have shown promise of extending the period during which the toxicant is released into the atmosphere or into an aqueous media. Probably the best known plastic formulations are the dichlorvos resin strips that release toxicant vapors into the atmosphere over extended periods. Smittle and Burden (1965) used formulations of dichlorvos in polyvinyl chloride to demonstrate the control of a number of insects of public health importance. Harvey and Ely (1968) used polyvinyl chloride strips or blocks that contained 20% dichlorvos to control some flying insects in homes and other buildings where strong drafts or extreme ventilation could be avoided and obtained good control of the short-nosed cattle louse (*Haematopinus eurysternus* Nitzsch.) when infested cattle were enclosed and exposed to the resin strips. Bailey *et al.* (1971) reported up to 7 weeks control of houseflies (*Musca domestica* L.) in poultry houses with dichlorvos applied in slow-release plastic (polyvinyl chloride) granules. In addition to these formulations that released toxicant vapors, several workers have described plastic formulations that released toxicants into aqueous media. For example, control of houseflies lasting as long as 5 months was obtained by formulating dichlorvos in wax or in a mixture of urea and formaldehyde and placing the blocks in aqueous sugar solution in chicken-watering devices (Kilpatrick *et al.*, 1962); pellets of polyvinyl chloride and polyamide containing Abate [*O,O,O',O'*-tetramethyl *O,O'*-(thiodi-*p*-phenylene) phosphorothioate], naled, and malathion showed promise of extending the activity of mosquito larvicides (Whitlaw and Evans, 1968); and Miles and Woehst (1969) reported releasing Abate from foamed polyvinyl chloride formulations into water for control of *Aedes aegypti* (L.). The larvae of the southern house mosquito (*Culex pipiens quinquefasciatus* Say) have been controlled up to 26 weeks with slow release formulations of Dursban [*O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl)] (Wilkinson *et al.*, 1971).

Stokes *et al.* (1970) and Coppedge (1970) reported previously the chemical and biological evaluation of controlled release formulations of aldicarb. They found that certain compacted carbon formulations released aldicarb more slowly in laboratory water immersion tests than standard corn cob formulations and also extended the systemic activity of this insecticide against the boll weevil (*Anthonomus grandis* Boheman) after treatment of cotton plants with side dress applications. Therefore, a similar chemical and biological evaluation of the rates of release of aldicarb and dimethoate from selected plastic formulations was made.

MATERIALS AND METHODS

Formulations of Aldicarb. All granular formulations of aldicarb contained approximately 10% active ingredient by weight and were sized 10/20 mesh. The corn cob granules (Union Carbide Corp., Clayton, N. C.) and the petroleum charcoal granules (Great Lakes Research Corp., Elizabethton, Tenn.) were used as the fast and slow release standards, respectively.

The urea-formaldehyde formulations were prepared by the methods described by Geary (1963) or by slight modifications of these methods. The specified quantities of aldicarb, urea-formaldehyde, and additives were mixed, 2

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