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

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[^{14}C]Phorate, 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 postapplication and composited. The extraction efficiency obtained by blending, washing, and leaching with methanol or acetonitrile was determined. The combined methanol extracts contained more ^{14}C than the combined acetonitrile extracts for all crop-time combinations, e.g., methanol 84.2% and acetonitrile 72.2% of the total ^{14}C of the 15-day potato composite. A slight decrease in extractable ^{14}C was observed with potatoes and radishes with longer incorporation periods. More [^{14}C]phorate residues were extractable by methanol from carrots than from potatoes and radishes, e.g., carrots 96.0%, potatoes 84.2%, and radishes 82.8% of the total ^{14}C in the 15-day sample. These extracts were combined, reduced in volume, and shaken with methylene chloride. Of the ^{14}C in the 15-day sample extracts, 95.8, 46.4, and 35.3% were partitioned into methylene chloride for the carrot, potato, and radish samples, respectively. A significant increase in water-soluble ^{14}C was observed with increasing incorporation periods for potatoes and radishes but not for carrots. Phorate sulfone and phorate sulfoxide were the major residues in the methylene chloride extracts of potatoes and radishes. In carrots, phorate sulfoxide, phorate sulfone, and phorate were the major residues.

Analytical methods used for the determination of pesticide residues in food commodities must be supported by sufficient evaluation of any procedure used. The efficiency of extraction of pesticide residues from sample matrices is not always separately evaluated even though it is one of the most important parts of a method. Recovery studies using samples fortified with pesticides at the time of analysis only evaluate analytical procedures after the extraction of pesticide residues. Sample fortification does not give a true picture of the method's ability to quantitatively determine incurred residues.

Although several reports have been published on the evaluation of extraction procedures for organophosphorus pesticides, there is still a need for the development of extraction procedures that would be generally applicable for use in future multiresidue method development. An extraction procedure can be evaluated only if the chemical form and quantity of the total residues in the sample are determined. Samples in which radioactively labeled pesticides are biologically incorporated can be used to clearly demonstrate the efficiency of an extraction procedure.

Bowman et al. (1968) determined the ability of benzene, chloroform, and/or 10% methanol in chloroform to remove residues of several organophosphorus pesticides and some of their metabolites from grass or corn by blending and/or Soxhlet extraction. They concluded that Soxhlet extraction of the finely chopped crop with 10% methanol in chloroform was the most reliable and efficient means of extracting these residues. Burke et al. (1971) evaluated the efficiency of blending with acetonitrile compared to that of Soxhlet extraction with chloroform–methanol (1:1) in extracting parathion and diazinon from kale. They reported that blending and extracting with acetonitrile gave efficiencies of 92–108% of those obtained by exhaustive Soxhlet extraction. Watts (1971) studied the efficiencies of extraction of ^{14}C-labeled malathion and phosphamidon from laboratory-grown and treated bean plants. He found that blending with ethyl acetate or acetonitrile and exhaustive Soxhlet extraction with 10% methanol in chloroform were all equally efficient (more than 92% extraction of total ^{14}C) in removing these organophosphorus pesticides. White et al. (1973) examined the efficiency of blending with acetonitrile to extract ring-^{14}C-labeled parathion residues from bean plants. They reported that a Polytron blending–extraction and a Soxhlet extraction were equally efficient. Saha et al. (1973) compared three extraction and cleanup procedures for determining ^{14}C-labeled fonofos residues from wheat plants. They reported that blending with ethyl acetate was the best procedure for the extraction of fonofos residues, yielding 71% of the [^{14}C]fonofos residues in the crop.

In all of the above reports that used radioactively labeled pesticides, total ^{14}C was used as the only indicator of extraction efficiency. To date, there has been no examination of both total residues and individual metabolites directed toward the establishment of a convenient, relatively rapid extraction procedure that is applicable to the multiresidue determination of organophosphorus pesticides.

The study reported here examines some extraction procedures for use in multiple organophosphorus pesticide residue analysis of food commodities. Phorate (Thimet), *O,O*-diethyl *S*-[(ethylthio)methyl]phosphorodithioate, was chosen for this study because its extraction efficiency from crops has not been reported and it is a widely used organophosphorus pesticide. Information on the efficiency of phorate extraction (parent and metabolites) may provide some idea about the efficiency of extraction of other organophosphorus pesticides and/or their metabolites, such as demephion, fenthion, demeton, disulfoton, dasanit, and fenamiphos, which contain thioether linkages.

Three root crops—carrots, potatoes, and radishes—were selected for this study to determine if the difficulty of extracting carbaryl from radish roots reported by Wheeler et al. (1978) is experienced with other pesticide/root crop combinations. Methanol and acetonitrile were selected as extraction solvents because these water-miscible, polar solvents are used in a number of analytical methods and are considered to be good candidates for use as multiresidue extraction solvents. Blending with methanol or acetonitrile was evaluated for the extraction of [^{14}C]phorate residues from the three root crops. Subsequent washing, leaching, and Soxhlet extraction steps were also evaluated.

With pesticides that have been reported to undergo extensive metabolism in plants, such as organophosphorus

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and carbamate compounds, the extracted residues must be characterized to evaluate extraction efficiency of the individual metabolites. Therefore, individual phorate metabolites were also quantitated.

EXPERIMENTAL SECTION

Chemicals. ^{14}C-Labeled phorate (*O,O*-diethyl *S*-[(ethylthio)methyl]phosphorodithioate), labeled at the methylene carbon (-S-$^{14}CH_2$-S-) and with a specific activity of 3.26 mCi/mmol, was obtained from New England Nuclear Co., Boston, MA. It was found to be 99% radioactively pure by thin-layer chromatography (TLC). Authentic samples of phorate sulfone, phorate sulfoxide, phoratoxon, phoratoxon sulfone, and phoratoxon sulfoxide were obtained from American Cyanamid Co., Princeton, NJ. Thimet 600 liquid formulation was obtained from a commercial source.

Treatment of Plants. Carrots and radishes were grown 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 seeds had sprouted, the pots were placed outdoors. After the plants began to produce edible portions, the pots were placed in a greenhouse with temperature control for treatment with [^{14}C]phorate. An aqueous suspension of [^{14}C]phorate was prepared by adding a small quantity of an acetone solution of [^{14}C]phorate to the commercial formulation (Thimet 600). The aqueous suspension of [^{14}C]phorate (specific activity 1.37 mCi/mmol) was then pipetted onto both the soil and the partly exposed tubers at an application rate of 2.25–3.35 kg of active ingredient/ha. The actual phorate application exceeded the recommended rate because it was applied on and near the partly exposed plant roots and was not applied to the soil by recommended application techniques. Also, phorate was not applied at or before planting as is recommended. Phorate is registered in the United States for use on potatoes but not on radishes and carrots.

Harvesting and Compositing of Root Crops. Crops were harvested 5, 10, and 15 days after phorate application. The foliar portions were cut off and 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 were 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 extraction procedure consisted of six steps as shown in Figure 1.

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 half-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 (60-mm diameter). 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.

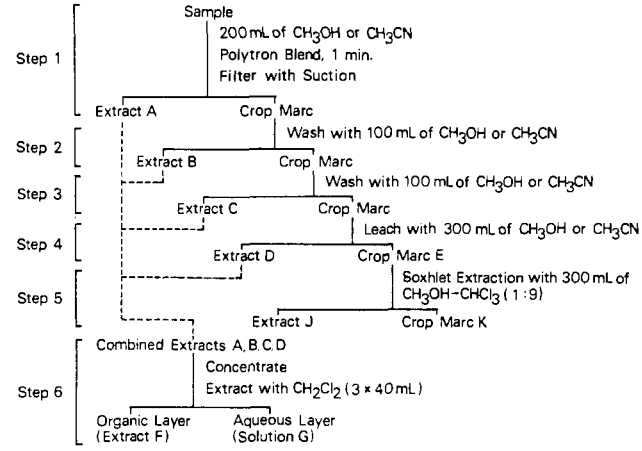

Figure 1. Flow diagram of the extraction procedure.

Step 3: Second Washing and Collection of Extract C. This 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 extraction apparatus (No. 9, 556B, Fisher Scientific Co., Pittsburgh, PA) by using 300 mL of chloroform–methanol (9:1). This extract is referred to as extract J, and the residual solids are referred to as crop marc K.

Step 6: Determination of Organic-Extractable and Water-Soluble [^{14}C]Phorate 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 was saved and is referred to as solution G. The loss of ^{14}C from extracts A–D during concentration was negligible.

Determination of ^{14}C. Liquid scintillation counting was performed by using a Mark III liquid scintillation spectrometer (Searle Inc., Des Plains, IL). Aliquots of extracts A–D, F, and J and solution G 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 2–11. The external standard technique

was used to correct for quenching. The ^{14}C in crop marc K (50–200 mg) was counted as described above following combustion of the sample and entrapment of the CO_2 using a Packard 306B sample oxidizer. The reagents specified in the manufacturer's manual were used in the sample oxidizer. The recovery of ^{14}C in the combustion procedure was between 98 and 100% when the crop marc was fortified with Spec-Chec (^{14}C-labeled 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. Walter and Purcell (1966) reported the use of a solution of *N*-bromosuccinimide in glacial acetic acid to eliminate the color quenching by carotenoids. In our study, a methanolic solution of *N*-bromosuccinimide (**caution**: highly irritating to skin) was used to eliminate color quenching. The total ^{14}C in each extract, solution, or crop marc was 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 mark 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.

TLC Radioautography. Precoated silica gel TLC plates (LKD Type, Kontes, Vineland, NJ) were used. Aliquots of extract F were streaked on TLC plates, and the chromatograms were developed with 1.75% methanol in chloroform. The TLC plates were then placed in contact with Kodak No-Screen X-ray film. Exposure time was 1–3 weeks. Exposed films were processed as usual. Each radioactive band was scraped into a scintillation vial, and 10 mL of Insta-gel was added. So that a suspension could be obtained, 5 mL of water was added, and ^{14}C was counted as described above. The phorate residues were identified by comparison of the R_f values obtained by radioautography. The reference standard spots of the phorate metabolites were visualized as described by Blinn (1963). Since the phorate residues from the sample composites were extracted in triplicate as described above, the phorate residues were quantitated individually in the triplicate sample extracts by using TLC radioautography.

Gas Chromatography. Gas chromatographic analysis of phoratoxon, phoratoxon sulfoxide, and phoratoxon sulfone was performed with a Shimadzu 3BFP (Tokyo) gas chromatograph equipped with a flame photometric detector (FPD). A 2 m × 3 mm o.d. glass column packed with 4% OV-101 on 60–80-mesh Gas-Chrom Q was used, and complete separation of these phorate analogues was attained. The column oven temperature was 200 °C, and the injection temperature was 240 °C. Nitrogen (carrier gas) flow was 60 mL/min, hydrogen 200 mL/min, and air 65 mL/min.

RESULTS

The data presented in Figures 2–4 and 9–11 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 5–8 are shown as percentages of the calculated total ^{14}C present in particular extracts or combinations of extracts. Differences in the extraction efficiency of methanol vs. acetonitrile were statistically evaluated, and these results are shown in the figures.

Total phorate residues, both extractable and unextractable, in sample composites of the three root crops were calculated as phorate and ranged from 0.86 to 12.6 ppm.

Extractable [^{14}C]Phorate Residues. *Extraction of [^{14}C]Phorate Residues by Blending, Washing, and*

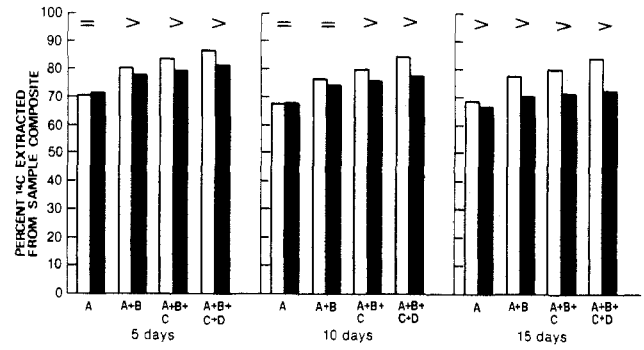

Figure 2. Comparison of extraction efficiency of methanol vs. acetonitrile for [^{14}C]phorate residues in potatoes. The fractions were analyzed for ^{14}C separately and the cumulative results are shown. The open column indicates ^{14}C obtained with methanol; the solid column indicates ^{14}C obtained with acetonitrile. 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 in the figure as follows: (=) indicates no significant difference found for these extracts when comparing methanol vs. acetonitrile; (>) indicates total ^{14}C in methanol is statistically greater than the total ^{14}C in acetonitrile; (<) indicates total ^{14}C in acetonitrile is statistically greater than the total ^{14}C in methanol.

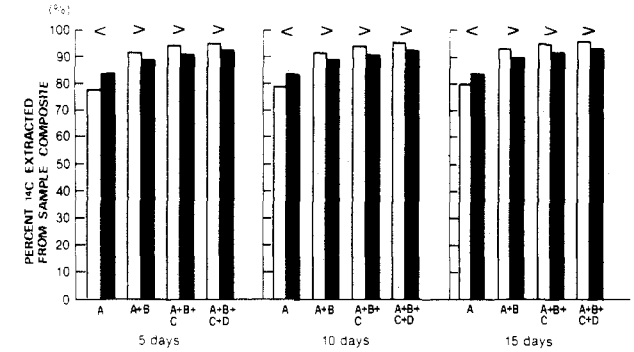

Figure 3. Comparison of extraction efficiency of methanol vs. acetonitrile for [^{14}C]phorate residues in carrots. The fractions were analyzed for ^{14}C separately and the cumulative results are shown. The open column indicates ^{14}C obtained with methanol; the solid column indicates ^{14}C obtained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

Leaching (Steps 1–4). Figure 2 shows the results of the extraction of [^{14}C]phorate residues from potatoes. The quantity of ^{14}C extracted is shown cumulatively from steps 1 to 4. Both methanol and acetonitrile extracted essentially the same quantity of ^{14}C in the first blending. The average ^{14}C extracted by methanol and acetonitrile was 70.9, 67.5, and 67.6%, respectively, for the 5-, 10-, and 15-day samples. Methanol was found to be the better solvent when the subsequent washing and leaching steps were also considered. The difference between methanol performance and acetonitrile performance in steps 1–4 was statistically significant. In the combined extracts (A + B + C + D), methanol extracted 86.6, 84.2, and 84.2% of the total ^{14}C and acetonitrile extracted 81.2, 77.6, and 72.2% of the total ^{14}C with 5-, 10-, and 15-day samples, respectively. The quantity of acetonitrile-extractable ^{14}C decreased slightly with increasing incorporation time, but this trend was less significant for methanol.

Figure 3 shows the results of the extraction of [^{14}C]-phorate residues from carrots. Unlike potatoes, but similar to radishes, more ^{14}C was collected in the blending filtrate with acetonitrile than methanol. However, methanol extracted more ^{14}C than did acetonitrile in the washing and

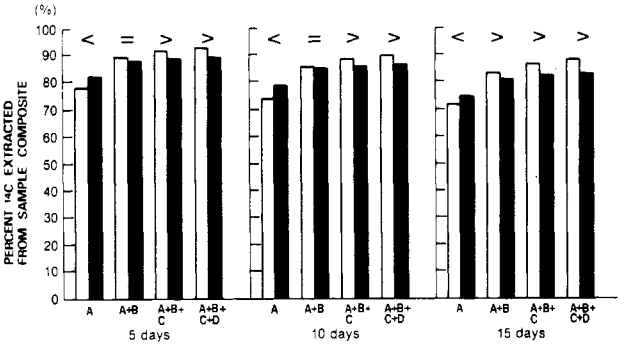

Figure 4. Comparison of extraction efficiency of methanol vs. acetonitrile for [14C]phorate residues in radishes. The fractions were analyzed for 14C separately and the cumulative results are shown. The open column indicates 14C obtained with methanol; the solid column indicates 14C obtained with acetonitrile.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

leaching steps, to the extent that the cumulative 14C extracted by methanol in steps 1–4 was statistically greater than that extracted by acetonitrile. In the combined extracts (A + B + C + D), methanol extracted 95.0, 95.3, and 96.0% of the total 14C present in the sample composite and acetonitrile extracted 92.5, 92.3, and 93.1% for the 5-, 10-, and 15-day samples, respectively. The percentage of extractable 14C was largest for carrots among the three root crops examined. No decrease in extractable 14C was observed with increasing incorporation time.

The results for radishes are shown in Figure 4. The extraction pattern was similar to that for carrots. Acetonitrile extracted more 14C than methanol in the first blending, but methanol was the better extractant in the subsequent washing and leaching steps. In the combined extracts (A + B + C + D), methanol extracted 92.4, 89.8, and 88.0% of the total 14C in the composite and acetonitrile extracted 89.0, 86.2, and 82.8% of the total 14C in the composite of 5-, 10-, and 15-day samples, respectively. A slight decrease in extractable 14C was observed for both solvents with increasing incorporation time.

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 14C associated with the methanol retained in the crop marc was eventually washed and/or leached and collected. The acetonitrile, however, could not remove the remaining 14C from the crop marc as effectively as methanol. This may explain why acetonitrile gave higher extraction efficiencies than methanol for step 1 and lower overall extraction efficiencies for the combined steps 1–4.

Determination of Water-Soluble vs. Organic-Soluble 14C in Extracts A + B + C + D. The concentrated combined extracts (A + B + C + D) for each sample were extracted with methylene chloride so that the methylene chloride extractable [14C]phorate residues and water-soluble [14C]phorate residues could be determined. The results are shown in Figure 5.

A great difference in the nature of [14C]phorate residues was observed between carrots and the other two root crops. With carrots, more than 96% of the total 14C in the combined extracts was methylene chloride extractable for all harvest-time combinations, and the ratio of the water-soluble 14C-labeled residues did not increase with increasing incorporation time. But with potatoes and radishes, the ratio of water-soluble 14C-labeled residues was much greater than with carrots and increased significantly with an increase in incorporation time. For example, at 15 days postapplication, 95.8% of the total 14C extracted from carrots was partitioned into methylene chloride

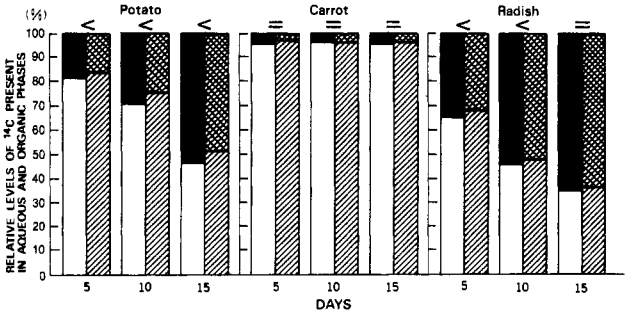

Figure 5. Comparison of methylene chloride extractable vs. water-soluble [14C]phorate residues. The open column indicates 14C in extract F obtained from the combined methanol extracts (A + B + C + D); the solid column indicates 14C in solution G obtained from combined methanol extracts (A + B + C + D); the shaded column indicates 14C in extract F obtained from the combined acetonitrile extracts (A + B + C + D); the crosshatched column indicates 14C in solution G obtained from the combined acetonitrile extracts (A + B + C + D). Total 14C in the combined extracts (A + B + C + D) is determined by summing the quantity of 14C found in each individual extract. See note 2 in Figure 2 for an explanation of symbols, which compare 14C in extract F in methanol and acetonitrile extraction.

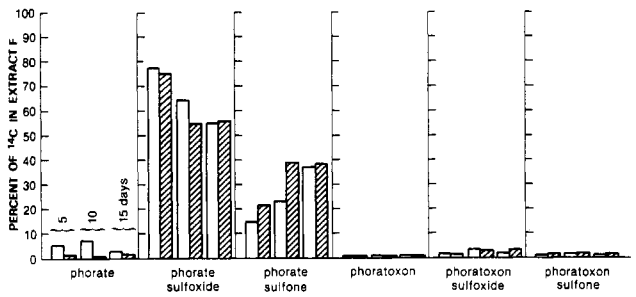

Figure 6. Quantitation of phorate residues in potatoes. The open column indicates 14C obtained with methanol; the shaded column indicates 14C obtained with acetonitrile. Total 14C in individual extracts F is 100%.

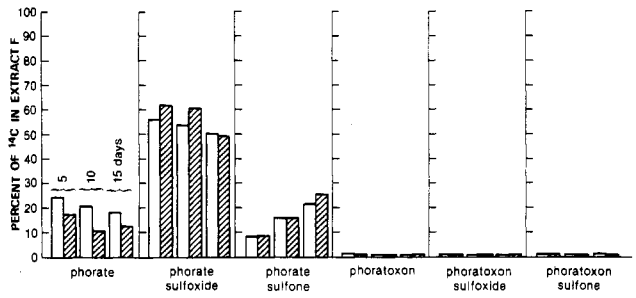

Figure 7. Quantitation of phorate residues in carrots. The open column indicates 14C obtained with methanol; the shaded column indicates 14C obtained with acetonitrile. Total 14C in individual extracts F is 100%.

compared to 46.4 and 35.3% for potatoes and radishes, respectively. The maximum water-soluble 14C was 64% of the total 14C present in extracts A + B + C + D and was observed in the 15-day radish sample. For potatoes and radishes, methanol extracted more water-soluble 14C than acetonitrile.

Quantitation of Individual [14C]Phorate Residues. Phorate residues in the methylene chloride extracts were further quantitated by using TLC radioautography and 14C counting. Characterization of the residues present in the aqueous layer was not attempted. In the methylene chloride extracts of potatoes, phorate sulfoxide and phorate sulfone were the major residues (Figure 6). For carrots, phorate, phorate sulfoxide, and phorate sulfone were the major residues (Figure 7). For radishes, phorate sulfoxide

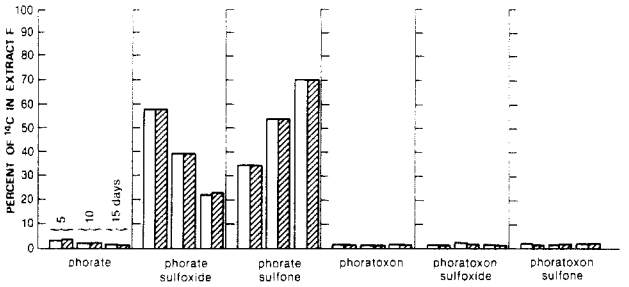

Figure 8. Quantitation of phorate residues in radishes. The open column indicates ^{14}C obtained with methanol; the shaded column indicates ^{14}C obtained with acetonitrile. Total ^{14}C in individual extracts F is 100%.

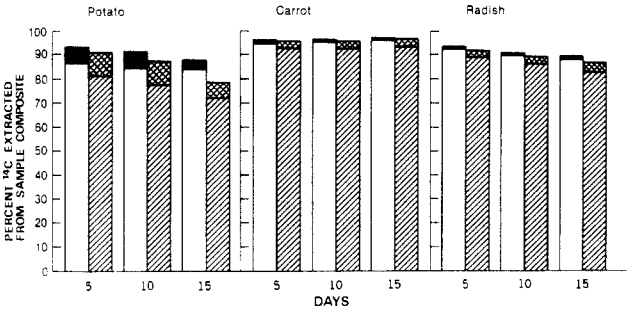

Figure 9. Soxhlet extraction of [^{14}C]phorate residues from crop marc. See note 1 in Figure 2. The open column indicates ^{14}C in the combined extracts (A + B + C + D) obtained by methanol extraction; the solid column indicates ^{14}C in extract J obtained after methanol extraction; the shaded column 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.

and phorate sulfone were the major residues (Figure 8). A common trend observed in these three root crops was that the ratio of phorate and phorate sulfoxide in the methylene chloride extracts decreased with incorporation time. However, the level of phorate sulfone, the more highly oxidized phorate metabolite, increased with increasing incorporation time. Phoratoxon, phoratoxon sulfoxide, and phoratoxon sulfone were found at trace levels with all crop composites (maximum 3.2% of the total ^{14}C in extract F). The six phorate residues quantitated accounted for more than 99.0% of the total ^{14}C in extract F for both methanol extraction and acetonitrile extraction.

The distribution of these six phorate residues into the aqueous layer (solution G) was considered to be negligible. Phoratoxon, phoratoxon sulfoxide, and phoratoxon sulfone, the more polar analogues of phorate, were added to composites of potatoes at the 1-ppm level individually. The composites were extracted with methanol according to the procedure in Figure 1, and the methylene chloride extracts (extract F) were obtained. Phorate residues in extract F were determined by FPD gas chromatography. The recoveries were 95.3% for phoratoxon and 98.2% for phoratoxon sulfone. Phoratoxon sulfoxide was recovered as phoratoxon sulfone by conversion, and the recovery was 94.1%.

Soxhlet Extraction. Crop marc E, which had been extracted with methanol or acetonitrile (steps 1–4), was further extracted in a Soxhlet extraction apparatus. The ^{14}C in the Soxhlet extracts is considered to be the quantity of solvent-extractable ^{14}C remaining in crop marc E, and its quantity is associated with the performance of the previous extraction procedure (steps 1–4). The quantity of ^{14}C in the Soxhlet extracts is shown in Figure 9 as well as the total ^{14}C extracted in the previous procedure (steps 1–4). With the carrot and radish crop marcs that were

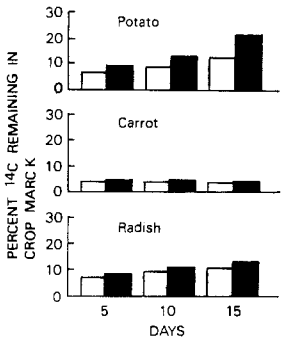

Figure 10. Unextractable (bound) [^{14}C]phorate residues. See note 1 in Figure 2. The open column indicates ^{14}C in crop marc K after methanol and Soxhlet extraction; the solid column indicates ^{14}C in crop marc K after acetonitrile and Soxhlet extraction.

previously extracted with methanol, little ^{14}C was removed by Soxhlet extraction (0.83, 1.14, and 0.95% of the total ^{14}C in the carrot composite of 5, 10, and 15 days postapplication, respectively; 0.94, 1.32, and 1.65% of the total ^{14}C in the radish composite of 5, 10, and 15 days postapplication, respectively). Thus, methanol is extremely efficient for the extraction of [^{14}C]phorate residues in carrots and radishes.

Soxhlet extraction of crop marcs previously extracted with acetonitrile removed from 2.0 to 3.1% more ^{14}C than was obtained from those previously extracted with methanol.

Unextractable (Bound) [^{14}C]Phorate Residues. The definition of bound pesticide residues of Dorough (1976) was used in this study; i.e., bound or unextracted metabolites are derivatives of the pesticides that cannot be removed from the substrate by thorough extraction. The ^{14}C present in crop marc K was considered to be bound pesticide residues, and this quantity was determined by ^{14}C analysis of the crop marc following combustion. The results are shown in Figure 10. Carrots have the smallest amount of bound residues for all incorporation periods, always less than 4.3%, and potatoes and radishes have more bound residues (from 7.2 to 21.7%), increasing as the incorporation period increases. Carrots did not show increased bound residues with the longer incorporation periods. More bound residues were found in the acetonitrile-extracted potato and radish crop marcs than in the methanol-extracted crop marcs. Acetonitrile blending, washing, and leaching may have extracted less water-soluble [^{14}C]phorate residues than methanol. Subsequent Soxhlet extraction might fail to extract the water-soluble [^{14}C]phorate residues, which were more readily extracted with methanol, because the solvent used in the Soxhlet extraction is more suitable for the extraction of lipid-soluble rather than water-soluble [^{14}C]phorate residues.

DISCUSSION

The data from this study show that methanol extracts from 1.6 to 12% more of the total [^{14}C]phorate residues from the three crops than acetonitrile, when the combined blend–wash–leach procedures (steps 1–4) are used. The filtrate from the initial crop–acetonitrile blend (solution A) was generally found to contain more ^{14}C than that obtained with methanol. This is at least partially due to the larger acetonitrile filtrate volumes obtained. Acetonitrile did not appear to be as effective as methanol in extracting additional ^{14}C-labeled residues from the crops with the wash-leach procedure (steps 2–4).

Soxhlet extraction of the crop marcs provided additional evidence that methanol extracts more [^{14}C]phorate residues than acetonitrile. In all cases, more ^{14}C was obtained

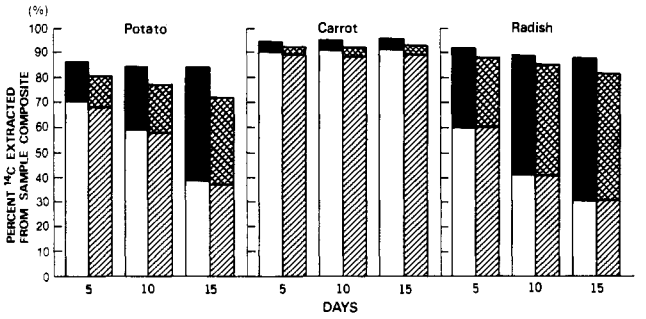

Figure 11. Comparison of methylene chloride extractable vs. water-soluble [^{14}C]phorate residues. See note 1 in Figure 2. The open column indicates ^{14}C in solution G obtained from the combined methanol extracts (A + B + C + D); the solid column indicates ^{14}C in solution G obtained from the combined acetonitrile 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).

by Soxhlet extraction of crop marcs previously extracted with acetonitrile than was obtained from those previously extracted with methanol. This is a good indication that acetonitrile is less efficient than methanol in extracting total phorate residues from these crops.

Burke et al. (1971) reported that 35% water in acetonitrile is the optimum solvent mixture for extracting pesticide residues from low moisture crops. In our study, most of the crop moisture was removed in the initial filtration of the crop–acetonitrile homogenate. The mixed solvent used by Burke et al., although not tried in this work, may be more effective in releasing additional ^{14}C-labeled residues.

Figure 11 illustrates the proportions of methylene chloride extractable (extract F) and water-soluble (solution G) ^{14}C-labeled residues which were extracted from the samples by steps 1–4. Methanol is equal to or slightly superior to acetonitrile in removing methylene chloride extractable [^{14}C]phorate residues from the samples and considerably superior to acetonitrile in extracting water-soluble [^{14}C]phorate residues, thus accounting for the superiority of methanol over acetonitrile.

The results of Soxhlet extraction following leaching indicate that methanol extracts almost all of the solvent-extractable residues from carrots and radishes. The potato crop marc retains more [^{14}C]phorate residues after leaching than the crop marc of the other two root crops, indicating that extraction of phorate residues from potatoes is more difficult.

Considering convenience, time, and cost, a suggested extraction procedure can be described as follows: blending of the root crop composite with 200 mL of methanol, followed by washing of the blender, sample container, and crop marc with two 100-mL portions of methanol. This procedure will extract more than 95% of the total ^{14}C extracted in steps 1–4.

Among the three root crops investigated, the metabolism of phorate in carrots is considerably different than in the other two crops when the ratio of methylene chloride extractable and water-soluble [^{14}C]phorate residues is compared. This is also true for the metabolism of carbofuran in these same root crops (Sonobe et al., 1982). The [^{14}C]phorate residues in carrots are mostly methylene chloride extractable. However, significant quantities of water-soluble residues were found in both potatoes and radishes. In their study of absorption and translocation of [^{14}C]phorate residues in soy and mung beans, Talekar et al. (1977) reported that a major portion of ^{14}C was associated with water-soluble and bound residues.

A literature search failed to find any reports on conjugated phorate residues. In our study, the characterization of the water-soluble [^{14}C]phorate residues was not attempted because of the difficulty in releasing these compounds by hydrolysis or oxidation from crop constituents without destroying the principal moieties of any toxic residues derived from phorate. Water-soluble ^{14}C-labeled residues of organophosphorus pesticides apparently have not been sufficiently investigated, but they should not be neglected as pesticide residues until their chemical form, toxicity, and bioavailability are clarified.

The major phorate residues in the methylene chloride extracts (phorate sulfoxide and phorate sulfone) are those usually found as major residues in plants. The metabolism of phorate in plants was studied by Bowman and Casida (1957). They found phorate sulfoxide, phorate sulfone, phoratoxon sulfoxide, and phoratoxon sulfone in plants as metabolites of phorate, with phorate sulfoxide and phorate sulfone as the major residues. They reported anticholinesterase activity in all of these compounds (the pI_{50} value ranged from 3.17 to 7.02). Accordingly, the efficient extraction of these compounds is important. In potatoes, corn, and carrots, phorate sulfone was the major residue (Lichtenstein et al., 1973). The ratio of phorate sulfone, the oxidized form of phorate and phorate sulfoxide, increased with longer incorporation periods. The slight difference in the ratio of the phorate metabolites extracted by methanol vs. acetonitrile may be due to a difference in the metabolites' susceptibility to oxidation in the two solvents.

Bound pesticide residues have only become a concern of residue chemists in the last 12–15 years. These compounds should not be ignored since they may be potentially hazardous chemicals. The level of bound residues in the three root crops treated with [^{14}C]phorate ranged from a minimum of 3.1% of the total ^{14}C present in carrots (always less than 4.3% irrespective of extraction solvent or harvest interval) to a maximum of 21.7% of the total ^{14}C present in potatoes harvested 15 days after phorate application. Carrots also contained the smallest quantities of bound residues when [^{14}C]carbofuran residues were extracted from these three root crops (Sonobe et al., 1982). This suggests that metabolism of pesticides in carrots is different from that in other root crops. We did not attempt to identify these residues. Our observations support the findings of other researchers [e.g., Wheeler et al. (1978, 1980)] that the percentage of bound residues in the total ^{14}C-labeled residues of crop samples differs with the variety of pesticides and crops.

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LITERATURE CITED

Blinn, R. C. *J. Assoc. Off. Agric. Chem.* **1963**, *46*, 952.
Bowman, J. S.; Casida, J. E. *J. Agric. Food Chem.* **1957**, *5*, 192.
Bowman, M. C.; Beroza, M.; Leuck, D. B. *J. Agric. Food Chem.* **1968**, *16*, 796.

Burke, J. A.; Porter, M. L.; Young, S. J. V. *J. Assoc. Off. Anal. Chem.* **1971**, *54*, 142.
Dorough, H. W. In "Bound and Conjugated Pesticide Residues"; Kaufman, D. D.; Still, G. G.; Paulson, G. D.; Bandel, S. K., Eds.; American Chemical Society: Washington, DC, 1976; Chapter 2.
Lichtenstein, E. P.; Fuhreman, T. W.; Shulz, K. R.; Liang, T. T. *J. Agric. Food Chem.* **1973**, *66*, 863.
Saha, M.; Sumner, A. K.; Saha, J. G. *J. Assoc. Off. Anal. Chem.* **1973**, *56*, 45.
Sonobe, H.; Carver, R. A.; Krause, R. T.; Kamps, L. R., submitted for publication in *J. Agric. Food Chem.*, 1982.
Talekar, N. S.; Lee, E. M.; Sun, L. T. *J. Econ. Entomol.* **1977**, *70*, 685.
Walter, W. M., Jr.; Purcell, A. E. *Anal. Biochem.* **1966**, *16*, 466.
Watts, R. L. *J. Assoc. Off. Anal. Chem.* **1971**, *54*, 953.
Wheeler, W. B.; Thompson, N. P.; Andrade, P.; Krause, R. T. *J. Agric. Food Chem.* **1978**, *26*, 1333.
Wheeler, W. B.; Thompson, N. P.; Andrade, P.; Krause, R. T. *J. Assoc. Off. Anal. Chem.* **1980**, *63*, 1286.
White, E. R.; Al-Adil, K. M.; Winterlin, W. L.; Kilgore, W. W. *Bull. Environ. Contam. Toxicol.* **1973**, *10*, 140.

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Determination of Chlorobutanol in Milk, Serum, and Tissues by a One-Step Cleanup and Gas–Liquid Chromatography

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A quantitative procedure for the determination of chlorobutanol in milk, serum, and tissues is described. A special distillation/extraction apparatus was designed to isolate the chlorobutanol in a single step. The chlorobutanol was quantitated on a 5-ft, 5% XE-60 column by electron capture detection with a 2-mCi ^{63}Ni source. The average percent recovery ± the standard deviation for chlorobutanol in milk over the 10–60-ppb range was 95.2 ± 2.7%. The average recovery for all tissues and serum at the 10-ppb level was 86.6 ± 3.8%. No interfering peaks were observed at the retention time of chlorobutanol. The analysis time for a single assay was 2 h.

Chlorobutanol is used as a preservative in products for the treatment of acute and chronic mastitis in dry cows. Since the route of administration is via the udder, an analytical method was needed to determine whether residual amounts of chlorobutanol were present in milk posttreatment and tissues upon slaughter. One published method for milk described a steam distillation technique (Wiskerchen and Weishaar, 1972). Because of the slow step with the Kurderna-Danish concentrator in their method, an all-glass distillation/extraction apparatus was designed, which carried out simultaneous steam distillation and transfer of chlorobutanol into a small volume of isooctane, therby eliminating the Kurderna-Danish concentrator.

The special steam distillation/extraction apparatus is a modification of the Bleidner-Heizler type described in the literature by Geissbuhler et al. (1971). However, several changes in design were necessary to ensure that the separation chamber remained cool throughout the distillation step. A temperature rise in the separation chamber of the Bleinder-Heizler design led to viscosity and density changes which resulted in phase boundary changes that were detrimental to a successful distillation/extraction.

This paper describes the design and use of the distillation/extraction apparatus for the assay of chlorobutanol in milk, serum, and tissues with a lower limit of detection of 10 ppb.

EXPERIMENTAL SECTION

Apparatus. The apparatus used were the following: special steam distillation/extraction apparatus (Figure 1); Waring Blendor, Model 702B, 1-L glass bowl refitted with gaskets cut from polyethylene; Beckman Zeromatic pH meter, Model SS-3; boiling chips, Fisher Scientific Co.; glass beads, No. 3000, 3 mm, chemically resistant, Fisher Scientific Co.; gas chromatograph, Hewlett-Packard, Model 402, equipped with a 2-mCi ^{63}Ni electron capture detector, a 5 ft × 3 mm i.d. glass column packed with 5% XE-60 on 60–80-mesh Diatoport S; Model 5A Kitchen Aid grinder.

Reagents. The reagents used were the following: chlorobutanol standard (1,1,1-trichloro-2-methyl-2-propanol, Matheson Coleman and Bell (mp 76–78 °C); silicotungstic acid, Fisher Scientific Co.; solvents, "distilled in glass" grade, Burdick & Jackson Laboratories; glassware cleaner, Orbit, Dubois Chemicals; silicotungstic acid solution (100 g/L).

Stock Standard Solutions. Chlorobutanol was prepared in isooctane, stock standard A (100 ppm) and stock standard B (1.0 ppm).

Fortification Standards. Aliquots of stock A (1.0, 2.0, and 3.0 mL) were pipetted into 100-mL volumetric flasks, and aliquots of stock A (2.0 and 3.0 mL) were pipetted into 50-mL volumetric flasks. All flasks were brought to volume with isooctane. This series of standards contained 1.0, 2.0, 3.0, 4.0, and 6.0 ppm of chlorobutanol, respectively.

GLC Standards. Aliquots of stock solution B (0.5, 1.0, 1.5, 2.0, and 3.0 mL) were pipetted into separate 100-mL volumetric flasks and brought to volume with isooctane. This series of GLC standards contained 5, 10, 15, 20, and 30 ng/mL and were equivalent to 10, 20, 30, 40, and 60 ppb of chlorobutanol, respectively, in a 50-g sample.

Sample Preparation. Fifty-milliliter aliquots of milk and serum from control and treated cows were placed in plastic containers and stored at −20 °C. The samples were

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