their request, for analysis and placement in the Institute's permanent collection.

The depression of cholinesterase activity by carbofuran is not excessive, as shown in both Figures 3 and 4. Organophosphorus compounds used caused severe inhibition of this enzyme's activity down to 0.01% of the control value for over 50 days. Using these compounds, however, mortality was only 7%. It would appear that the ability of carbofuran to inhibit cholinesterase is not the reason for its toxicity to *L. terrestris*. It is obvious, however, that the enzyme activity depression promotes many of the reactions described. Worms given both small (0.5 mg/kg) and severe doses of paraoxon (15 mg/kg) showed coiling and rigidity. Even with the highest dose, these symptoms seldom persisted more than 2 days, although cholinesterase levels remained below normal throughout the experimental period of 6 days (Figure 3).

Carbofuran metabolism has been studied by several authors, including Metcalf *et al.* (1968), Dorough (1968a,b), Ivie and Dorough (1968), and Ashworth (1969). *Lumbricus terrestris* would appear to metabolize the insecticide initially in a similar fashion to both plants and animals. Hydroxylation at the 3 position causes formation of 3-hydroxycarbofuran (2,3-dihydro-2,2-dimethyl-3-hydroxybenzofuran-7-yl *N*-methylcarbamate) and subsequent cleavage of the carbamine group produces 3-hydroxycarbofuranphenol (2-3-dehydro-2,2-dimethyl-3,7-dihydroxybenzofuran). Traces of other allied compounds normally found after metabolism of carbofuran by insects were detected but not qualified or quantified. The metabolite(s) "Unknown I" found excreted was very polar and was not further separated, but moved as a single spot in the acetonitrile–water solvent system.

Excretion of less than 10% of the total amount of insecticide taken up originally would appear to be connected with the reabsorption of excreted materials by the worms. The nature of the tissue bound radioactive material probably derived from previously excreted metabolites of [^{14}C]carbofuran requires further study. Reabsorption and maintenance of excreted material, especially carbofuran and 3-hydroxycarbofuran, will increase the effectiveness of this insecticide as a toxicant to the earthworm. Efforts to find the rates of production of metabolites and a quantitative analysis of these compounds in the worm will be made.

LITERATURE CITED

Ashworth, R. J., Ph.D. Thesis, North Carolina State University, Department of Crop Science, Raleigh, N. C., 1969.
Aspoeck, H., an der Lan, H., *Z. Angew. Zool.* **50**, 343 (1963).
Davidson, J. D., Oliverio, V. T., Peterson, J. J., in "Liquid Scintillation Counting," Bransome, E. D., Jr., Ed., Grune and Stratton, New York, London, 1970, p 222.
Dorough, H. W., *Bull. Environ. Contam. Toxicol.* **3**, 164 (1968a).
Dorough, H. W., *J. Agr. Food Chem.* **16**, 319 (1968b).
Ellman, G. L., Courtney, K. D., Anders, V., Jr., Featherstone, R. M., *Biochem. Pharmacol.* **7**, 88 (1961).
Ivie, G. W., Dorough, H. W., *J. Agr. Food Chem.* **16**, 849 (1968).
Metcalf, R. L., Fukuto, T. R., Collin, C., Borck, K., Abd El-Aziz, S., Munoz, R., Cassil, C. C., *J. Agr. Food Chem.* **16**, 300 (1968).
Nurnberg, E., *Deut. Apoth. Zt.* **101**, 268 (1961).
Stenersen, J., *J. Chromatogr.* **54**, 77 (1971).
Thompson, A. R., *Bull. Environ. Contam. Toxicol.* **5**, 577 (1970).

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Method for High-Speed Liquid Chromatographic Analysis of Benomyl and/or Metabolite Residues in Cow Milk, Urine, Feces, and Tissues

Joseph J. Kirkland

Residues of benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] and/or methyl 2-benzimidazolecarbamate, methyl 5-hydroxy-2-benzimidazolecarbamate, and methyl 4-hydroxy-2-benzimidazolecarbamate may be simultaneously determined in cow milk, tissues, urine, and feces. The first step in the method consists of hydrolyzing the sample in aqueous acid to convert benomyl to methyl 2-benzimidazolecarbamate and to free the metabolites from conjugates. The freed materials are then extracted into an organic solvent, the extract is cleaned up by a solvent–solvent partitioning process, and the components are determined in a single scan by high-speed strong cation exchange liquid chromatography. Recoveries of the various components average about 80% in cow milk and urine, with average recoveries of about 50–80% obtained from tissue samples and feces. Recoveries for the various compounds have been demonstrated at the 0.01–0.02-ppm level in cow milk, at the 0.05–0.1-ppm level in tissues and feces, and at the 0.2-ppm level in urine. No interference with the method was found from a number of other pesticides with tolerances in milk and meat tissues.

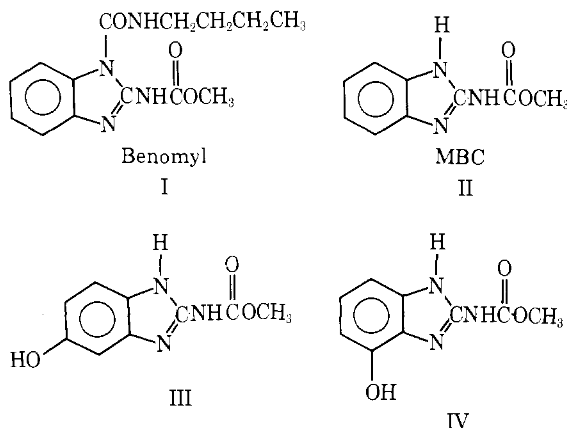
Analytical methods have previously been given for determining residues of benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate], the active ingredient in DuPont Benlate benomyl fungicide (Pease and Gardiner, 1969; Pease and Holt, 1971). These procedures involve the quantitative conversion of benomyl to methyl 2-benzimidazolecarbamate, sometimes called MBC, then to 2-aminobenzimidazole (2-AB) by a two-stage acid-base hydrolysis procedure. Hence, benomyl, its principal degradative product, MBC, and a minor component of the residue in plants, 2-AB, are measured as a composite value by fluorometry or an alternate colorimetric procedure.

Methyl 2-benzimidazolecarbamate has been identified as a major benomyl breakdown product in aqueous solution and within plants (Clemons and Sisler, 1969; Fuchs *et al.*, 1970; Peterson and Edgington, 1969; Sims *et al.*, 1969). This compound has also been proposed as a possible intermediate in the conversion of benomyl to methyl

Biochemicals Department, Experimental Station, E. I. du Pont de Nemours & Company, Wilmington, Delaware 19898.

5-hydroxy-2-benzimidazolecarbamate identified as a major metabolite in the urine of rats after enzyme hydrolysis to liberate glucuronide and/or sulfate conjugates (Gardiner *et al.*, 1968). Another minor metabolite, methyl 4-hydroxy-2-benzimidazolecarbamate, has also been identified in the urine of animals fed a diet containing benomyl (Gardiner *et al.*, 1972).

This paper will describe a routine, high-speed liquid chromatographic method for simultaneously determining residues of benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] (I) and/or methyl 2-benzimidazolecarbamate (II), methyl 5-hydroxy-2-benzimidazolecarbamate (III), and methyl 4-hydroxy-2-benzimidazolecarbamate (IV) in cow milk, urine, feces, and tissues.



The first step in the analytical method consists of heating the sample with aqueous acid to hydrolyze (I) to (II) and free the metabolites from conjugates. The freed materials are extracted into an organic solvent, and this crude extract is cleaned-up by a solvent partitioning process. The various components of interest are then measured in a single strong cation exchange high-speed liquid chromatographic separation. Depending on the time needed for sample preparation, 2–4 samples per day can be routinely analyzed by an operator using the procedure herein described.

EXPERIMENTAL SECTION

Apparatus and Reagents. Liquid chromatographic measurements were performed with a custom-built apparatus equivalent to that previously described (Kirkland, 1968, 1969a,b). A highly sensitive ultraviolet photometer operated at 254 nm was used as the detector. Commercial liquid chromatographic equipment capable of carrying out this method is now available from the Instrument Products Division, E. I. du Pont de Nemours & Company, Wilmington, Del. (Model 820 or 830 liquid chromatographs). A more complete description of the chromatographic equipment and some of the experimental parameters are given below.

Centrifugation was carried out with an International Model uv, or equivalent, suitable for handling 250-ml bottles. Measurements of pH were made with a meter equipped with a glass-calomel electrode (standardized to pH 7) and a magnetic stirrer. Samples were prepared in a Waring Blendor with a semimicro stainless steel jar having a 360-ml capacity. Solvent concentration was performed in a Calab (Berkeley, California) or Buchler (Model PTFE-1GN) rotary evaporator equipped with a 500-ml flask and a water bath for heating the evaporator flask to 60°. Extractions were carried out in 125- or 250-ml separatory funnels equipped with Teflon stopcocks. Certain phase transfers were made with 100-ml glass syringes with 12-gauge square-tipped needles.

Reference samples were obtained from the Biochemicals Department, Agrichemicals Sales Division, E. I. du Pont de Nemours & Co., Inc., Wilmington, Del. The solvents used were distilled-in-glass, purchased from Burdick and Jackson Laboratories, Inc., Muskegan, Mich.

Hydrolysis and Extraction of Milk. Weigh 25.0 g of milk into a 250-ml beaker and add 10 ml of 15 *N* phosphoric acid (1 vol of 85% phosphoric acid into 2 vol of distilled water). Cover the beaker and heat on top of a steam bath for 1 hr. Cool the solution and adjust to pH 6.5 by adding 50% sodium hydroxide dropwise to the solution, stirred magnetically, while monitoring with a pH meter. The 50% sodium hydroxide should be added slowly so that the temperature of the solution does not exceed 70°. Losses of metabolites III and IV can occur at higher temperatures because of the labile nature of these compounds. Losses of sample due to spattering can also take place. Should the pH be overrun to the basic side during this adjustment, 2 *N* hydrochloric acid should be added promptly to establish the desired pH 6.5.

If the milk sample contains a very high fat content, it may be necessary to make a single clean-up extraction of the fatty material from this hydrolyzed solution before adjusting the pH. This is accomplished by placing the sample in a 250-ml centrifuge tube, adding 50 ml of hexane, and mixing well by continuously inverting the tube for 1 min *without* vigorously shaking the contents of the tube. The mixture is then centrifuged for 10 min at 2000 rpm. Remove the upper phase with a 100-cm^3 hypodermic syringe and discard.

After adjusting the pH to 6.5, cool the solution and quantitatively transfer to a 250-ml centrifuge bottle. At this point the solution should be sufficiently cool to allow extraction with ethyl acetate without difficulty of pressure buildup in the centrifuge bottle as a result of the increased vapor pressure from the solvent. Extract the sample with 100 ml of ethyl acetate by continuously inverting the centrifuge tube for 1 min, but *without* vigorously shaking the contents of the tube. Excessive agitation at this point may result in the formation of emulsions which cannot be broken by normal centrifugation. Centrifuge the mixture for 10 min at 2000 rpm and remove the upper phase with a 100-cm^3 hypodermic syringe. Care should be taken so that none of the intermediate phase containing water is removed with the upper phase in this step.

Pass this organic phase through a 1½-in. bed of anhydrous sodium sulfate contained in a 45° funnel, and collect in a 500-ml round-bottomed evaporator flask. Repeat the extraction with two additional 100-ml portions of ethyl acetate.

Evaporate the combined dried ethyl acetate extract to about 15 ml in the rotary evaporator and quantitatively transfer the concentrated extract to a 125-ml separatory funnel using ethyl acetate washes. Extract the organic phase (now about 25 ml) with two 25-ml portions of 0.1 *N* hydrochloric acid, transferring the aqueous acidic phases to a 125-ml separatory funnel. Back-wash the combined aqueous acid phase with 50 ml of ethyl acetate, discarding the organic layer after phase separation. Quantitatively transfer the acidic phase to a 150-ml beaker and adjust to pH 6.5 by adding 1 *N* sodium hydroxide dropwise while stirring and monitoring with a pH meter. If the pH is overrun during adjustment at this point, 0.1 *N* hydrochloric acid should be added promptly to adjust to the desired pH 6.5.

Quantitatively transfer the pH-adjusted solution to a 250-ml separatory funnel and extract this material with four 50-ml portions of ethyl acetate. Pass each organic extract successively through a 1.5-in. bed of anhydrous sodium sulfate contained in a 45° funnel and deliver the dried extract into a 500-ml round-bottomed rotary evaporator flask. Concentrate the combined extract to about 5 ml in the rotary evaporator and quantitatively transfer this concentrate to a 30-ml beaker with ethyl acetate washes. Evaporate the organic solvent just to dryness using a

stream of dry nitrogen. Dissolve the residue in 0.1 *N* phosphoric acid (by slightly heating on a steam bath to place all residue in the solution if necessary) and quantitatively transfer to a 1-ml volumetric flask using 0.1 *N* phosphoric acid washes. Dilute the flask to volume with 0.1 *N* phosphoric acid.

Samples should be analyzed as quickly as possible after completion. Should a delay be necessary, the final extracts should be retained in a refrigerator or preferably a freezer until analysis. If samples have been stored in this manner, they should be warmed sufficiently to ensure that all materials contained in the sample are again dissolved. If it becomes necessary to interrupt the extraction scheme because of time limitations, the samples should be retained at a step in which the sample is in an acidic aqueous medium. It is preferred that the samples be taken as far through the extraction scheme as possible before such an interruption is made. Interrupted samples should always be stored in a freezer, preferably only for an overnight period, before continuing the extraction scheme.

Hydrolysis and Extraction of Cream and Feces Samples. The same procedure is used as for milk as described above, except that to the 25-g sample is added 50 ml of distilled water and 25 ml of 15 *N* phosphoric acid in the initial step. Continue the analysis as described for the analysis of milk above.

Hydrolysis and Extraction of Urine. The same procedure is used as for milk described above, except that to the 25 g of urine is added 25 ml of 15 *N* phosphoric and 1 g of solid sodium bisulfite in the first step. The analysis is continued as described for milk.

Hydrolysis and Extraction of Animal Tissues. Reduce the tissue into small pieces no larger than ½-in. cubes. Weigh out 25.0 g of this material and add the pieces slowly to 75 ml of 15 *N* phosphoric acid contained in a Waring Semimicro Blendor operated at full speed. [Note: Occasional low recoveries of III and IV from liver and kidney may be avoided by adding 1 g of solid sodium bisulfite at this point.] Continue the blending for about 1 min after the last piece of meat is added to the blender; the total time for addition and grinding should amount to about 3 min. Transfer the heavy slurry to a 250-ml beaker, using a small amount of 15 *N* phosphoric acid as wash liquid. Cover the beaker and heat for 1 hr *inside* a steam bath. Cool the mixture and adjust the pH to 6.5 with 50% sodium hydroxide solution, while stirring, using the pH meter as a monitor. Addition of the sodium hydroxide should be made slowly, since this slurry is sometimes rather thick and mixing may be slow. The temperature of the slurry during this pH adjustment step can be maintained below 70° by cooling in an ice bath.

Should the tissue sample contain a high level of fatty material, it may be necessary to preextract the sample with hexane prior to this pH adjustment. Subcutaneous beef fat must be treated in this manner. This preextraction is accomplished by dividing the sample slurry into approximately equal quantities and transferring it into two 250-ml centrifuge bottles. Seventy-five milliliters of hexane is added to each tube, the tubes are capped, and the mixture is shaken vigorously for 1 min. Centrifuge the tubes at 2000 rpm for 10 min, remove the upper phase with a 100-ml syringe, and discard.

After adjustment of the thick slurry to pH 6.5 as described above, cool the mixture and transfer approximately equal quantities into two 250-ml centrifuge bottles. Add 75 ml of ethyl acetate to each tube, cap, and shake vigorously for 1 min. Centrifuge the tubes at 2000 rpm for 10 min. Remove the upper phase with a 100-ml syringe and pass it through a 1.5-in. bed of anhydrous sodium sulfate contained in a 45° funnel. Repeat the extractive operation on the contents of both tubes using two more 75-ml portions of ethyl acetate.

Continue with the extraction procedure, starting with the fifth paragraph under the procedure for milk described above, beginning, "Evaporate the combined . . . ."

Liquid Chromatographic Analysis. Liquid chromatographic analysis is performed with a 1000 mm by 2.1 mm i.d. stainless steel column containing Zipax SCX strong cation exchange packing. This packing or a prepacked column may be obtained from the Instrument Products Division, E. I. du Pont de Nemours & Co., Inc., Wilmington, Del. The column is equilibrated at 60° with a carrier composed of seven parts 0.15 *N* sodium acetate and three parts of 0.15 *N* acetic acid (pH 5.15).

Calibration. To calibrate the chromatographic system, 80 μl of a solution containing 20, 10, and 4 μg per milliliter of compounds II, III, and IV, respectively, in 0.10 *N* phosphoric acid are injected into the column held at 60° with a carrier flow rate of 0.5 cm^3 per minute and a detector sensitivity of 0.04 absorbance, full-scale. Under these conditions, IV and III elute in about 7.2 and 8.6 min, respectively. Fifteen minutes after sample injection, the flow rate is increased to 1.5 ml per minute; II elutes in about 22 min from the start of the chromatogram.

Calibration factors are determined for each compound by measuring peak heights and dividing the micrograms per milliliter in the calibration solution by the peak height in chart divisions. This calculation produces a calibration factor in micrograms/milliliter/chart division. Calibration solutions should be chromatographed daily. A range of concentrations of the desired subject compounds produces linear calibration plots, the slopes of which show less than 10% variation from day to day. Therefore, a single calibration point is usually adequate to establish the slope of the curve for any day.

Liquid Chromatographic Analysis of Extract from Unknown Sample. An 80-μl aliquot of the final extract from the unknown sample obtained from the extraction procedures described above is chromatographed, using the conditions described in the Calibration Section. [If the final extract exhibits turbidity, it should first be filtered through a 1.5-μ Millipore membrane (URWPO1300 or equivalent) held in a Swinney filter, both available from Millipore Corporation, Bedford, Mass.]

RESULTS

As shown in Tables I–V, recoveries of benomyl and its metabolites average about 80% in cow milk and urine, with average recoveries of about 50–80% obtained from tissue samples and feces. Method sensitivity is estimated at 0.02 ppm in milk for I and/or II, and at 0.01 ppm each for III and IV. In the urine, method sensitivity is estimated at 0.1 ppm for all compounds due to variability of backgrounds for different samples. In feces, recoveries at the 0.05-ppm level were shown for III and IV, and recoveries at the 0.1-ppm level were obtained for I and/or II. For cow tissues, estimated sensitivity limits are 0.1 ppm for I and/or II in liver, kidney, fat, and lean muscle. For compounds III and IV, sensitivity limits are estimated at 0.05 ppm in these same tissues. Interferences were sometimes encountered for compound IV in just the liver and kidney samples, such that a sensitivity of 0.05 ppm could not always be reached. No such interferences were encountered for compound III, the major metabolite, in any of the tissues.

Representative chromatograms obtained from various recovery studies are shown in Figures 1–3. It should be noted that all of these chromatograms were obtained with the custom-built apparatus.

Simplified studies have indicated that 28 other pesticides with tolerances in milk and meat tissues cause no interference with the analytical procedure for determining residues of benomyl and its hydroxylated metabolites. The equivalent of 0.5 ppm of residues of each of these

Table I. Milk Recovery Study

25-g sample					
Level of fortification, ppm			% recovery		
I/II[a]	III[b]	IV[c]	I/II	III	IV
0	0	0			
0	0	0			
0.02	0.01		100	115	
0.02	0.01		120	77	
0.02	0.01		100	77	
0.04	0.02		60	58	
0.04	0.03		90	90	
0.02	0.05		100	100	
0.08	0.08		90	70	
0.08	0.08		95	95	
0.20	0.10		64	66	
0.20	0.10		88	81	
0.8	0.8		90	82	
1.0	1.0		72	69	
1.0	1.0		88	73	
8.0	8.0		83	95	
		0.02		83	83
		0.10		79	79
0.05	0.05	0.05	120	64	100
0.10	0.10	0.06	70	70	67
		Average	86%	83%	82%

[a] II = methyl 2-benzimidazolecarbamate. [b] III = methyl 5-hydroxy-2-benzimidazolecarbamate. [c] IV = methyl 4-hydroxy-2-benzimidazolecarbamate.

Table II. Separated Milk (Milk Fat) Recovery Study

25-g sample					
Level of fortification, ppm			% recovery		
II[a]	III[b]	IV[c]	II	III	IV
0.04	0.02		100	96	
0.04	0.04		83	85	83
		0.02			
		Average	91%	90%	

[a] II = methyl 2-benzimidazolecarbamate. [b] III = methyl 5-hydroxy-2-benzimidazolecarbamate. [c] IV = methyl 4-hydroxy-2-benzimidazolecarbamate.

Table III. Feces Recovery Study

25-g sample					
Level of fortification, ppm			% recovery		
II[a]	III[b]	IV[c]	II	III	IV
0.10	0.05	0.05	64	31	25
0.20	0.52		62	75	
2.0	4.2		78	88	
		Average	68%	65%	

[a] II = methyl 2-benzimidazolecarbamate. [b] III = methyl 5-hydroxy-2-benzimidazolecarbamate. [c] IV = methyl 4-hydroxy-2-benzimidazolecarbamate.

compounds was injected directly into the liquid chromatograph. No detector response was found in the region of interest for 21 of these compounds shown in Table VI. The seven remaining compounds, as given in the table, showed some positive detector response, usually in the form of elevated baselines and shoulders of peaks in the region of interest. Therefore, these seven compounds were then added to milk at levels of 0.4–0.5 ppm, and carried through the entire extraction, isolation, and liquid chromatographic measurement procedure. No interference from any of these

Table IV. Tissue Recovery Study

25-g sample									
Tissue sample	Level of fortification, ppm			% recovery			Average % recovery		
	II[a]	III[b]	IV[c]	II	III	IV	II	III	IV
Liver	0.08	0.08		65	60		57	54	
	0.08	0.08		75	55				
	0.10	0.05		28	37				
	1.0	1.0	0.1	60	65	*d*			
Kidney	0.08	0.08		85	60		76	59	
	0.08	0.08		80	55				
	0.05	0.10		69	46				
	1.0	1.0		68	73				
Subcutaneous fat	0.08	0.08		110	75		77	85	63
	0.10	0.10		80	73				
	1.0	0.52		40	108				
			0.05			75			
	0.40	0.40	0.40	72	80	50	69	67	
Lean muscle	0.05	0.05		69	62				
	0.08	0.08		80	80				
	0.48	0.21		58	58				

[a] II = methyl 2-benzimidazolecarbamate. [b] III = methyl 5-hydroxy-2-benzimidazolecarbamate. [c] IV = methyl 4-hydroxy-2-benzimidazolecarbamate. [d] Background interference; not able to measure.

Table V. Urine Recovery Study

25-g sample					
Level of fortification, ppm			% recovery		
II[a]	III[b]	IV[c]	II	III	IV
0.20	0.52		72	60	
0.48	1.0		*d*	88	
0.80	0.80		95	90	
0.80	0.80		90	85	
1.0	4.2		*d*	87	
2.0	10		*d*	91	
		0.19			82
		1.9			73
		Average	86%	74%	78%

[a] II = methyl 2-benzimidazolecarbamate. [b] III = methyl 5-hydroxy-2-benzimidazolecarbamate. [c] IV = methyl 4-hydroxy-2-benzimidazolecarbamate. [d] Background interference; not able to measure.

compounds was shown. These data indicate that the compounds of interest are free from interference and verify the unique selectivity of the total method.

DISCUSSION

At the onset of this work, the liquid chromatographic analysis of benomyl metabolites involved the use of a custom-built or "breadboard" chromatograph and column made up of component parts, since commercial equipment for high-speed liquid chromatography was not available (Kirkland, 1968, 1969a,b). Most of the analytical data obtained in this study were taken with this system. However, for this analytical procedure to be used effectively by other workers, the utilization of commercially available apparatus and columns is desirable. Consequently, the original procedure was later slightly modified to adapt it to equivalent equipment and columns now commercially available. The recommended procedure described in the Experimental Section involves the use of the commercial apparatus. Identical analytical information has been obtained from both experimental systems, even though the experimental conditions were slightly different in the two cases.

A comparison of the separations performed by the cus-

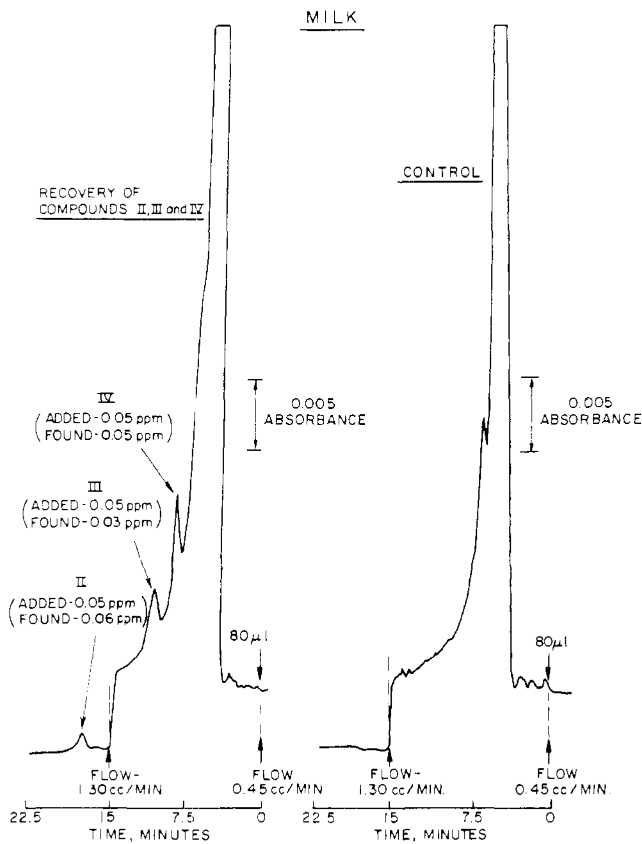


Figure 1. Liquid chromatograms of cow milk extracts. Column, 1 m × 2.1 mm strong cation exchange (Zipax SCX); carrier, 0.1 *M* acetic acid–0.1 *M* sodium acetate; temperature, 60°.

Table VI. Interference Study

Compound tested	
Methomyl	Chloroneb metabolite (phenol)
Thiabendazole	Lindane
Dicamba	Ronnel
Monuron	Dalapon
Lasso	Heptachlor
2,6-Diethylaniline	Guthion
Ethion	*N*-Isopropylaniline (related to Ramrod)
Strobane	Diphenyl amine[a]
Malathion	Atrazine[a]
DDT	Sevin[a]
Simazine	Diuron[a]
Tetradifon	Linuron[a]
Methoxychlor	Methyl parathion[a]
Chloroneb	Ramrod[a]

[a] Compounds showing a positive detector response upon direct injection into the liquid chromatograph usually in the form of elevated baselines and shoulders in the regions of interest. No interference from these materials found when carried through the entire isolation-analysis procedure.

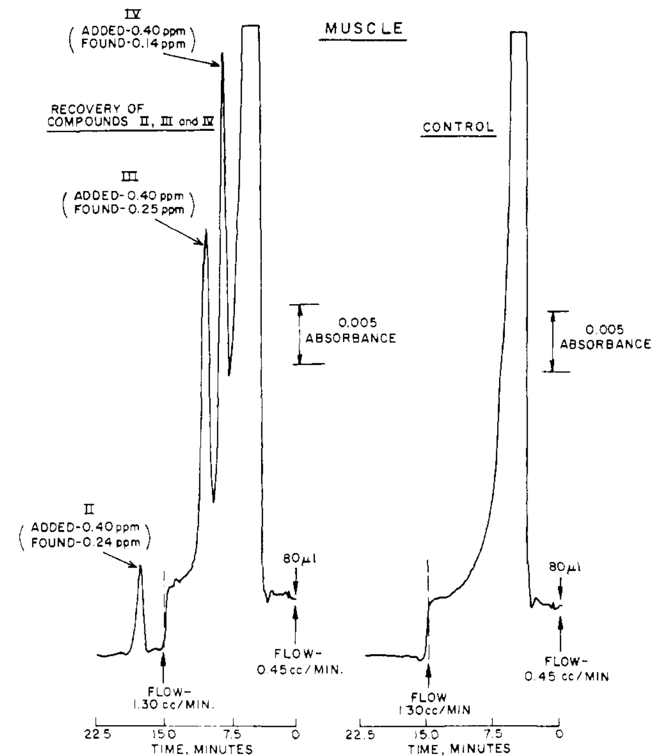


Figure 2. Liquid chromatograms of cow muscle extract. Conditions same as Figure 1.

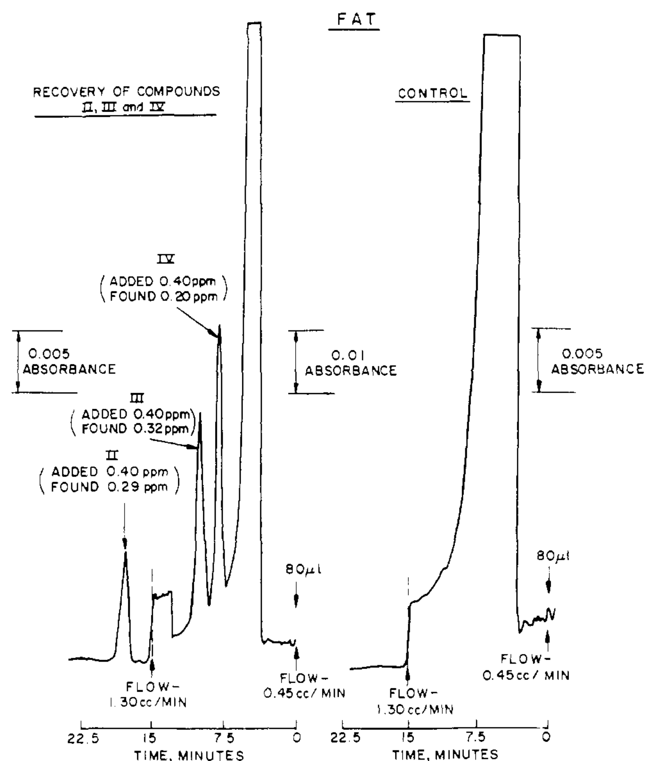


Figure 3. Liquid chromatograms of cow fat extracts. Conditions same as Figure 1.

tom and commercial liquid chromatographic equipment is shown in Figure 4. Minor adjustments in the strength of the eluent and flow rate have resulted in similar retention times and resolutions for the compounds of interest with the two approaches. (Note the difference in sample size in the two separations.) However, increasing the flow to elute compound (II) with the commercial liquid chromatographic unit results in a shifting baseline at the high detector sensitivity used. This baseline drift is related to a change in temperature in the detector cell at the increased flow. Less baseline shift is evident with the custom unit, since the detector cell was thermostated at the temperature of the column. The drifting baseline in the chromatogram from the commercial unit does result in a factor of about 2, lower detectability for compound II. However, the baseline for the hydroxy-substituted metabolites III and IV in extracts is usually somewhat better with the commercial instrument. Also, certain tissue ex-

Table VII. Comparison of Milk Samples Analyzed by Custom-Built and Commercial Liquid Chromatographic Apparatus

Level of fortification, ppm			Found, ppm					
			II		III		IV	
II[a]	III[b]	IV[c]	Custom	Commercial	Custom	Commercial	Custom	Commercial
	Control		n.d.,[d] <0.02	n.d., <0.02	n.d., <0.01	n.d., <0.01	n.d., <0.01	n.d., <0.01
	Control		n.d., <0.02	n.d., <0.02	n.d., <0.01	n.d., <0.01	n.d., <0.01	n.d., <0.01
0.05	0.05	0.02	0.06	0.05	0.03	0.03	0.03	0.02
0.10	0.10	0.10	0.07	0.08	0.07	0.06	0.05	0.03

[a] II = methyl 2-benzimidazolecarbamate. [b] III = methyl 5-hydroxy-2-benzimidazolecarbamate. [c] IV = methyl 4-hydroxy-2-benzimidazolecarbamate. [d] n.d. = not detected.

tracts, particularly liver and kidney, often show a better separation of the hydroxylated metabolite peaks from the extract background when using the commercial apparatus. The end result reflects no clear advantage for either the custom or the commercial systems. Table VII shows the type of crosscheck data which is obtained between the two systems with milk samples fortified with II and the two hydroxylated metabolites III and IV. Even though some of the samples were kept for over a month and subjected to repeated freezing and thawing cycles, there is reasonably good agreement between data obtained with the two analytical systems.

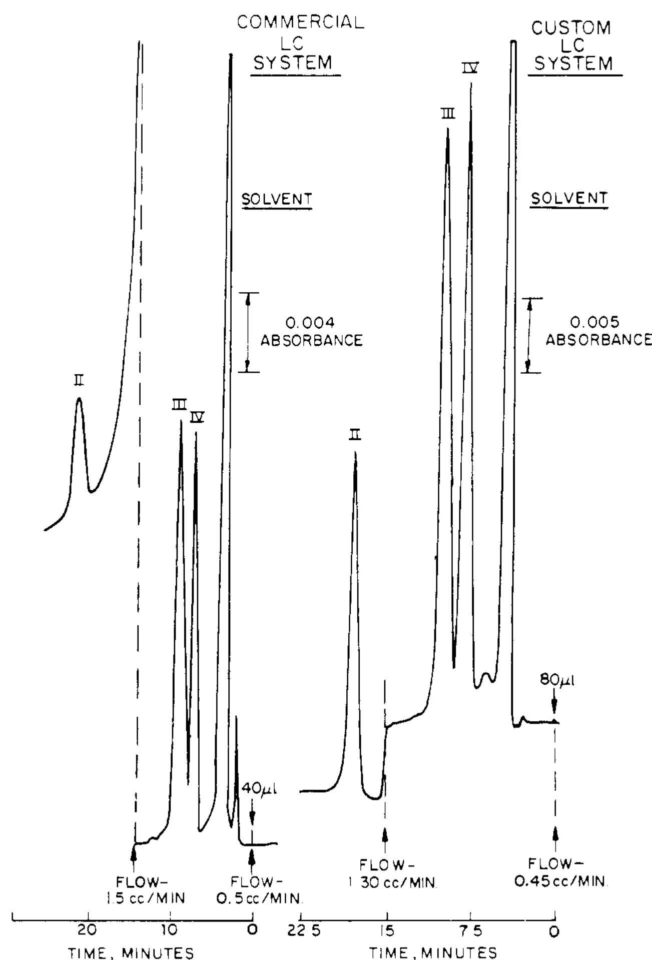


Figure 4. Comparison of standard chromatograms from custom and commercial instruments. Right: custom lc system; column, 1 m × 2.1 mm experimental Zipax SCX; carrier, equal parts 0.1 *M* acetic acid–0.1 *M* sodium acetate; temperature, 60°. Left: Commercial lc system (DuPont Model 820 liquid chromatograph); column, 1 m × 2.1 mm i.d. Zipax SCX; carrier, 3 parts 0.15 *M* acetic acid–7 parts 0.15 *M* sodium acetate; temperature, 60°.

The hydroxylated metabolites III and IV have substituted phenolic structures which are easily oxidized when unduly exposed to air and other oxidizing media. Compound IV is the more sensitive of these two compounds. As a result of this situation, the isolation procedure has been devised to minimize losses of these metabolites by oxidation reactions. Bisulfite is added as a reducing agent to certain matrices, and prompt execution of the isolation procedure without interruption is recommended to reduce oxidation losses. Nevertheless, casual handling of the isolation procedure and the final extracts can result in a loss in compounds III and IV, particularly the latter because of its higher susceptibility to oxidation. Some of the lower yields reported for compound IV in the accompanying tables and figures undoubtedly reflect this condition.

The high selectivity of the method is obtained due to: (1) the unusual selectivity of the partitioning–clean-up step; (2) the classifying nature of the ion exchange liquid chromatographic separation employed; and (3) the selective response of the ultraviolet photometric detector used. The isolation and clean-up steps in the analytical method are highly selective, since the procedure is specifically designed to recover only those compounds which have much higher solubility in aqueous acid than in ethyl acetate. Therefore, acidic compounds are not recovered in the final extract and organosoluble compounds are eliminated during the various partitioning steps.

Additional selectivity is afforded in the method by the liquid chromatographic measurement. To be retained and chromatographed by the strong cation exchange system used, components must be soluble in the carrier and contain an ionized or ionizable basic functional group for the cationic exchange retention process. In addition, any potential interfering material must also respond to the ultraviolet detector. One of the best indications of the high selectivity of the method is the ability to obtain "clean," reproducible control scans from meat tissues and milk, both of which contain a very large number and variety of potentially interfering substances.

Preliminary studies indicated that the use of a photometric detector system operating at 280 nm results in greater freedom from interferences than a 254-nm detector, because of the better selectivity afforded by the longer wavelength device. Operation at 280 nm also provides a somewhat higher sensitivity for the subject compounds, since this wavelength is closer to the absorption maxima for these materials. With the 280-nm detector, there is also less background absorption at the region in which compound IV elutes. Therefore, added selectivity is afforded for the hydroxylated metabolites with this detector. Unfortunately, photometric detectors with a 280-nm source are less widely used, more expensive, and generally have a substantially narrower linear dynamic range of operation. Thus, while the 280-nm photometric detector may offer advantages in some situations, use of this device is not required since the 254-nm detector operates satisfactorily with the system described.

The high-speed ion exchange chromatography permitted in this method is the result of using a column containing porous layer bead-type particles. The theoretical chromatographic advantages of column packings for liquid chromatography having a porosity limited to its outer shell have already been discussed (Horne *et al.*, 1966; Kirkland, 1969b, 1971a; Knox, 1966). Liquid chromatographic separations have already been demonstrated with solid-core "pellicular" and superficially porous ion-exchange particles prepared by coating glass beads with ion-exchange resins of the same particle size (Horvath *et al.*, 1967; Kirkland, 1969b, 1970). These studies clearly indicate the speeds up to an order of magnitude faster which can be obtained with solid-core spherical particles coated with a thin film of ion exchange medium compared to chromatography with conventional resin ion exchangers of the same particle size. The sites of ion exchange interaction are readily accessible in the superficially porous particles, and mass transfer can be effected more rapidly because of the lack of deep pores of stagnant mobile phase which are present in conventional ion exchange resins. In addition, the normal particle-diffusion (stationary phase) rate controlling process, the process normally dominating the efficiency characteristics of ion-exchange liquid chromatographic columns, is readily improved by the use of thin ion-exchange films.

The packing used in this study consists of Zipax controlled surface porosity support coated with a thin film of a polyfluorinated aliphatic sulfonic acid. This strong sulfonic acid polymer is highly stable chemically because of its polyfluorinated character, and is a stronger acid than the usual sulfonic acid ion exchangers. A description of this ion exchanger has been given in other publications (Kirkland, 1969b, 1970, 1971b).

Long-term routine analyses of sample extracts have shown that the strong cation exchange column used in this procedure is very durable and can be used for a long period of time before a replacement is required. Up to 18 months of continuous service has been obtained on these columns. However, if samples containing undissolved particulate matter are injected into the column, the column packing can become so contaminated that it will not perform the desired analysis. Therefore, samples containing insoluble matter should be filtered before they are chromatographed. If the column does become overly contaminated because of heavy retention of soluble impurities, it can easily be regenerated by pumping 1 M nitric acid at 60° through the column for about 2 hr at a rate of approximately 1 cm^3/min. After reequilibration with the carrier phase, the column should return to its normal operating condition. If this treatment does not result in the proper elution pattern for the compounds of interest, the column should then be replaced.

This study has demonstrated the usefulness of modern high-efficiency liquid chromatography for conducting a sensitive, selective analysis for certain pesticide residues and metabolites that do not lend themselves to analysis by widely-used gas chromatographic techniques because of lack of volatility or instability at the high temperatures required. The versatile column techniques of liquid-partition, liquid-solid (adsorption), and ion-exchange chromatography appear uniquely suited for solving problems involving such materials.

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LITERATURE CITED

Clemons, G. P., Sisler, H. D., *Phytopathology* **59**, 705 (1969).
Fuchs, A., Homans, A. L., de Vries, F. W. *Phytopathol. Z.* **69**, 330 (1970).
Gardiner, J. A., Brantley, R. K., Sherman, H., *J. Agr. Food Chem.* **16**, 1050 (1968).
Gardiner, J. A., Kirkland, J. J., Pease, H. L., Wall, E. N., Morales, R., unpublished studies, 1972.
Horne, D. S., Knox, J. H., McLaren, L., *Separ. Sci.* **1**, 53 (1966).
Horvath, C. G., Preiss, B. A., Lipsky, S. R., *Anal. Chem.* **39**, 1422 (1967).
Kirkland, J. J., *Anal. Chem.* **40**, 391 (1968).
Kirkland, J. J., *J. Chromatogr. Sci.* **7**, 7 (1969a).
Kirkland, J. J., *J. Chromatogr. Sci.* **7**, 361 (1969b).
Kirkland, J. J., *J. Chromatogr. Sci.* **8**, 72 (1970).
Kirkland, J. J., "Modern Practice of Liquid Chromatography," Wiley-Interscience, New York, N. Y., 1971a.
Kirkland, J. J., U. S. Patent 3,577,266 (May 4, 1971b).
Knox, J. H., *Anal. Chem.* **38**, 253 (1966).
Pease, H. L., Gardiner, J. A., *J. Agr. Food Chem.* **17**, 267 (1969).
Pease, H. L., Holt, R. F., *J. Ass. Offic. Anal. Chem.* **54**, 1399 (1971).
Peterson, C. A., Edgington, L. V., *J. Agr. Food Chem.* **17**, 898 (1969).
Sims, J. J., Mee, H., Erwin, D. C., *Phytopathology* **59**, 1775 (1969).

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