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Extraction Efficiencies for Pesticides in Crops. 1. [14C]Carbaryl Extraction from Mustard Greens and Radishes

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^{14}C-labeled carbaryl (1-naphthyl *N*-methylcarbamate) suspended in a commercial carbaryl formulation was sprayed on mustard greens and radishes. At three intervals, postapplication, the crops were extracted using methanol, acetonitrile, or acetone. Crops were either blended and leached or repetitively blended followed by Soxhlet extraction. Essentially all of the extractable radioactivity was removed by blending. The ^{14}C was more difficult to extract from radishes than from mustard greens and with increasing time after application. For mustard greens 92, 83, and 77% of the ^{14}C at harvest was extractable at 3, 7, and 14 days, respectively; for radishes 91, 76, and 58% was extractable at the same intervals. Methanol was generally the best solvent and the blend–Soxhlet process was superior to the blend–leach process. Thin-layer chromatography of the organic soluble extracts indicated that the majority of ^{14}C was carbaryl. Acid hydrolysis of the extracted tissues released 40–50% of the residual ^{14}C.

Quantitive data for many pesticide residue analytical extraction methods consist primarily of determinations made on representative sample types fortified in the laboratory with the compounds of interest. Such studies provide data on recovery of the pesticide through the various manipulations of the method, but fail to provide the equally essential information of the ability of the extraction step of a method to remove "field-incurred" residues from the sample. This problem is well understood by pesticide analysts but relatively little work has been reported to provide an estimate of the magnitude of the problem or solutions to it.

One of the earliest reports was that by Klein et al. (1959) who reported a nonextractable residue of radioactivity remaining in spinach after it was sprayed with labeled methoxychlor [2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane].

Mumma et al. (1966), Wheeler et al. (1967), and Wheeler and Frear (1966) reported that root absorbed and translocated [^{14}C]dieldrin [1,2,3,4,10,10-hexachloro-*exo*-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo*,*exo*-5,8-dimethanonaphthalene] was not efficiently extracted by blending with *n*-hexane–isopropyl alcohol (2:1, v/v). A subsequent exhaustive extraction using chloroform–methanol (1:1, v/v) in a Soxhlet extractor recovered 20 to 40% of the total dieldrin residue. Several subsequent reports (Burke and Porter, 1966; Burke et al., 1971; and Caro, 1971) compared various extraction systems to the exhaustive extraction procedure of Mumma et al. (1966).

Bowman et al. (1968) evaluated nine procedures for the extraction of six organophosphate insecticides and their metabolites from field-treated crops. They detected the highest total residues when the samples were extracted by using 10% methanol in chloroform in a Soxhlet extractor.

Very little work has been reported on the evaluation of the efficiency of extraction of carbamate insecticides. Watts (1971) applied [^{14}C]carbaryl [1-naphthyl *N*-methylcarbamate] to bean larves and was able to extract 100% of the applied radioactivity 48 h later.

Van Middelem and Peplow (1973) studied the extraction of [^{14}C]carbofuran [2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate] from cabbage after soil application of the pesticide. At postapplication intervals up to 35 days, 90% of the ^{14}C was extractable by acid digestion, Soxhlet extraction using methanol, or by blending in methanol.

Although a number of investigators agree that one of the most effective means to evaluate extraction efficiency is

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through the use of radioactive pesticides, this procedure is not often used. In order to more closely approximate field conditions, pesticides should be applied as a formulation rather than in purified form dissolved in a pure solvent.

This is the first of a series of reports describing the extraction of ^{14}C pesticide applied to plants in a commercial formulation of the same pesticide. The amounts of ^{14}C that can be extracted and the amounts that cannot be extracted are measured. This paper describes the extraction efficiency of blend–leach and blend–Soxhlet extraction procedures using methanol, acetone, and acetonitrile for mustard greens and radishes treated with carbaryl.

MATERIALS AND METHODS

Plant Growth. Mustard greens and radishes were grown from seed in a Florida soil (low organic matter, low clay content) held in 15-cm Styrofoam pots. Generally the plants were started and grown outdoors until approximately 1 week prior to maturity. The plants were then moved into growth chambers [Scherer-Gillet Model (CEL 512-37)]. Day and night periods were 14 h at 32 °C and 10 h at 27 °C, respectively. When space allowed, plants were started and kept in growth chambers throughout the growth process. Water, fertilizer, and pest control measures were applied as needed.

Carbaryl Application. Aromatic ring ^{14}C-labeled carbaryl was custom synthesized by New England Nuclear Corporation. It was shown to be 98+% pure by thin-layer chromatography (TLC). For application to plants, approximately 500 μCi was diluted with Sevin No. 5 Aqua (64%) (Miller Chemical and Fertilizer Corporation) and the appropriate amount was sprayed using an aerosol can powered device similar to those used for spraying chromogenic reagents on TLC plates. The sprayer delivery rate (mL/min) was determined experimentally, and the volume of pesticide necessary to provide an application rate of 2.2 kg of active ingredient/ha was applied. For radishes, the root tops and surrounding soil were sprayed; for mustard greens the foliage was sprayed.

Plants were treated in chambers that were similar to bacteriological hoods. They were completely enclosed by polyethylene sheets and were equipped with glove ports on one side. After the spraying was completed, the vapors were allowed to settle on the plant material for 30 min. A vacuum pump was then attached to a port and air was pulled through the spraying chamber for 15 min prior to opening the chamber. Plants were then transferred to the growth chambers to await harvest. The polyethylene sheets were discarded after each spraying operation.

Harvest. Crops were harvested 3, 7, and 14 days postapplication. Radishes were pulled from the soil and the tops were cut off and discarded. The roots were rinsed with water to remove adhering soil. Mustard greens were cut approximately 2 cm above the soil surface.

Chopping and Subsampling. At each harvest interval, approximately 2000 g of crop was required. The crop was placed in a Hobart Chopper (Model 84141, Hobart Mfg. Co., Troy, Ohio) and the sample was chopped and mixed for 10 min). Samples (100 g each) were weighed into each of 18-tared 1-qt Mason jars.

Extraction Procedures. Two extraction procedures were used, each using nine samples.

1. Blend–Leach. Three samples were extracted with acetone, three with acetonitrile, and three with methanol. Each extraction was performed as follows: Two hundred milliliters of solvent was added to the 100 g of crop; the sample was blended for 0.5 min at 3000 rpm and then for 2.0 min at 7000 rpm (all blender speeds were determined by using a calibrated reflecting tachometer, Power Instruments, Inc., Skokie, Ill., Model B-891) in a Lourdes Model VM blender. The blended sample was then suction-filtered through Whatman No. 1 filter paper held in a 600-mL glass, coarse fritted filter funnel. The blender blades were rinsed with 50 mL of solvent; the blender jar and filter cake were rinsed with three additional 50-mL portions of solvent. After the filtration was complete, the tissue residue and the filter paper were transferred to a leaching column (2.5 × 20 cm leaching portion plus a 12 × 6.5 cm solvent reservoir), and the same solvent used in blending was added to the column. One 500-mL portion was collected at a leaching rate of approximately 5 mL/min (the rate with methanol was much slower, ranging from 20 to 30% of the rate of the other solvents). The blended and leached tissues were allowed to dry in the leaching column, then transferred to tared beakers and dried at 45 °C to a constant weight.

2. Blend–Soxhlet. Extraction was performed as described above up to the transfer of tissue residue to the leaching column. For this procedure, the tissue residue was scraped from the filter paper and back into the original blender jar; 300 mL of solvent was added to the blender jar and the mixture was blended for 2.5 min at 3000 rpm. This process was repeated until a total of four blend extracts were collected. The extracted tissue residues and the filter paper were placed into glass extraction thimbles and extracted for 16 h in a Soxhlet extractor at a rate of five to six cycles per hour. The "methanol" and "acetone" samples were extracted with 250-mL portions of methanol and acetone, respectively. Chloroform–methanol (9:1 v/v) was used to extract the "acetonitrile" samples. The extracted tissues were transferred to tared beakers and dried to constant weight at 45 °C.

After a small portion of the "blend–Soxhlet" tissue residue had been taken to determine the ^{14}C residue value, the remaining portion of that sample was placed in a 500-mL boiling flask. Fifteen drops of Tween 20 (to reduce foaming) and 200 mL of 0.25 N HCl were added, and the mixture was refluxed for 1 h. After refluxing, the solution was filtered through a Buchner funnel containing filter paper and Hyflo Supercel. The extract was diluted to a volume of 250 mL and the radioactivity in a 1-mL aliquot was determined.

Analysis of Extracts. Each extract and tissue residue was analyzed in duplicate for radioactivity. All samples were combusted in a Teledyne Interchnique IN4101 liquid scintillation sample oxidizer.

To prepare the samples for combustion, aliquots of the extracts were pipeted into 3 in. × 3 in. squares of Cellophane (Carolina Biological Supply "Cello-Flex") held in small beakers and allowed to evaporate to dryness. The Cellophane squares were then folded and placed into polycarbonate capsules designed for the sample oxidizer. Recovery of [^{14}C]carbaryl through the evaporation and combustion steps averaged 94%.

In the case of tissue residue, small portions were weighed and combusted.

The sample oxidizer automatically adds a pre-mixed scintillation cocktail (phenethylamine–methanol–toluene–water–Permafluor, 33:22:36:5:4). Each sample was then counted in a Packard Instrument Company, Model 3375 Liquid Scintillation Spectrometer. The following instrumental settings were used: windows, 50–1000; 25.0% amplification; present time 10 min; present count 2000. All sample counts were corrected for background counts. No corrections were made for quenching effects because

Table I. Percentage ^{14}C Extracted from Mustard Greens by the Blend-Leach Procedure

		% ^{14}C		
	fraction	meth-anol	acetone	aceto-nitrile
harvest 1	blend	88.2	87.2	82.3
	leach	4.7	5.4	4.8
	residual	6.7	7.3	12.5
harvest 2	blend	80.2	76.5	77.5
	leach	4.0	3.2	2.0
	residual	15.5	19.7	20.0
harvest 3	blend	67.8	70.6	61.6
	leach	10.0	5.3	6.9
	residual	21.8	23.4	31.2

Table II. Percentage ^{14}C Extracted from Mustard Greens by the Blend-Soxhlet Procedure

		% ^{14}C		
	fraction	meth-anol	acetone	aceto-nitrile
harvest 1	blend 1	85.0	87.8	84.0
	blend 2	9.0	4.1	4.3
	blend 3	1.0	0.5	0.6
	blend 4	0.2	0.2	0.4
	Soxhlet	0.1	0.9	2.6
	residual	7.0	6.4	7.8
harvest 2	blend 1	77.6	80.8	74.1
	blend 2	3.8	2.6	5.3
	blend 3	0.7	0.4	0.8
	blend 4	0.3	0.2	0.3
	Soxhlet	0.8	0.5	3.2
	residual	16.4	15.1	15.7
harvest 3	blend 1	72.4	67.0	69.4
	blend 2	6.9	7.8	5.2
	blend 3	1.1	0.9	0.8
	blend 4	0.4	0.4	0.5
	Soxhlet	0.6	1.9	3.7
	residual	18.2	23.7	19.6

the automatic external standard ratios were similar for all samples. The total radioactivity in each fraction was determined by multiplying the average quantity of radioactivity in each aliquot by the dilution factor.

Thin-Layer Chromatography Analysis of Extracts for Carbaryl and Metabolites. A portion of the first blend extracts were streaked on silica gel TLC plates. After applying authentic standards as reference materials (obtained from the Environmental Protection Agency, Washington, D.C.), the plate was developed in acetone–benzene (1:4, v/v) or in other solvent systems described by Locke (1972). After the chromatogram was developed, the TLC plate was placed against X-ray film (Kodak No-Screen) to locate radioactive zones, the film was developed, and the radioactive areas were detected. R_f values were then measured, radioactive zones were scraped, and the radioactivity in each zone was determined.

Statistics. An analysis of variance was performed on the "residue" term of the data. The effects of the extracting solvent, crop, harvest interval, and extraction process were evaluated. The following interactions were also tested for statistical significance: extracting solvent by extraction process, crop by harvest interval, crop by extraction process, and harvest interval by extraction process.

RESULTS

Tables I, II, III, and IV present the extraction data in terms of the percentage ^{14}C in each fraction at each harvest interval and for each extracting solvent. Table I describes the blend–leach data for mustard greens. The average percentage ^{14}C extracted by blending at the first harvest interval was approximately 86%. The leaching process

Table III. Percentage ^{14}C Extracted from Radishes by the Blend-Leach Procedure

		% ^{14}C		
	fraction	meth-anol	acetone	aceto-nitrile
harvest 1	blend	87.3	83.8	83.7
	leach	5.5	5.3	3.2
	residual	7.0	10.4	12.8
harvest 2	blend	74.0	68.0	63.2
	leach	6.0	5.4	3.5
	residual	19.5	25.7	32.4
harvest 3	blend	59.6	50.6	41.9
	leach	6.8	2.7	3.9
	residual	37.9	45.3	53.2

Table IV. Percentage ^{14}C Extracted from Radishes by the Blend-Soxhlet Procedure

		% ^{14}C		
	fraction	meth-anol	acetone	aceto-nitrile
harvest 1	blend 1	88.0	83.1	79.4
	blend 2	4.9	6.1	5.5
	blend 3	0.4	0.9	0.6
	blend 4	0.2	0.4	0.4
	Soxhlet	0.3	0.8	2.3
	residual	6.1	8.3	11.5
harvest 2	blend 1	77.2	65.7	69.6
	blend 2	3.3	4.2	1.7
	blend 3	0.7	1.0	0.8
	blend 4	0.5	0.6	0.8
	Soxhlet	0.5	1.6	3.8
	residual	17.3	25.9	22.9
harvest 3	blend 1	63.6	46.7	55.4
	blend 2	4.0	1.7	2.1
	blend 3	1.1	0.2	0.2
	blend 4	0.3	0.3	0.1
	Soxhlet	1.1	1.2	2.5
	residual	29.1	48.6	38.7

recovered another 5% with approximately 9% "residual" or unextractable ^{14}C. The ^{14}C was more efficiently extracted with methanol and acetone than with acetonitrile.

At harvest 2, the percentage ^{14}C in the blend extract was approximately 78%. The leach process recovered approximately 3% and the residue term contained an average of 18%. In this case, methanol more efficiently extracted ^{14}C than the other two solvents.

By harvest 3, the blend process removed approximately 67% of the ^{14}C, the leach extracted some 7% and the unextractable portion averaged 25%. Acetonitrile did not extract as much of the radioactivity as did methanol and acetone.

Table II presents the blend–Soxhlet data for mustard greens. In general, these data are similar to the blend–leach data. The unextractable "residue" being 7, 16, and 20% at harvest 1, 2, and 3, respectively. The sum of ^{14}C in the first and second blend fractions represents essentially all that was extractable. The deviation from this rule was the Soxhlet extract of the acetonitrile samples. Substantially more ^{14}C was extracted with chloroform–methanol in the Soxhlet process than with the other two solvents because less ^{14}C was extracted by blending with acetonitrile than by blending with acetone or methanol. The similarity of the "residual" values supports this interpretation.

The blend–leach data for radishes are presented in Table III. The mean percentage ^{14}C in the blend extract was 85% at harvest 1. The leach fraction contained an average of 4.7% and the residual 10%. Methanol was superior to the other two solvents extracting a total of 93% of ^{14}C present at harvest 1 as compared to 90% for acetone and 87% for acetonitrile. At harvest 2, the overall percentage

Table V. ^{14}C Released by Acid Digestion of Extracted Tissues

extraction solvent	harvest	mean % radioactivity in tissue residue prior to digestion	mean % radioactivity in acid digest
	Radish		
acetone	1	8.3	2.3
	2	25.9	6.8
	3	48.6	40.9
acetonitrile	1	11.4	3.2
	2	22.9	9.5
	3	38.8	39.0
methanol	1	6.1	1.2
	2	17.3	3.2
	3	29.1	25.0
	Mustard Greens		
acetone	1	6.4	3.0
	2	15.1	17.6
	3	23.7	8.3
acetonitrile	1	7.8	2.9
	2	15.7	9.3
	3	19.6	8.9
methanol	1	7.1	1.4
	2	16.3	10.7
	3	18.2	4.5

that was extractable had dropped to 80, 75, and 68% for methanol, acetone, and acetonitrile, respectively. The trend of methanol as the superior solvent continued through harvest 3. The unextractable ^{14}C residues from methanol, acetone, and acetonitrile extractions were 38, 45, and 53%, respectively.

As was seen for mustard greens, the ^{14}C was increasingly difficult to extract with time. This trend was more pronounced in radishes than in mustard greens.

The blend–Soxhlet data for radishes are presented in Table IV. These data are similar to the mustard green data in that after the first two blending fractions, very little additional radioactivity was removed. The radioactivity in the Soxhlet fraction of the acetonitrile-extracted samples was higher than that for the other solvents as was the case with mustard greens, but the differences were less dramatic. By harvest 3, considerable differences between solvents were noted. Methanol was not able to extract 29% of the ^{14}C present at harvest while the residual term for acetone and acetonitrile was 49 and 39%, respectively.

TLC was employed in an effort to identify the ^{14}C that was extractable. For both mustard greens and radishes, the major extractable component detected, representing 50–75% of the radioactivity, was parent carbaryl. Smaller quantities of other metabolic products were also detected. These are most likely hydroxy carbaryl derivatives, based on reported TLC R_f values (Locke, 1972). α-Naphthol was not detected.

In some cases, considerable ^{14}C remained unextractable from plant tissues. In an effort to solubilize this radioactivity, the tissues remaining after Soxhlet extraction were refluxed in 0.25 N HCl for 1 h. Table V presents the results of the acid hydrolysis. While as much as 100% of the residual ^{14}C was solubilized by this treatment, 50 to 75% was more typical for both radishes and mustard greens. For mustard greens, the acidic extracts were partitioned with methylene chloride. Essentially all of the ^{14}C remained in the aqueous phase, indicating that the uncharacterized materials that were released were polar in nature.

The analysis of variance of the data revealed a number of conclusions: On an overall basis, methanol is the best solvent, leaving only 16.9% residual ^{14}C. Acetone left 21.6% and acetonitrile failed to extract 23.5%.

There is a difference between the extractability of carbaryl from radishes and from mustard greens. The overall residual ^{14}C was 25.1% for radishes and 16.1% for mustard greens.

There was also a significant difference in the amount of carbaryl extracted with harvest intervals. The residual ^{14}C was 8.5, 20.5, and 32.6% at the first, second, and third harvests, respectively.

The overall extraction procedure made a difference. The blend–Soxhlet process left 18.8% residual while the blend–leach process left 22.5%.

A solvent by crop interaction occurred. This indicates that the solvents did not behave in a predictable manner between the two crops. Methanol was superior for both crops. Acetone was the poorest solvent for radishes while acetonitrile was poorest for mustard greens.

There was solvent by harvest interaction. Methanol was best at all harvests. Acetone was second best at harvest 1, being superior to acetonitrile. By harvest 3, however, methanol was far better than acetone and acetonitrile, and the latter two solvents were almost indistinguishable in their extraction efficiencies.

A solvent by extraction process interaction was detected. Methanol was best using either the blend–leach or the blend–Soxhlet process. However, for the blend–leach process, acetonitrile was the poorest solvent and in blend–Soxhlet process acetone was least effective in extracting ^{14}C.

There was also a crop-by-harvest interaction. At harvest 1, radishes and mustard greens were extracted with almost equal efficiency (9.3 and 7.7% residual, respectively). By the second harvest the difference between crops had widened (radishes, 24% residual ^{14}C and mustard greens 17%). At the third harvest (14 days after application) the residual ^{14}C was 42.1% for radishes and 23.0% for mustard greens.

DISCUSSION

A practical consideration is how these extraction data relate to the extraction efficiency when applied to actual carbaryl residues in field grown radishes, mustard greens, and other crops. It is clear from these data that not all of the ^{14}C present at harvest, which had been applied as [^{14}C]carbaryl, was extractable. Watts (1971) reported 100% recovery of [^{14}C]carbaryl and Van Middelem and Peplow (1973) reported 90% or greater recovery of [^{14}C]carbofuran. This report shows considerably lower ^{14}C recoveries. The major difference between this work and that of Watts (1971) and Van Middelem and Peplow (1973) is the form in which the pesticide was applied. The other investigators applied the pure compound dissolved in organic solvent whereas we used [^{14}C]carbaryl diluted in a commercial formulation. Thus it appears the formulation has considerable influence on plant–pesticide interactions. To evaluate other possible factors, the application of pure compound vs. pure compound in formulation followed by extraction should be done under comparably controlled conditions. Various formulations might also be compared for their potential to facilitate the formation of bound residues.

The concern about bound residues exists for several reasons. Since they are not extractable by conventional pesticide residue methodology, such residues cannot be quantitatively measured. Furthermore, these bound residues might potentially be released (e.g., through food processing, cooking, human or animal digestive processes, microbial hydrolysis, etc.) and thus become biologically

available. In the case of carbaryl reported here, part of the unextracted ^{14}C was solubilized by acid hydrolysis of the tissues although only a small portion of this solubilized material consisted of carbaryl-related chemicals. Solubilization of bound radioactivity did not always occur with other pesticides in this laboratory. This could mean that the ^{14}C remaining in tissues is no longer structurally related to the parent compound; it may even be incorporated into naturally occurring biological compounds and, therefore, be of no toxicological significance.

Another practical consideration is the actual residue levels detected in this work. In our system, the bound plus extractable ^{14}C represented approximately 6 ppm carbaryl in radishes and approximately 40 ppm in mustard greens at both 3 and 7 days postapplication. The tolerance for carbaryl on radish and mustard green are 5 and 12 ppm, respectively. The average (for all three solvents) unextractable ^{14}C in radishes was 8.6, 22.0, and 38.8% (for the more rigorous blend–Soxhlet process) at 3, 7, and 14 days, respectively, postapplication. If one assumes that all of the ^{14}C present is toxicologically significant, then the routine analysis would reveal above-tolerance carbaryl residues at 3 days and below-tolerance levels at 7 and 14 days when, in fact, the 5 ppm tolerance was exceeded at all three harvest intervals.

The data show that the major proportion of pesticide can be extracted by a single blend; some additional ^{14}C was recovered, however, by the leaching process or by additional blends.

The Soxhlet process was used to be certain that exhaustive extraction had occurred. The chloroform–methanol solvent combination reported to be highly efficient for the extraction of organophosphates (Bowman et al., 1968) was substituted for acetonitrile owing to its proven effectiveness.

From a statistical viewpoint, methanol was the superior extraction solvent. Part of the reason for this may be related to the relatively slow filtration and/or leaching rate of methanol compared to the other solvents. The slower rate allowed for greater contact time between crop and solvent which may have resulted in more pesticide partitioning into the methanol and thus the higher extraction efficiency. If this theory has any basis, then a more thorough rinse of the blended tissues could result in equal extraction efficiencies among all three solvents.

The reasons for radishes being more difficult to extract than mustard greens are obscure. Radish roots are storage organs and might, therefore, have greater potential for "bound" residues. Radishes are perhaps far more metabolically active than are mustard greens. Other work in this laboratory (unpublished) has shown this observation is typical for several other carbamate pesticides. It is not currently known if the age, size, or physiological state of the radish influences extraction efficiency, or if the radish is typical of root crops. This could be verified in experiments with other root crops.

All the data reported here should be considered broadly. The entire potential toxicologically significant carbaryl residue present at harvest is determined by measuring the amount extracted and the amount remaining in the tissue marc. The mechanism of binding, the chemical structure of bound materials, and the potential for them becoming biologically available should be determined.

This report is the first of a series designed to evaluate the effectiveness of the extraction methodology by measuring the ^{14}C that is extractable and that which is "bound". In the future, attempts will be made to characterize the "bound" materials with reference to its potential toxicological significance.

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