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DETERMINATION OF CARBOFURAN AND ITS METABOLITES*

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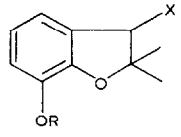
SUMMARY

High-performance liquid chromatographic (HPLC) methods for the determination of carbofuran and its metabolites are described. Results obtained for carrot samples by HPLC are compared with those obtained using gas chromatography and nitrogen-specific detection for carbofuran and 3-hydroxy carbofuran and gas chromatography–mass spectrometry with selected ion monitoring for the determination of the three 7-phenol metabolites. The effect upon observed residue levels of conjugate formation by the 3-hydroxy and/or the 7-phenoxy groups of the four metabolites is demonstrated, and appropriate acid hydrolysis techniques are described. Variations in the HPLC parameters for different commodities are presented.

INTRODUCTION

Carbofuran, 2,3-dihydro-2,2-dimethylbenzofuranyl N-methylcarbamate (I), is a broad spectrum insecticide–nematicide for which an ever increasing number of new agricultural uses are being proposed. Because of the required time intervals between application and harvesting, residues of carbofuran on eatable agricultural commodities are frequently present as the 3-hydroxy carbamate metabolite, 2,3-dihydro-3-hydroxy-2,2-dimethylbenzofuranyl N-methylcarbamate (II), and the three phenolic metabolites, 2,3-dihydro-2,2-dimethyl-7-benzofuranol (III), 2,3-dihydro-



	R	X
I	$CONHCH_3$	H
II	$CONHCH_3$	OH
III	H	H
IV	H	(=O)
V	H	OH
VI	H	OC_2H_5
VII	$CONHCH_3$	(=O)

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2,2-dimethyl-3-oxo-7-benzofuranol (IV) and 2,3-dihydro-2,2-dimethyl-3,7-benzofurandiol (V). Residue data for registration purposes are required by the United States Environmental Protection Agency for both the parent compound and these four metabolites[1].

The original analytical methods for carbofuran and other methyl carbamate pesticides required extensive clean-up procedures prior to the final spectrophotometric quantitation steps[2,3]. Gas chromatographic (GC) advances made measurements more feasible, but derivatizations of the metabolites were required which added to the difficulty of these methods[4]. Determination of the carbamates by GC using a nitrogen-specific thermionic detector[5], in conjunction with a mass spectrometric selected ion mode (MS–SIM) determination of the phenolic metabolites, is useful[6], but the equipment required for the latter is not generally available for routine analyses. Likewise, the use of GC–MS–SIM in the chemical ionization mode for determination of the carbamate series is expensive and requires the formation of the heptafluorobutyryl derivatives[7].

The polar nature of these compounds, particularly the metabolites, makes their analysis by high-performance liquid chromatography (HPLC) attractive, and the aromatic moiety assures reasonable responses to UV detection. Several studies of this nature have been reported, including one utilizing fluorigenic labeling of the carbamates[8–10]. More recently, a simultaneous HPLC separation of these materials with both isocratic and gradient solvent systems using a reversed-phase column illustrated the potential of this general technique[11]. However, much of this work has been with standards and/or at relatively high concentrations; methodologies for sub-ppm determinations in complex matrices are not well developed.

Accordingly, we have developed a set of analytical methods for these compounds which are readily adapted to a variety of commodities. In this paper we report the analysis of field-treated carrot samples using our own HPLC methods, and GC with nitrogen–phosphorus-specific detection plus GC–MS methods for comparison. We also conducted the HPLC analyses with and without an acid hydrolysis step to assess the extent to which conjugate formation with the free 3-hydroxy and 7-phenoxy groups was occurring, and to what extent it could influence analytical results.

EXPERIMENTAL

Instrumentation

A Micromeritics Model 7000B liquid chromatograph equipped with a Tracor Model 970A UV-VIS variable wavelength detector and a Rheodyne Model 7120 valve loop injector (100-μl loop) was used. Water Associates μBondapak C_{18} (10 μm particle size) reversed-phase columns (30 cm × 3.9 mm I.D.) were used initially, with Waters Radial Compression Separation System (RCSS) C_8 Radial-PAK (10 μm particle size) reversed-phase columns (10 cm × 8 mm I.D.) being used later. Columns and solvents were maintained at ambient temperatures.

Nitrogen-specific GC was performed on a Tracor Model 222 GC equipped with a Perking Elmer N-P (thermionic) detector run in the nitrogen mode at 205°C: H_2 flow-rate 10.5 ml/min; air flow-rate 2.5 ml/min; injector temperature 210°C with N_2 carrier. Columns were 5% OV-3 on Chromosorb W HP (80–100 mesh), 4 ft. × 1/8 in., at 175°C.

GC–MS studies were done on a Hewlett-Packard Model 5985 gas chromatograph–mass spectrometer–data system coupled to a Hewlett-Packard Model 5840 capillary gas chromatograph with a multiple purge capillary injector and a 10-m Carbowax 20M wall-coated column (0.32 mm I.D.). Samples were run using the selected ion monitor (SIM) option of the specific ion detector batch processor program (BATCH). The injector temperature was 250°C, column temperature 200°C and detector electron multiplier voltage 2800 V.

Reagents

All solvents were spectral or liquid chromatography grade; acetone was redistilled from glass. Standards of carbofuran and metabolites were obtained from FMC Corporation (Middleport, NY, U.S.A.). Florisil (80–100 mesh) was activated at 140°C for 24 h, cooled and deactivated with 4.0% water added as a fine mist with constant mixing. The container was sealed for at least 48 h and shaken occasionally before use. Buffer solution (pH 12) was made by mixing 6.4 g citric acid, 3.54 g boric acid, 2.12 ml 89% orthophosphoric acid and 13.72 g sodium hydroxide, and diluting to 1.0 l in distilled water. Precipitating solution was prepared by mixing 1.25 g ammonium chloride and 2.5 ml 89% orthophosphoric acid and diluting to 1.0 l in distilled water. Anhydrous granular sodium sulfate was obtained from Mallinckrodt (St. Louis, MO, U.S.A.).

Carrot samples were produced by incorporating 0, 3 and 6 lbs. of granular carbofuran (Furadan 10G) per acre at planting. Carrots were harvested 112 days later (10-lb. random samples), shipped frozen on dry-ice and chopped frozen in a Hobart Food Chopper; 100-g subsamples were taken and held at −20°C until analyzed.

HPLC of carbofuran

Carrots, 100 g, were blended with 250 ml of acetone for 3 min and filtered with suction through Whatman No. 1 paper fitted over a coarse glass filter. The filtrate was transferred to a 1000-ml separating funnel, 250 ml of chloroform were added, the funnel was shaken and the phases were allowed to separate. The chloroform layer was drawn off through a 3-cm thick bed of sodium sulfate in a 60-ml coarse sintered glass filter funnel. After repeating the chloroform washings twice with 100 ml chloroform, and draining through the sodium sulfate, the collected solvent was reduced under vacuum (50–60°C) to *ca.* 5 ml. Evaporation was completed at ambient temperature with a gentle air stream. After adding 15 ml of acetone followed by 50 ml of the precipitating solution with swirling, the flask was allowed to stand for 30 min. The resulting slurry was filtered through a 3-mm pad of Celite filter-aid in a Büchner funnel which had been prewetted with 25 ml of precipitating solution. The filtrate and 20 ml of the buffer solution were combined and transferred to a 250-ml separating funnel containing 100 ml of carbon tetrachloride. The lower phase was passed through a 3-cm pad of sodium sulfate. This extraction was repeated with two more 100-ml portions of carbon tetrachloride, and the combined organic phases were reduced to *ca.* 5 ml on a rotary vacuum evaporator at 50–60°C. Evaporation was completed with an air stream and the sample was dissolved in HPLC-grade methanol for injection. Samples were chromatographed using the C_{18} μBondapak reversed-phase column. The mobile phase was methanol–water (50:50) at a flow-rate of 1 ml/min; detector wavelength 200 nm.

Recoveries were determined by spiking check samples in the blender at 0.1 and 1 ppm; recoveries were 80 and 95% respectively at these levels. No carbofuran was detected in any samples (<0.1 ppm). Injections of 17.5 ng of standard in 7.0 μl of check sample gave 8% full-scale deflection (f.s.d.) using the C_{18} column, while 6.8 ng in 4.0 μl gave 11% f.s.d. with the C_8 column (0.02 absorbance units/f.s.d.).

HPLC without hydrolysis

3-Hydroxy carbofuran. Extraction was the same as for carbofuran except that 50 ml of acetone (not 15 ml) were added at the precipitation step, and the subsequent extraction from the buffer solution was done with chloroform rather than carbon tetrachloride —the quantities remained the same. The mobile phase was changed to methanol–water (30:70); recovery was 83% at 0.25 ppm. Injections of 17.5 ng of standard in 7.0 μl of check sample gave 9% f.s.d.

Three phenolic metabolites. Extraction and clean-up were identical to that described for 3-hydroxy carbofuran, and injections were made from the same final solution in methanol. Chromatography was on a Waters RCSS C_8 column, 10 cm × 8 mm I.D., using water–methanol (50:50) at a flow-rate of 2.0 ml/min. The detector wavelength was 210 nm. Recoveries at 0.5 ppm were 71, 75 and 81% for the 7-phenol, 3-keto-7-phenol and 3-hydroxy-7-phenol metabolites respectively. F.s.d. values (ng injected) were respectively: 6.5% (20), 7.5% (19.5) and 5.5% (18.0). No metabolite residues were detected in any samples (<0.1 ppm).

HPLC with hydrolysis

3-Hydroxy carbofuran. Fifty grams of finely chopped carrots were placed in a 1-l round bottom flask containing 200 ml of 0.25 *N* hydrochloric acid, and fitted with a reflux condenser. After refluxing for 1 h, the flask and contents were cooled and filtered through a 9-cm Büchner funnel which had been prepared by placing a 1-cm layer of sea-sand over a circle of Whatman No. 1 paper. If filtering was difficult, the slurry was blended for 1 min with 15 g of Hyflo-Supercel before refiltering. The aqueous phase was collected and adjusted to a volume of 300 ml with 0.25 *N* hydrochloric acid. A 60-ml aliquot (equivalent to a 10-g sample) was placed in a 250-ml separating funnel with 60 ml of methylene chloride, and 0.5 g of sodium dodecyl sulfate were added to the aqueous phase. After shaking, the phases were allowed to separate and the methylene chloride layer was run through a 3-cm pad of sodium sulfate and collected. The washing was repeated with two fresh 60-ml portions of methylene chloride and the combined organic phases were evaporated to *ca.* 1 ml under vacuum. A 2-ml volume of hexane was added to the flask.

To a 1.9 cm I.D. chromatography column, fitted with a stopcock and a glass wool plug, were added a 1-cm layer of sea-sand, 2 g of deactivated Florisil and a final 1-cm layer of sea-sand. The column was prewashed with 50 ml of hexane. The sample was transferred to the column with a total volume of 15 ml hexane. The column was eluted first with 100 ml of 10% ethyl acetate in hexane (discarded), and then with 100 ml of 50% ethyl acetate in hexane (collected). The collected fraction was evaporated on a rotary evaporator to 2 ml, and finally to dryness with an air stream. After being redissolved in methanol the sample was ready for HPLC. Recovery at the 0.4 ppm level was 95%. A 4-μl injection containing 9.8 ng of standard gave a 15.5% f.s.d.

Three phenolic metabolites. A 60-ml aliquot of the above described hydrolysis filtrate was diluted to 200 ml with 0.25 *N* hydrochloric acid and placed in a 500-ml separating funnel with 0.5 g sodium dodecyl sulfate. 150 ml of methylene chloride–diethyl ether (3:1, v/v) were added and the funnel was shaken for 1 min. The lower

phase was filtered through a 3-cm pad of sodium sulfate and collected. Two further washes with fresh portions of the solvent mixture were performed, and the combined organic phases were evaporated to *ca.* 2 ml. The solution was transferred to a 125-ml round bottom flask fitted with a reflux condenser; 25 ml of absolute ethanol and six drops of concentrated hydrochloric acid were added. The mixture was refluxed for 30 min and cooled. Sodium chloride (5 g) and 100 ml of water were placed in a 250-ml separating funnel; the reflux mixture was transferred to the funnel using 50 ml of methylene chloride. The funnel was shaken for 1 min and the lower phase transferred to a 500-ml separating funnel. After twice repeating the methylene chloride extraction, 50 ml of 0.25 *N* sodium hydroxide solution were added to the combined organic phases in the 500-ml funnel, shaken, and the sodium hydroxide phase was placed in a clean 250-ml separating funnel. The sodium hydroxide extraction of the methylene chloride was repeated, and to the combined alkaline fractions were added 100 ml of hexane, which, after shaking, was discarded. The hexane washing procedure was repeated then 6 ml of concentrated hydrochloric acid and 1 g of sodium chloride were added to the alkaline phase. This was extracted three times with 50-ml portions of methylene chloride, each portion being run through a 3-cm bed of sodium sulfate and collected in the same flask. After evaporation under vacuum to 2 ml, the solution was taken to dryness under an air stream. The sample was dissolved in methanol and transferred to a 2.0-ml volumetric flask. Samples were chromatographed on a C_8 column with methanol–water (40:60) at 2 ml/min; detector wavelength 203 nm. Recoveries of the 7-phenol, 3-keto-7-phenol and 3-hydroxy-7-phenol (as its 3-ethoxy derivative) were 73, 100 and 100% at the 0.6 ppm level. The detector sensitivity was 36%, 17% and 21% f.s.d. respectively for 12-ng injections in 4 μl of check solution.

GC of carbofuran and 3-hydroxy carbofuran

Carrot samples were analyzed for carbofuran and 3-hydroxy carbofuran as described by Nelson and Cook[5], except that methylene chloride was substituted for hexane as the first extracting solvent, no column clean-up was necessary for carbofuran and instrument parameters were as cited under *Instrumentation.* No carbofuran was detected in any of the samples (<0.1 ppm); recovery was 80% at 1.0 ppm. The recovery of 3-hydroxy carbofuran was 78% at 0.4 ppm; analytical results are presented in Table I.

GC–MS of three phenolic metabolites

The extraction, clean-up and derivatization procedure differed from that described for the HPLC analysis of the metabolites in that the sample solution of 2 ml methylene chloride was used for injection rather than being evaporated for final dissolution in methanol. These conditions were similar to those recommended in Nelson *et al.*'s[6] GC–MS method. Each metabolite was monitored at three atomic mass units (a.m.u.) as follows, the number in parentheses being the per cent relative abundance for each member of the set: 7-phenol metabolite, 122 (23.5), 149 (89.9), 164 (100); 3-keto-7-phenol, 110 (59.1), 135 (100), 178 (91.3); 3-ethoxy-7-phenol, 137 (100), 162 (14.3), 208 (35.6). Ethyl anthranilate, the internal standard (std), was monitored at 119 a.m.u. Retention times (min) and per cent recoveries for the four components were 5.3/75, 7.8/100, 6.7/102 and 5.7/std, respectively. Calibration and standardization was with a solution containing each of the metabolites at 2 ppm and the internal standard at 10 ppm. Recoveries were determined using a check sample spiked before hydrolysis at 2 ppm with all three metabolites. Concentrations were calculated by utilizing only the responses of the major peak for each of the metabo-

TABLE I

RESIDUES OF CARBOFURAN METABOLITES IN CARROT ROOTS (ppm)

Samples in the first HPLC series for 3-hydroxy carbofuran were not acid hydrolyzed; all other samples were acid hydrolyzed. GC–NPD = gas chromatography with nitrogen specific detection.

No.	*Rate*	*3-Hydroxy carbofuran*			*7-Phenol*		*3-Keto-7-phenol*		*3-Ethoxy-7-phenol*	
		HPLC	*HPLC*	*GC NPD*	*HPLC*	*GC MS*	*HPLC*	*GC–MS*	*HPLC*	*GC–MS*
941	0	<0.10	<0.20	<0.10	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
942	0	<0.10	<0.20	<0.10	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
945	3 lb./acre	0.38	1.3	0.96	<0.05	0.09*	<0.05	0.18*	0.23	0.50*
946	3 lb./acre	<0.10	0.75	0.85	0.11	0.11*	<0.05	0.20*	0.28	0.42*
947	3 lb./acre	<0.10	0.84	0.60	<0.05	0.11*	<0.05	0.09*	0.27	0.37
948	3 lb./acre	<0.10	1.1	0.78	0.16	0.10*	0.40	0.25*	0.31	0.44
949	6 lb./acre	<0.10	0.77	0.76	<0.05	0.13*	0.22	0.16	<0.05	0.37
950	6 lb./acre	0.34	2.4	0.58	0.17	0.26	0.24	0.47	0.44	1.3
951	6 lb./acre	0.23	1.6	1.1	0.26	0.08*	0.35	0.17	0.44	0.42
952	6 lb./acre	0.22	1.2	1.1	0.26	0.11*	0.58	0.10*	0.37	0.41

* Peak ratio deviated from standard values.

lites, the minor peaks being used to obtain peak ratios to support the identities of the fragment(s). Values in Table I marked with an asterisk showed sufficient deviation in peak ratios for that sample to cause suspicion as to the qualitative make-up of the fragments at the specific a.m.u.

RESULTS AND DISCUSSION

The absence of any detectable residues of carbofuran in the carrot samples was not surprising given the 112-day treatment-to-harvest interval and in light of published metabolism/decomposition studies[12,13]. These studies have also shown that only a fraction of the 3-hydroxy carbofuran, and usually none of the phenolic metabolites, are present in the unconjugated form. Our data (Table I) substantiated this in that only 20–50% of compound II and none of III, IV or V were detected in the absence of an acid hydrolysis step to free the 7-phenoxy and 3-hydroxy glycosides.

Analysis for the parent compound did not require that the sample be hydrolyzed, therefore, were were able to take advantage of an old extraction/clean-up technique used for precipitating methyl anthranilate and other interfering plant materials[3]. The complexity of the mixture increased with hydrolysis and this required the addition of a Florisil column clean-up for some analyses of conjugate-forming metabolites. Because of the variation in Florisil activity with changes in age, mesh, batch, etc., each supply must be standardized and appropriate adjustments made when problems are encountered with the elution of fractions from the deactivated Florosil columns[14].

The ethoxylation of compound V to VI was not necessary for the HPLC determination, but it was found that it lengthened the retention time and improved the separation. Several of the cited works make reference to 3-keto carbofuran (VII). Its occurrence in field samples is unlikely since its rate of hydrolysis to compound IV is 170 times faster than the corresponding hydrolytic transformation of I to III[12]. The RCSS C_8 column system was found to give better results at higher flow-rates. However, either the C_8 or C_{18} column may be used with appropriate parameter adjustment.

The agreement between HPLC and GC with nitrogen–phosphorus specific detection for compound II is excellent, with all but one sample (950) falling within a factor of two. The results obtained for the phenolic metabolites are comparable when the usual variations between subsamples are considered. Deviations in GC–MS peak ratios from values obtained from standards do not automatically negate a determination, but serve as a subjective indication that there might be some fragments at the monitored a.m.u. values that are being formed from compounds other than the one(s) under investigation. Moreover, as the threshold of sensitivity for a fragment of low per cent relative abundance is reached, the ratio becomes erratic.

No argument for the superiority of one method over the other is presented; the HPLC determination, however, may be performed on readily available equipment that can be dedicated to analytical programs. Both approaches require that careful consideration be given to the preliminary chemistry before any reliable analytical results are obtained. Table II gives the retention times for the compounds, as well as summary information on the crops we have investigated.

TABLE II

HPLC PARAMETERS FOR CARBOFURAN AND METABOLITES

The hydrolysis step was included for all compounds except I.

Commodity and compound		*Column*	*Mobile phase methanol–water*	*Flow-rate (ml/min)*	*Retention time (min)*	*Recovery*		*Detector wavelength (nm)*
						ppm	*%*	
Carrot roots	I	C_{18}	50:50	1	5.0	1.0	95	200
	I	C_8	35:65	2	3.4	0.1	100	200
	II	C_{18}	30:70	1	5.7	0.4	95	200
	II	C_8	35:65	2	3.8	0.4	95	200
	III	C_8	40:60	2	5.5	0.6	73	203
	IV	C_8	40:60	2	3.1	0.6	100	203
	VI	C_8	40:60	2	6.8	0.6	100	203
Carrot tops	I	C_{18}	50:50	1	6.1	–	–	200
	II	C_8	45:55	2	3.0	0.4	98	200
	III	C_8	40:60	2	7.6	–	–	203
	IV	C_8	40:60	2	4.1	–	–	203
	VI	C_8	40:60	2	9.8	–	–	203
Blueberries	I	C_8	45:55	2	3.0	0.4	79	200
	II	C_8	45:55	2	5.3	0.4	100	200
	III	C_8	40:60	2	6.3	50	203	
	IV	C_8	40:60	2	3.4	0.6	75	203
	VI	C_8	40:60	2	7.9	0.6	60	203
Grapes	I	C_{18}	60:40	1	4.5	0.1	88	200
	II	C_{18}	30:70	1	5.9	0.5	86	200
Spinach	I	C_{18}	50:50	1	6.4	0.2	100	200
Cabbage	I	C_{18}	50:50	1	6.5	0.2	85	200

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