rate of application of the granular carbofuran. The residues of carbofuran were at a maximum concentration 6–8 days after seeding and then decreased quickly between 11 and 14 days after seeding. By 28 days after seeding, the residues of carbofuran were trace or not detectable. The concentrations of a 3-ketocarbofuran residues were always lower than those of either carbofuran or 3-hydroxycarbofuran. The highest concentrations of 3-ketocarbofuran, 0.8 ppm, occurred in the period 8–11 days after seeding in plants collected from the plots receiving the high treatment rate of carbofuran. Concentrations of 3-ketocarbofuran decreased after the period so that by 28 days after seeding residues of 3-ketocarbofuran were not detectable.

The maximum concentration of 3-hydroxycarbofuran residues, conjugated and nonconjugated, were found in the samples collected 11 days after seeding with 6.48 ppm found in plants grown with the high rate of treatment. The concentration of 3-hydroxycarbofuran decreased throughout the remainder of this study so that residues of 0.20 and 0.44 ppm were detected in plants grown in plots treated at the low and high rate, respectively, 28 days after seeding. The relatively high level of 3-hydroxycarbofuran compared to that of carbofuran and 3-ketocarbofuran is likely the result of the rapeseed plants conjugating this metabolite, thus slowing its degradation (Knaak et al., 1970).

The detection of carbofuran and its metabolites in emerging seedlings of rapeseed indicates that absorption of carbofuran by the seedlings starts prior to emergence, thus providing chemical protection against flea beetle feeding.

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Extraction of Biologically Incorporated [^{14}C]Carbofuran Residues from Root Crops

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[^{14}C]Carbofuran, suspended in a commercial formulation, was added to the soil and exposed roots of growing potatoes, carrots, and radishes. The treated crops were harvested at 5, 10, and 15 days post-application and composited. Carbofuran residues in the composites were extracted by blending with methanol or acetonitrile, followed by washing and leaching with the same solvent used in the blending. With potatoes and radishes, both solvents extracted approximately equal percentages of [^{14}C]carbofuran residues (85% for potatoes and 37% for radishes from 15-day samples) for all harvest-time combinations. With carrots, methanol extracted more ^{14}C than acetonitrile for all harvest-time combinations (e.g., 95.8 vs. 90.7% from the 15-day sample). Methanol was as efficient as acetonitrile for extracting ^{14}C from radishes and potatoes. Residues extracted from samples with methanol or acetonitrile were partitioned into methylene chloride and characterized. Carbofuran was the major residue in potatoes and radishes; the level decreased with longer incorporation periods. The angelic acid ester of 3-hydroxycarbofuran, carbofuran, and 3-hydroxycarbofuran were the main residues in carrots. The aqueous phase of each extractant, after partitioning with methylene chloride, contained a significant quantity of ^{14}C, which increased with incorporation period. Acid hydrolysis of the aqueous phase released the following residues as major aglycons of conjugated carbofuran metabolites: 3-keto-7-phenol for potatoes, 3-hydroxycarbofuran for carrots, and an unidentified metabolite for radishes. The crop marc remaining after Soxhlet extraction contained a significant amount of bound residues (up to 62.1% of total) only in radishes. Acid hydrolysis of these crop marcs released some bound residues into aqueous solution, but only a minute quantity of ^{14}C could subsequently be extracted into methylene chloride.

The introduction and increasing use of carbamate pesticides have resulted in a need for analytical methodology which can be used to determine carbamate residues in foods and environmental samples. Analytical methods for determining multiple carbamate residues in samples are highly desirable. These methods must provide efficient extraction of residues from the sample matrix.

The extraction of pesticide residues can be evaluated by using samples in which radioactively labeled pesticides are biologically incorporated. This allows the determination of extracted and unextracted residues; conjugated and bound pesticide residues can also be measured. Although

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interest in bound and conjugated pesticide residues has been increasing in recent years, relatively little information is available to evaluate the hazard they may present. Information on the chemical forms and quantities of conjugated and bound pesticide residues which may be present in foods is necessary for risk evaluation of these compounds.

Few studies on the evaluation of the extraction efficiencies of carbamate pesticides have appeared in the literature compared to similar studies on organochlorine pesticides. Watts (1971) evaluated blending with ethyl acetate or acetonitrile as well as exhaustive Soxhlet extraction with 10% methanol in chloroform for their relative efficiencies in the removal of ^{14}C-labeled carbaryl from laboratory-grown bean plants and field-treated kale. He observed that the three extraction procedures were equally effective for the removal of [^{14}C]carbaryl residues on bean leaves (96–100% efficiency).

Van Middelem and Peplow (1973) evaluated blending with methanol, acetonitrile, or ethyl acetate, Soxhlet extraction with methanol, and acid digestion as extraction procedures for the removal of [^{14}C]carbofuran residues from cabbage. They reported that acid digestion, blending with methanol, and Soxhlet extraction were each approximately 90% efficient.

Cook et al. (1969) reported the extraction of carbofuran residues by refluxing the crop sample with 0.25 N HCl. This method was developed because solvent extraction failed to remove conjugated carbofuran residues from treated samples. Hydrolysis with 0.25 N HCl does not destroy the carbamoyl moiety, which is the toxicologically active (cholinesterase inhibitive) portion of the carbofuran molecule. Cook (1973) demonstrated the use of acid hydrolysis for extraction of carbofuran residues from various environmental samples.

Recently, in contrast to acid hydrolysis extraction, solvent extraction of carbamate pesticide residues has been reported. Wheeler et al. (1978) evaluated the extraction efficiency of blending with methanol, acetonitrile, or acetone for foliar-applied [^{14}C]carbaryl residues from mustard greens and radishes. Methanol was the best extractant. A significant quantity of bound residues was found in radishes. Acid hydrolysis removed 50–75% of those bound residues. In only one of the above reports were both total residues and individual metabolites examined as indicators of extraction efficiency (Van Middelem and Peplow, 1973). In the other reports, total ^{14}C was used as the only indicator of extraction efficiency. The Van Middelem and Peplow study examined [^{14}C]carbofuran extraction from cabbage leaves following systemic movement of the parent pesticide from the roots to the leaves.

In the present study, the extraction of [^{14}C]carbofuran residues resulting from the application of [^{14}C]carbofuran to the soil and partially exposed potato, carrot, and radish roots was evaluated. This evaluation was undertaken to determine the extent of effort required to extract carbofuran residues and the various levels of efficiency obtained by these efforts. Ultimately, the data should enable any scientist to determine the effectiveness of these procedures vs. the amount of effort required to obtain various extraction efficiencies. This study examines some extraction procedures which are used in multiple pesticide residue analysis of food commodities. Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate), a widely used carbamate pesticide, was chosen as the model carbamate. Root crops were selected because significant quantities of bound [^{14}C]carbofuran residues were observed in radishes after foliar application (Wheeler and Thompson, 1977). Radishes were also used in this study to see if there would be a significant difference between soil application and foliar application. Carbofuran is registered for use on potatoes in the United States but not for radishes and carrots.

Two water-miscible solvents, methanol and acetonitrile, which are widely used as extractants in the determination of organochlorine and organophosphorus pesticide residues in crops, were compared for their ability to extract biologically incorporated carbofuran residues. [^{14}C]Carbofuran residues and conjugated carbofuran residues in root crops were also determined.

EXPERIMENTAL SECTION

Chemicals. [^{14}C]Carbofuran, uniformly labeled at the benzene ring carbons, was obtained from New England Nuclear Co., Boston, MA. It had a specific activity of 8.2 mCi/mmol and was found to be 98% radioactively pure by high-performance liquid chromatography (HPLC) as described below. The following carbofuran metabolites and commercial formulation were obtained from FMC Corp., Middleport, NY: 2,3-dihydro-2,2-dimethyl-3-hydroxy-7-benzofuranyl methylcarbamate (3-hydroxycarbofuran), 2,3-dihydro-2,2-dimethyl-3,7-dihydroxybenzofuran (3-hydroxy-7-phenol), 2,3-dihydro-2,2-dimethyl-3-keto-7-benzofuranyl methylcarbamate (3-ketocarbofuran), 2,3-dihydro-2,2-dimethyl-3-keto-7-hydroxybenzofuran (3-keto-7-phenol), and 2,3-dihydro-2,2-dimethyl-7-hydroxybenzofuran (7-phenol); Furadan 4 Flowable (a commercial carbofuran formulation). The purity of the metabolites was verified by thin-layer chromatography (TLC) as described below.

Treatment of Plants. Carrot and radish plants were grown from seed in pots 21.6 cm diameter × 22.9 cm deep and 15.2 cm diameter × 17.8 cm deep, respectively. Potatoes were grown from seed potatoes with one plant per 35.6 cm diameter × 29.2 cm deep pot. The silt loam soil used was collected from a field at the U.S. Department of Agriculture Research Center, Beltsville, MD. Growth of crops was started in a greenhouse, and after the seedlings had emerged, the pots were placed outdoors. After the plants began to produce edible portions, the pots were returned to the greenhouse, which was temperature controlled (18–21 °C depending on the outside temperature). An aqueous suspension of [^{14}C]carbofuran was prepared from the commercial formulation (Furadan 4 Flowable) by adding an acetone solution (less than 3 mL) containing the [^{14}C]carbofuran to 200 mL of aqueous dispersed formulation. The specific activity of the final solution was 2.56 mCi/mmol. The suspension of [^{14}C]carbofuran was pipetted onto the soil and the partly exposed tubers at an application rate of 2.25–3.35 kg of carbofuran/ha.

Harvesting and Compositing of Root Crops. Crops were harvested 5, 10, and 15 days after carbofuran application. The foliar portions were discarded. The root portions were rinsed with cold water to remove adhering soil, placed in a Hobart chopper (Model 84141, Hobart Manufacturing Co., Troy, OH), and then chopped and mixed into a homogeneous composite. Sample portions (100 g for potatoes and radishes and 70 g for carrots) were weighed into separate 1-qt glass jars (obtained from Tropicana Products Inc., Bradenton, FL) and stored at −20 °C until they were analyzed.

Extraction Procedure. Triplicate portions of each composite were analyzed individually. The procedures used to determine extraction efficiency consisted of eight steps as shown in Figure 1. The volume of each extract was recorded, and small portions were used for radioactive counting.

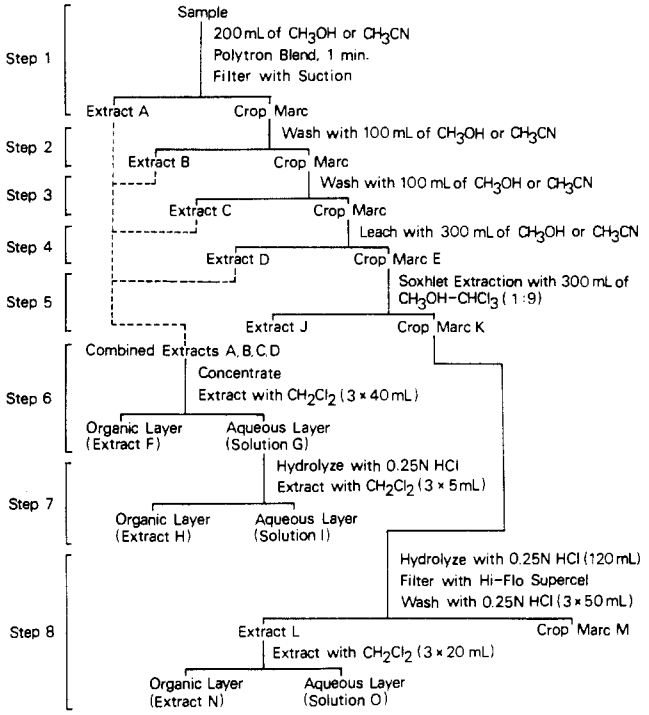

Figure 1. Flow diagram of the extraction procedure.

Step 1: Blending and Collection of Extract A. Each sample portion was blended with 200 mL of methanol or acetonitrile with a Polytron blender (Model PT 20, Brinkmann Instruments, Westbury, NY) for 0.5 min at $^1/_2$ maximum speed and 1 min at maximum speed. The blended composite was filtered with suction through Whatman No. 1 filter paper placed on a coarse glass filter. The volume of the extract collected from each sample was not the same, but each extract was collected until filtration became extremely slow (residual liquid collected at a rate of 1 drop/5 s). This filtrate is referred to as extract A.

Step 2: First Washing and Collection of Extract B. The residual solid was scraped from the blender and the glass jar. The blender and jar were rinsed with 100 mL of the same solvent used for blending. This solution, along with the residual solid, was poured onto the filter cake and the extract collected by suction as described in step 1. This filtrate is referred to as extract B.

Step 3: Second Washing and Collection of Extract C. Step 3 is a repeat of step 2. This filtrate is referred to as extract C.

Step 4: Leaching and Collection of Extract D. The crop marc was leached with 300 mL of the same solvent used in step 2. The flow rate of the leaching solvent was controlled (between 5 and 10 mL/min) so that the solvent had an increased, regulated contact time with the crop marc. After the leaching stopped, suction was applied to remove the solvent remaining in the crop marc. This filtrate is referred to as extract D, and the residual solids are referred to as crop marc E.

Step 5: Soxhlet Extraction and Collection of Extract J. Crop marc E was placed in a paper thimble and extracted for 16 h in a Soxhlet apparatus by using 300 mL of chloroform–methanol (9:1). This extract is referred to as extract J, and the remaining crop marc is referred to as crop marc K.

Step 6: Determination of Organic-Extractable and Water-Soluble [^{14}C]Carbofuran Residues. Extracts A–D were combined, concentrated to about 90 mL with a rotary evaporator, and transferred to a separatory funnel. The combined aqueous extracts were partitioned with three 40-mL portions of methylene chloride. For the acetonitrile extracts, 50 mL of water was added before the extraction with methylene chloride. Without the additional water, a single phase was occasionally obtained when the methylene chloride was added. The organic layers were collected and combined and are referred to as extract F. The aqueous layer is referred to as solution G. The loss of ^{14}C from extracts A–D during concentration was negligible.

Table I. Solubilization of Bound [^{14}C]Carbofuran Residues by Acid Hydrolysis

		^{14}C in extract L, %[a]		
days	solvent	potatoes	carrots	radishes
5	methanol	3.27 (39.4)	1.34 (38.8)	8.27 (24.5)
	acetonitrile	3.79 (41.2)	2.33 (53.7)	6.61 (20.8)
10	methanol	4.76 (35.2)	1.03 (31.1)	9.95 (18.7)
	acetonitrile	5.37 (41.2)	1.66 (41.2)	8.31 (16.0)
15	methanol	5.30 (46.0)	1.46 (41.4)	8.02 (13.0)
	acetonitrile	4.11 (35.6)	1.87 (43.3)	8.06 (13.1)

[a] As a percentage of the total ^{14}C in the sample composite (100%). The number in parentheses indicates ^{14}C in extract L as a percentage of the total ^{14}C in crop marc K (100%).

Step 7: Hydrolysis of Solution G. An aliquot (10 mL) of solution G was placed in a 30-mL test tube with a screw cap, and enough 1 N HCl was added to obtain a 0.25 N HCl solution. The capped tube was placed in a boiling water bath for 1 h. The tube was cooled in running water, and the aqueous solution was extracted with three 5-mL portions of methylene chloride. The combined methylene chloride layers are referred to as extract H, and the aqueous layer is referred to as solution I.

Step 8: Hydrolysis of Crop Marc K. Crop marc K was placed in a 500-mL round-bottom flask, and 120 mL of 0.25 N HCl was added. A few drops of Tween 20 (Sigma Chemical Co., St. Louis, MO) was added to prevent foaming. The flask was fitted with a condenser, and the sample was refluxed 1 h. The hydrolysate was filtered through Whatman No. 1 filter paper after 10 g of Hi-Flo Supercel (Johns-Manville Products Corp., Denver, CO) was added to the solution. The solids were washed with three 50-mL portions of 0.25 N HCl. The filtrates were combined and are referred to as extract L. The remaining solids are referred to as crop marc M. A 100-mL portion of extract L was extracted with three 50-mL portions of methylene chloride. A small quantity of powdered sodium lauryl sulfate was added to break emulsions. The methylene chloride extracts were separated and combined and are referred to as extract N. The aqueous layer is referred to as solution O.

Determination of ^{14}C. Liquid scintillation counting was performed by using a Mark III Model 6880 liquid scintillation spectrometer (Searle, Inc., Des Plaines, IL). Aliquots of extracts (5 mL for solution I; 1 or 2 mL for all others) were placed in glass vials, and 10 mL of Insta-gel (Packard Instrument Co., Downers Grove, IL) was added before counting. Since triplicate portions of sample were individually analyzed by the procedure described above for each solvent and each crop-time combination, three extracts, solutions, or crop marcs were obtained for each step. Duplicate aliquots of each extract, solution, or crop marc were analyzed for ^{14}C content, for a total of six determinations for each data point in Figures 2–8 and Table I. The external standard technique was used to correct for quenching. The ^{14}C in crop marcs K and M (50–200 mg) was counted as described above after combustion of the sample and entrapment of the CO_2 using a Packard 306B sample oxidizer. Crop marc M was a homogeneous mixture of crop tissues and Hi-Flo Supercel, which was

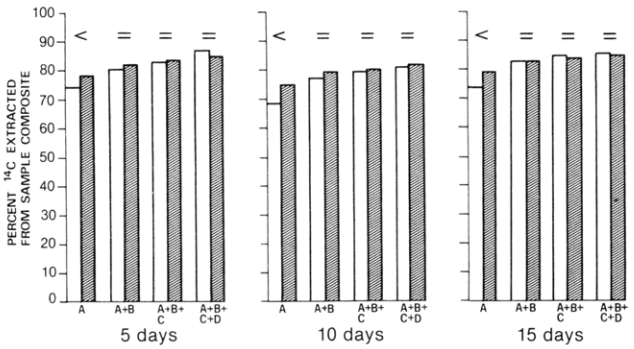

Figure 2. Comparison of extraction efficiency of methanol vs. acetonitrile for [^{14}C]carbofuran residues in potatoes. The fractions were analyzed for ^{14}C separately and the cumulative results are shown. The open column indicates methanol extraction; the shaded column indicates acetonitrile extraction. Note 1: Total ^{14}C in sample composite (100%) is determined by adding the quantity of ^{14}C in extracts A–D and J and crop marc K. Note 2: Statistical analyses (i.e., two-tailed t statistics at a confidence level of 5%) of the individual determinations made with each solvent were performed and the results are indicated as follows: (=) indicates no significant difference found for these extracts when comparing methanol vs. acetonitrile; (>) indicates the total ^{14}C in methanol is statistically greater than the total ^{14}C in acetonitrile; (<) indicates the total ^{14}C in acetonitrile is statistically greater than the total ^{14}C in methanol.

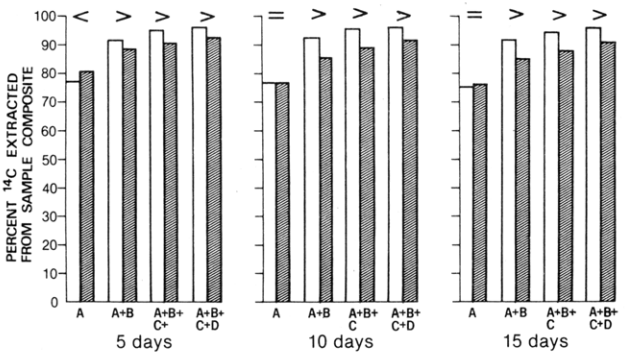

Figure 3. Comparison of extraction efficiency of methanol vs. acetonitrile for [^{14}C]carbofuran residues in carrots. The fractions were analyzed for ^{14}C separately and the cumulative results are shown. The open column indicates ^{14}C obtained with methanol; the shaded column indicates ^{14}C obtained with acetonitrile. See notes 1 and 2 in Figure 2.

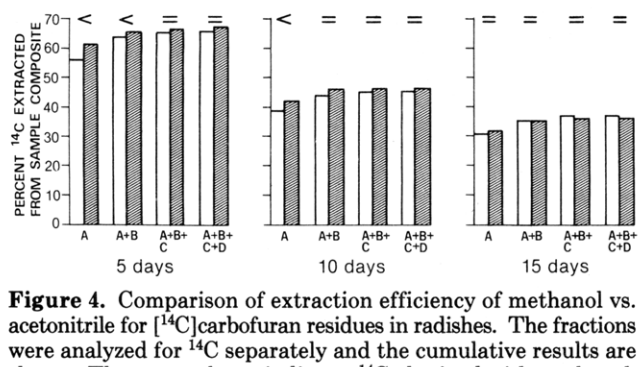

Figure 4. Comparison of extraction efficiency of methanol vs. acetonitrile for [^{14}C]carbofuran residues in radishes. The fractions were analyzed for ^{14}C separately and the cumulative results are shown. The open column indicates ^{14}C obtained with methanol; the shaded column indicates ^{14}C obtained with acetonitrile. See notes 1 and 2 in Figure 2.

prepared by grinding in a mortar. The recovery of ^{14}C in the combustion procedure was between 98 and 100% when the crop marc was fortified with Spec-Chek (^{14}C standard, Packard). The ^{14}C counting of the crop marc was not corrected for ^{14}C recovery. Several carrot extracts were highly colored and were severely quenched. They were counted after decolorization with a methanolic solution of

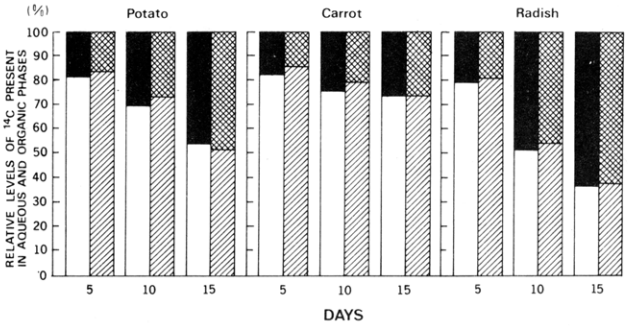

Figure 5. Comparison of methylene chloride extractable vs. water-soluble [^{14}C]carbofuran residues. The total ^{14}C in combined extracts A–D was determined by adding the quantity of ^{14}C found in each individual extract. The open column indicates ^{14}C in extract F obtained from combined methanol extracts (A + B + C + D); the solid column indicates ^{14}C in solution G obtained from the combined methanol extracts (A + B + C + D); the shaded column indicates ^{14}C in extract F obtained from the combined acetonitrile extracts (A + B + C + D); the crosshatched column indicates ^{14}C in solution G obtained from the combined acetonitrile extracts (A + B + C + D).

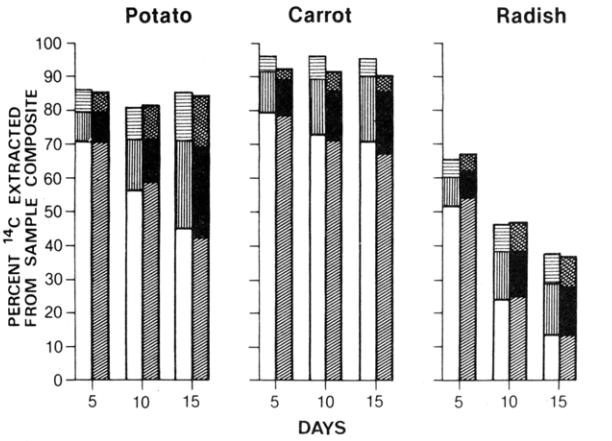

Figure 6. Comparison of results obtained from hydrolysis of water-soluble [^{14}C]carbofuran residues. The open column indicates ^{14}C in extract F, the vertical-shaded column indicates ^{14}C in extract H, and the horizontal-shaded column indicates ^{14}C in extract I obtained from the combined methanol extracts (A + B + C + D); the diagonal-shaded column indicates ^{14}C in extract F, the solid column indicates ^{14}C in extract H, and the crosshatched column indicates ^{14}C in extract I from the combined acetonitrile extracts (A + B + C + D).

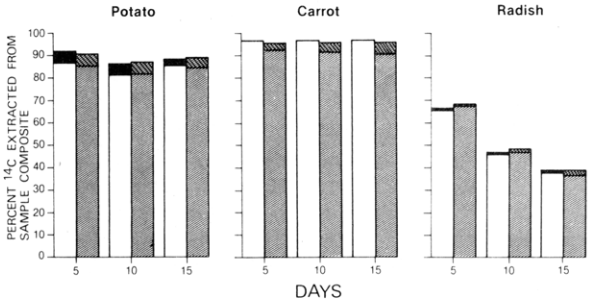

Figure 7. Soxhlet extraction of [^{14}C]carbofuran residues from crop marc. See note 1 in Figure 2. The open column indicates ^{14}C in the combined extracts (A + B + C + D) obtained by methanol extraction; the solid column indicates ^{14}C in extract J obtained after methanol extraction; the shaded column indicates ^{14}C in the combined extracts (A + B + C + D) obtained by acetonitrile extraction; the crosshatched column indicates ^{14}C in extract J obtained after acetonitrile extraction.

N-bromosuccinimide as described by Sonobe et al. (1982). The total ^{14}C of each extract, solution, or crop marc was

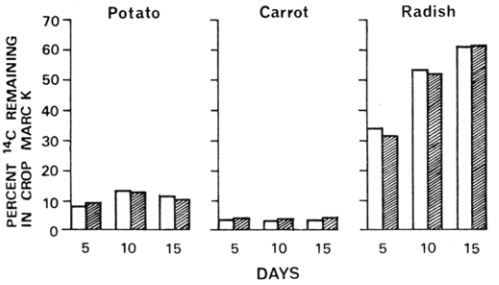

Figure 8. Unextractable (bound) [^{14}C]carbofuran residues. See note 1 in Figure 2. The open column indicates ^{14}C in crop marc K after methanol and Soxhlet extraction; the shaded column indicates ^{14}C in crop marc after acetonitrile and Soxhlet extraction.

calculated from the mean dpm of the duplicate aliquots and the fraction that the aliquot represented. An overall mean ^{14}C value for each extract, solution, or crop marc was determined by averaging this total ^{14}C for each of the triplicate extracts, solutions, or crop marcs for each solvent and each crop-time combination. Total ^{14}C in each sample was calculated by summing the overall mean ^{14}C for extracts A–D and J and crop marc K.

Determination of Carbofuran Residues Using HPLC. The reversed-phase HPLC procedure, using a Zorbax ODS column and methanol–water gradient elution, which was developed for the separation of carbofuran residues by Sonobe et al. (1981a), was followed. The retention times of carbofuran and its five metabolites (3-hydroxycarbofuran, 3-hydroxy-7-phenol, 3-ketocarbofuran, 3-keto-7-phenol, and 7-phenol) were determined, and collecting windows were determined by using standards to enable complete collection of each compound. A fraction containing the 100% methanol column flush following the elution of 7-phenol was also collected. All fractions of eluates collected were analyzed for ^{14}C as described previously. If two phases were observed after adding 10 mL of Insta-gel, water was added until a stable emulsion was obtained. In every case, the entire amount of the total ^{14}C injected into the HPLC system could be accounted for in the fractions collected.

Extracts F and H from each of the triplicate samples of each crop-time combination were analyzed by the HPLC procedure for carbofuran residues. HPLC of extracts F was performed separately for acetonitrile- and methanol-extracted samples. The methylene chloride in each extract was replaced with methanol before HPLC. Methylene chloride extracts were concentrated to about 5 mL, and 5 mL of methanol was added. The resulting solution was concentrated to about 5 mL, a second 5 mL of methanol was added, and the solution was again concentrated to less than 2 mL. The solution was diluted to 10 mL with methanol. Loss of ^{14}C in this solvent exchange procedure was negligible. The results presented in Figures 9 and 10 represent the means of each triplicate.

The angelic acid ester of 3-hydroxycarbofuran, the occurrence of which was reported in carrots by Sonobe et al. (1981b), elutes during the column wash with 100% methanol. For each crop-time combination, several column wash fractions were combined and concentrated to a few milliliters, and 10 mL of water was added. This solution was extracted with three 5-mL portions of methylene chloride. The methylene chloride layer contained 100% of the total ^{14}C in the combined column wash fractions. Methylene chloride extracts were concentrated and chromatographed with HPLC using a Zorbax-Sil column as reported by Sonobe et al. (1981b).

TLC and Radioautography. The identities of carbofuran residues were confirmed by TLC, although no

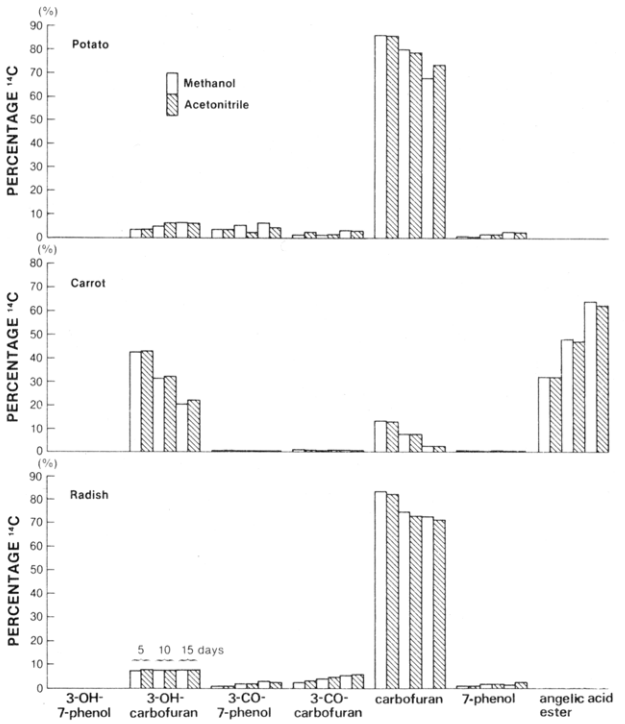

Figure 9. Carbofuran residues present in extract F. The ordinate indicates percentage of ^{14}C of each compound compared with the total ^{14}C in extract F (100%). The angelic acid ester of 3-hydroxycarbofuran was quantitated for carrots only (see the text). The sequence of HPLC elution is from left to right.

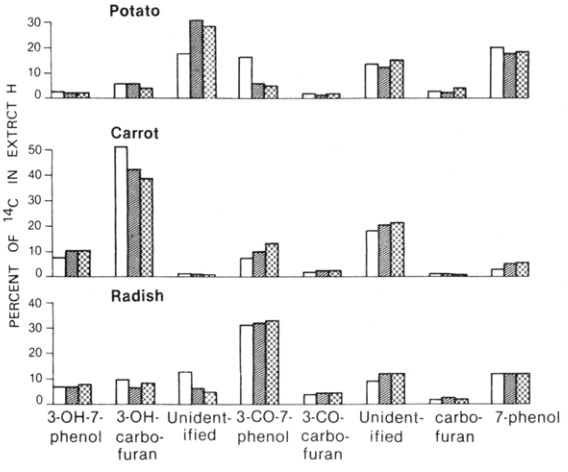

Figure 10. Carbofuran residues present in extract H. The open column indicates ^{14}C in individual compounds for 5-day samples; the shaded column indicates ^{14}C in individual compounds for 10-day samples; the crosshatched column indicates ^{14}C in individual compounds for 15-day samples. The sequence of HPLC elution is from left to right. Two fractions containing one or more unidentified compounds were collected in addition to other carbofuran residues.

quantitation was attempted. Sample extracts and nonlabeled carbofuran standards were spotted on precoated silica gel TLC plates (LKD Type, Kontes, Vineland, NJ). Plates were developed with ethyl ether–*n*-hexane (3:1). Radioactive compounds were detected by radioautography with Kodak No-Screen X-ray film. Exposed films (1–3 weeks) were processed according to the manufacturer's instructions. Standards were visualized by using a 0.1 N methanolic NaOH solution and a freshly prepared solution of 0.1% *p*-nitrobenzenediazonium fluoroborate as reported by Miskus et al. (1961). Compounds were identified by comparison of the R_f values of the standards and ra-

dioautograms of sample extracts.

RESULTS

The data presented in Table I and Figures 2–8 are shown as percentages of the calculated total ^{14}C present in each crop composite as described in the previous section. The data in Figures 5, 9, and 10 are shown as percentages of the calculated total ^{14}C present in particular extracts or combinations of extracts. Carbofuran residues, both extractable and unextractable, in sample composites of the three root crops were calculated as carbofuran and ranged from 1.6 to 7.2 ppm.

Extraction of [^{14}C]Carbofuran Residues by Blending, Washing, and Leaching (Steps 1–4). Figure 2 shows the extraction efficiencies of methanol and acetonitrile for [^{14}C]carbofuran residues in potatoes. The extraction efficiencies are shown cumulatively from steps 1–4. More ^{14}C was collected in a filtrate obtained by blending with acetonitrile than with methanol for the 5-, 10-, and 15-day samples. This difference was statistically significant (Figure 2). Often larger volumes of filtrates were collected with acetonitrile than with methanol. Less of the acetonitrile extract was apparently retained with the crop marc than the methanol extract. However, it was not determined whether the difference in the total ^{14}C in extract A comparing methanol and acetonitrile was simply due to the difference in the volume of solvents collected. Additional ^{14}C was extracted in the washing and leaching procedure, which resulted in a relatively equal extraction efficiency for both methanol and acetonitrile when the cumulative ^{14}C is compared. The extraction efficiency for methanol in steps 1–4 was 86.8, 81.2, and 85.4% for the 5-, 10-, and 15-day samples, respectively. The extraction efficiency for acetonitrile in steps 1–4 was 85.5, 81.5, and 84.6% for the 5-, 10-, and 15-day samples, respectively. The ratio of extractable ^{14}C did not decrease significantly with longer incorporation periods. The percentage of extractable ^{14}C was smallest for the 10-day sample.

The results of the carrot study are shown in Figure 3. Acetonitrile extracted more ^{14}C for the 5-day postapplication sample compared to methanol, but no difference was observed between solvents for the 10- and 15-day postapplication samples in the blending filtrate (step 1). Methanol was the better solvent in the washing and leaching procedures (steps 2–4); it extracted more ^{14}C than acetonitrile. This difference between solvents in steps 2–4 was statistically significant (two-tailed t test at the 5% confidence level). Approximately 96.1, 96.1, and 95.8% of the total ^{14}C in the sample composites were extracted by methanol in steps 1–4 for 5-, 10-, and 15-day samples, while 92.4, 91.5, and 90.7% were extracted with acetonitrile for 5-, 10-, and 15-day samples, respectively. No difference in the levels of extractable ^{14}C was observed with the various incorporation times. The percentage of extractable ^{14}C was highest for carrots among the three root crops examined.

The results for radishes are shown in Figure 4. The difference in extraction efficiency between methanol and acetonitrile was similar to that observed for potatoes. With the 5- and 10-day radish samples, acetonitrile extracted more ^{14}C than methanol in step 1. When washing and leaching were included, both solvents were equal in their ability to extract [^{14}C]carbofuran residues for the 5-, 10-, and 15-day samples. The extractable ^{14}C decreased significantly with longer incorporation time. This trend was not observed with potatoes and carrots. For the 5-, 10-, and 15-day samples, 65.7, 46.0, and 37.7% of the total ^{14}C, respectively, could be extracted by methanol in steps 1–4. The extraction efficiency for acetonitrile in steps 1–4 was 67.3, 46.9, and 36.7% for the 5-, 10-, and 15-day samples, respectively.

Generally, larger volumes were obtained in extract A (step 1) with acetonitrile than with methanol. The crop marc apparently retained more of the methanol extractant. The ^{14}C associated with the methanol retained in the crop marc was eventually washed and/or leached and collected. The acetonitrile, however, could not wash the remaining ^{14}C from the crop marc as effectively. This may explain why acetonitrile gave extraction efficiencies higher than or equal to methanol for step 1 but extraction efficiencies equal to or lower than methanol for the combined steps 1–4.

Methylene Chloride Extractable and Water-Soluble [^{14}C]Carbofuran Residues in Extracts A + B + C + D. To determine the quantity of organic solvent-extractable and water-soluble ^{14}C residues, extracts A–D were combined, evaporated, and partitioned with methylene chloride. The results of partitioning are shown in Figure 5. The total ^{14}C in the combined extracts (A + B + C + D) was considered to be 100%. Potatoes and radishes showed a similar pattern in the ratios of methylene chloride extractable and water-soluble ^{14}C residues. With these crops a significant decrease in methylene chloride extractable ^{14}C was observed with longer incorporation periods. With potatoes, methanol extracted from 81.8% for the 5-day sample to 53.0% for the 15-day sample. With radishes, methanol extracted from 78.6% for the 5-day sample to 37.4% for the 15-day sample. For carrots this trend was much less noticeable but still present (i.e., methanol extracted from 82.3% of the total ^{14}C for the 5-day sample to 73.8% for the 15-day sample). The increase in water-soluble [^{14}C]carbofuran residues, which is due to the formation of more polar metabolites, both conjugated and unconjugated, was especially noticeable for potatoes and radishes.

Nature of Water-Soluble [^{14}C]Carbofuran Residues. The quantity of ^{14}C in extract H (methylene chloride extract after acid hydrolysis) and solution I (aqueous layer) is shown in Figure 6. The amount of ^{14}C in extract F is also shown for comparison. The total ^{14}C in the sample composite was considered to be 100%. Each solution G contained a significant quantity of [^{14}C]carbofuran residues, conjugated or unconjugated, which became extractable in methylene chloride after acid hydrolysis. A significant increase in ^{14}C in extract H was observed in potatoes with longer incorporation periods (with methanol extractions, the ^{14}C in extract H increased from 8.4% for the 5-day sample to 25.3% for the 15-day sample). This trend was less noticeable with methanol-extracted radishes (from 8.8% for the 5-day sample to 15.4% for the 15-day sample) and much less noticeable with methanol-extracted carrots (from 12.9% for the 5-day sample to 19.4% for the 15-day sample). When the total ^{14}C in solution G is considered as 100%, 53–63% of this ^{14}C became extractable into methylene chloride after acid hydrolysis with potatoes, 74–78% with carrots, and 59–66% with radishes. Solution G of carrots contains a higher percentage of conjugated or unconjugated residues, which were water-soluble before acid hydrolysis.

Quantitation of Carbofuran Residues in Extract F. Figure 9 shows the result of the quantitation of carbofuran residues in extract F, determined by using HPLC fractionation. The total ^{14}C in individual extracts F is considered to be 100%. The residues were 3-hydroxy-7-phenol, 3-hydroxycarbofuran, 3-ketocarbofuran, 3-keto-7-phenol, carbofuran, 7-phenol, and the angelic acid ester of 3-hydroxycarbofuran (Sonobe et al., 1981b).

The angelic acid ester elutes from the HPLC column after the 7-phenol during the 100% methanol column wash. The ^{14}C which elutes with this fraction was less than 8.0% of the total ^{14}C present in extract F for potatoes and radishes. The small amounts of unidentified residues present in potatoes and radishes were not characterized and may contain the angelic acid ester of 3-hydroxycarbofuran. With potatoes and radishes, carbofuran was the major residue, and the percentage of carbofuran in extract F decreased with longer incorporation periods. A completely different pattern of carbofuran residues was obtained with carrots; the angelic acid ester of 3-hydroxycarbofuran, 3-hydroxycarbofuran, and carbofuran were the major residues. The percentage of the angelic acid ester of 3-hydroxycarbofuran increased and 3-hydroxycarbofuran and carbofuran decreased in extract F with longer incorporation periods.

The ratio of the angelic acid ester of 3-hydroxycarbofuran to the total ^{14}C in extract F in carrots increased with an increase in the incorporation time. The relative levels of this metabolite vs. the total ^{14}C in the sample composite were calculated by multiplying the percentage of this metabolite shown in Figure 9 by the percentage of the total ^{14}C in extract F of the corresponding sample. With methanol extraction, the angelic acid ester of 3-hydroxycarbofuran represents 29.0, 40.0, and 51.9% of the total ^{14}C in the sample composite for 5-, 10-, and 15-day samples, respectively. These percentages are representative of the absolute levels of the metabolite since the total ^{14}C per gram of sample composite was not significantly different among the 5-, 10-, and 15-day samples, ranging from 1.73×10^5 to 1.83×10^5 dpm. Therefore, the absolute level of the angelic acid ester of 3-hydroxycarbofuran increased in carrots with an increase in incorporation time. The absolute level of both 3-hydroxycarbofuran and carbofuran decreased with increasing incorporation time. Thus, 3-hydroxycarbofuran represents 33.3, 23.1, and 14.2% of the total ^{14}C in the sample composite and carbofuran represents 10.8, 5.77, and 1.93% of the total ^{14}C with methanol extraction for 5-, 10-, and 15-day samples, respectively.

Characterization of Carbofuran Residues in Extract H. Carbofuran residues in extract H were quantitated in the same manner as for extract F. The results are shown in Figure 10. The ^{14}C that elutes after 7-phenol was negligible with all crop samples. The total ^{14}C in methylene chloride extract H was considered as 100%. Only extract H from methanol-extracted samples was used for this study because no significant difference was observed in the total ^{14}C extracted by using methanol or acetonitrile, and as a result, the carbofuran residues present in extract H of the same crop should not be significantly different. The characteristics of the conjugated residues of carbofuran in each root crop were different. An unidentified compound (a single spot was observed with TLC of this fraction) was the major residue in potatoes, 3-hydroxycarbofuran was the major residue in carrots, and 3-keto-7-phenol was the major residue in radishes. These results were confirmed by TLC radioautography. The level of 3-hydroxycarbofuran in carrots decreased with longer incorporation time.

Soxhlet Extraction of Crop Marc E. In this study Soxhlet extraction was used to extract the ^{14}C remaining in the crop marc after leaching. The quantity of ^{14}C thus extracted should indicate how efficiently the previous extraction procedure removed [^{14}C]carbofuran residues. The results are shown in Figure 7. The total ^{14}C in the sample prior to step 1 is considered as 100%. With potatoes, Soxhlet extraction removed only a fraction of the ^{14}C not removed by either solvent (ca. 5% of the total ^{14}C in the sample composite). Less than 0.65% of the ^{14}C present in the sample prior to step 1 could be Soxhlet-extracted from the crop marc of carrots after the sample had been extracted with methanol. Soxhlet extraction removed an additional 5% of total ^{14}C from the crop marc of carrots previously extracted with acetonitrile. Thus, methanol attained a high extraction efficiency relative to that of acetonitrile for carrots. With radishes, very little additional (maximum 2%) ^{14}C was removed by Soxhlet extraction from the crop marc remaining after extraction with either solvent. As a result, both solvents have an equally high extraction efficiency for carbofuran residues in radishes for combined steps 1–4.

Quantitation of Bound Carbofuran Residues; Acid Hydrolysis of Bound Residues. According to Dorough (1976), residues of pesticides remaining in the crop marc after exhaustive solvent extraction are considered to be bound. The quantity of bound [^{14}C]carbofuran residues was determined by combustion of crop marc K followed by counting of ^{14}C. The results are shown in Figure 8. The results show that, of the three root crops examined, only radishes produced a significant quantity of bound residues, which increased with increasing incorporation time (with methanol extraction 34.1% for the 5-day sample, 51.8% for the 10-day sample, and 62.1% for the 15-day sample). Bound [^{14}C]carbofuran residues in potatoes were at a maximum for the 10-day sample (13.5% for methanol extraction). The smallest quantity of bound residues was found in carrots (less than 4.3%) with no difference observed in samples from the three incorporation periods.

So that the nature of bound [^{14}C]carbofuran residues could be investigated, an attempt was made to release the ^{14}C by hydrolysis with 0.25 N HCl. The levels of ^{14}C in extract L are shown in Table I. With the radish crop marc, in which a significant quantity of bound residues was found, 6.61–9.95% of the total ^{14}C residue was solubilized. The ratio of solubilized ^{14}C to bound residues decreased with an increase in incorporation time (from 24.5 to 13.0%), suggesting a change in the nature of the bound residues with time. With potatoes and carrots, the nature of the bound residues appears to be different from that of radishes since more ^{14}C in the bound residues was solubilized (31.1–53.7%), although the absolute quantity of bound residue was small.

The hydrolysate was extracted with methylene chloride, which should extract all of the previously reported nonconjugated carbofuran residues. The maximum quantity of ^{14}C found in the methylene chloride layer was 1.37% of the total ^{14}C in the sample composite (15-day postapplication sample of radishes with methanol extraction). The levels of these residues were especially low for potatoes ($\leq$0.70%) and carrots ($\leq$0.26%). When TLC of the methylene chloride layer (extract N) was performed, some carbofuran metabolites were detected by TLC radioautography. Identification of these compounds was not pursued because of the low levels present.

DISCUSSION

The data from this study show that methanol is approximately equal to acetonitrile in its ability to extract total [^{14}C]carbofuran residues from potatoes and radishes by using the combined blend–wash–leach procedures (steps 1–4). However, methanol extracts from 2.6 to 5.3% more of these residues from carrots by using the same procedures. Methanol is apparently more versatile than acetonitrile since methanol has also been found to be a better extractant than acetonitrile in extracting [^{14}C]phorate residues from root crops (Sonobe et al., 1982). An ex-

traction procedure involving steps 1–3 would be practical and sufficient for the extraction of carbofuran residues. The total ^{14}C extracted by step 4 (leaching) is insignificant (the maximum ^{14}C extracted by leaching was 3.88% for potatoes, 1.06% for carrots, and 0.55% for radishes of the total ^{14}C in the sample). These extraction steps are simple, rapid, and applicable as a multiple residue extraction procedure.

Soxhlet extraction of the crop marcs provides additional evidence that methanol is equal to or more efficient than acetonitrile in extracting [^{14}C]carbofuran residues from these crops. In every case, an equal or greater amount of ^{14}C was obtained by Soxhlet extraction of crop marcs previously extracted with acetonitrile than was obtained from those previously extracted with methanol. A similar result was also observed in a study involving [^{14}C]phorate residues in the same crops (Sonobe et al., 1982).

Most of the crop moisture was removed in the initial filtration of the crop–solvent homogenate. Burke et al. (1971) reported that 35% water in acetonitrile was the optimum solvent mixture for extracting pesticide residues from low moisture crops. This mixed solvent may be more effective than acetonitrile alone in releasing additional [^{14}C]carbofuran residues following the initial filtration of the blended sample–solvent mixture.

For the extraction of carbofuran metabolites conjugated as glycosides, acid digestion of the intact, composited crop sample was first developed by Cook et al. (1969), and this method has been widely applied to various types of crops (Van Middelem et al., 1971; Williams and Brown, 1973; Nelsen and Cook, 1980). Our results show that hydrolysis of the crop marc with hydrochloric acid following solvent extraction is not necessary for the extraction of carbofuran residues since negligible quantities of methylene chloride partitionable carbofuran residues are found. Conjugated residues of carbofuran were sufficiently extracted with methanol; the quantities of carbofuran residues extracted with the Soxhlet extraction procedure following blending, washing, and leaching were small. Acid digestion of crop material is a time-consuming procedure. Problems such as foaming during digestion and difficulty in filtration must be overcome. With the samples used in this study, methanol extracted polar carbofuran metabolites, conjugated and nonconjugated, and the quantity of carbofuran residues extracted with methylene chloride after acid digestion of the previously solvent-extracted crop marc was negligible.

Aqueous extracts (solution G) must be hydrolyzed with 0.25 N HCl since they contain significant quantities of conjugated carbofuran residues, and an analytical procedure is currently not available for the direct determination of these residues. 3-Hydroxycarbofuran, which may be released from the conjugates in carrots, is the most toxic carbofuran metabolite to form a conjugate. Therefore, a method that can separately determine conjugated hydroxycarbofuran residues should be used. Acid digestion of aqueous extracts is a simple procedure.

Metabolism, and thus toxicity, of conjugated residues may differ from that of unconjugated residues in humans and other species. Therefore, a procedure that allows separate determination of conjugated and unconjugated residues should be used, if possible, when determining toxic components in foods. Previously developed methods for carbofuran residues involving hydrolysis of the unextracted crop material do not permit the separate determination of conjugated and unconjugated residues, only total residues. A procedure for carbofuran residues involving methanol extraction, followed by evaporation and partitioning with methylene chloride, would allow separation of the polar (mostly conjugated) residues found in the aqueous phase and nonpolar (mostly unconjugated) residues found in the methylene chloride phase. Subsequent hydrolysis of the aqueous phases followed by methylene chloride partitioning would permit the determination of the polar (mostly conjugated) residues; however, even in this scheme all conjugated residues are not separated from the unconjugated ones and vice versa. For example, the angelic acid ester conjugate of 3-hydroxycarbofuran is relatively nonpolar and remains with the methylene chloride layer prior to the acid hydrolysis step.

The major residue in carrots, the angelic acid ester of 3-hydroxycarbofuran, is a conjugated residue with chemical properties different from those of the known glycosidic conjugates. It is not water soluble but is organic solvent soluble (methanol, acetonitrile, methylene chloride). It can be chromatographed by appropriate HPLC systems and it can be determined without hydrolysis to obtain an aglycon. The results of quantitation of carbofuran residues in carrots indicate that the level of the angelic acid ester of 3-hydroxycarbofuran increased, while the levels of 3-hydroxycarbofuran and carbofuran decreased with increasing incorporation time, strongly suggesting that the metabolic pathway is from carbofuran through 3-hydroxycarbofuran.

The level of the angelic acid ester of 3-hydroxycarbofuran increases with incorporation time in carrots. Carbofuran residues apparently accumulate and are stored in carrots as the angelic acid ester. The half-life of this compound in carrots appears to be significantly longer than the half-life of the other carbofuran residues. Of the three crops examined, this residue is observed only in carrots. The toxicity of this compound is unknown and may be appropriate for testing.

The [^{14}C]carbofuran residues in carrots were more efficiently extracted than those in potatoes and radishes, and the lowest levels of bound residues were in carrots. This same trend was observed with [^{14}C]phorate residues in root crops (Sonobe et al., 1982). The cause for this is unknown. Of the three root crops examined, only radishes had a significant quantity of bound [^{14}C]carbofuran residues with root incorporation. This was also observed with foliar application by Wheeler and Thompson (1977). Bound residues in radishes became more diffucult to free by acid hydrolysis with increasing incorporation time, suggesting that extensive and rapid metabolism of carbofuran had occurred.

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Registry No. 3-Keto-7-phenol, 17781-16-7; carbofuran, 1563-66-2; 3-hydroxycarbofuran, 16655-82-6; angelic acid ester of 3-hydroxycarbofuran, 79189-81-4; methanol, 67-56-1; acetonitrile, 75-05-8.

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Acute Toxicity, Bioconcentration, and Persistence of AC 222,705, Benthiocarb, Chlorpyrifos, Fenvalerate, Methyl Parathion, and Permethrin in the Estuarine Environment

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Six pesticides were evaluated in laboratory studies to determine acute (96-h) toxicity, octanol–water partition coefficient (log *P*), solubility, and persistence in seawater. In addition, three of the six pesticides (synthetic pyrethroids) were tested by using the eastern oyster (*Crassostrea virginica*) in long-term (28-day) tests to determine their respective bioconcentration factors (BCF). Acute toxicity tests provided the following decreasing order of toxicity to estuarine crustaceans and fishes: AC 222,705, fenvalerate, permethrin, chlorpyrifos, methyl parathion, and benthiocarb. The estuarine mysid (*Mysidopsis bahia*) was consistently the most sensitive species, with LC_{50} values as low as 0.008 μg/L. The sheepshead minnow (*Cyprinodon variegatus*) was generally the least sensitive (range of LC_{50} values = 1.1–1370 μg/L). log *P* values were inversely related to solubility in seawater. The following are the increasing order of log *P* values (range 1.8–6.5) and decreasing order of solubility (range >1000–24 μg/L): methyl parathion, benthiocarb, chlorpyrifos, AC 222,705, fenvalerate, and permethrin. Pesticide half-lives in sediment–water studies ranged from 1.2 to 34 days and were in the following order of increasing persistence: methyl parathion, permethrin, benthiocarb, AC 222,705, chlorpyrifos, and fenvalerate. The steady-state BCF's of the three synthetic pyrethroids were 1900 for permethrin, 2300 for AC 222,705 and 4700 for fenvalerate. After termination of the exposure, each insecticide was depurated by oysters to nondetectable concentrations within 1 week.

The manufacture and use of organochlorine pesticides in the United States have decreased in the last decade, in part due to their adverse effects on fish and wildlife and the tendency of these chemicals to bioconcentrate. Replacement of these pesticides in the agricultural industry fell initially on the organophosphate insecticides and, more recently, the synthetic pyrethroid insecticides. Chlorpyrifos [*O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate] and methyl parathion [*O,O*-dimethyl *O*-(4-nitrophenyl) phosphorothioate] (Figure 1) are organophosphate insecticides that have been in use for many years. Permethrin [3-phenoxybenzyl (±)-*cis,trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate], fenvalerate [(*RS*)-α-cyano-3-phenoxybenzyl (*RS*)-2-(4-chlorophenyl)-3-methylbutyrate], and AC 222,705 [(*RS*)-α-cyano-3-phenoxybenzyl (*RS*)-2-[4-(difluoromethoxy)phenyl]-3-methylbutyrate] (Figure 1) are synthetic pyrethroid insecticides that were introduced during the 1970s (Miester, 1980) and are in wide use throughout western Europe and Japan. The registration of AC 222,705 (Payoff), permethrin, and fenvalerate by the U.S. Environmental Protection Agency (EPA) is limited essentially to cotton application. the herbicide benthiocarb [*S*-(4-chlorobenzyl) diethylthiocarbamate] (Figure 1) is now registered by EPA for use in rice fields to control weed growth.

Evaluation of the relative hazards of these chemicals to aquatic environments requires that information on toxicity, accumulation potential, and expected environmental concentrations be compared. We therefore initated a series of studies on these six pesticides to determine (1) the acute

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