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RESOLUTION, SENSITIVITY AND SELECTIVITY OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC POST-COLUMN FLUOROMETRIC LABELING TECHNIQUE FOR DETERMINATION OF CARBAMATE INSECTICIDES

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SUMMARY

N-Methyl carbamate insecticides and carbamate metabolites are separated on octylsilane and cyanopropylsilane bonded packings with an acetonitrile–water gradient mobile phase. The eluted carbamates are detected at the nanogram and subnanogram levels with a fluorescence detector after in-line hydrolysis to methylamine and subsequent reaction with *o*-phthalaldehyde and 2-mercaptoethanol to form the fluorophore. Using this high-performance liquid chromatographic (HPLC) technique, no response was observed for 182 pesticides including various amides, anilides, anilines, carboximides, N-methyl carbamates, triazines, ureas and other nitrogen-containing pesticides. The HPLC post-column fluorometric system provides the resolution, sensitivity and selectivity desired for the determination of multicarbamate insecticide residues.

INTRODUCTION

The increasing use of N-methyl carbamate insecticides in agriculture has created a need for a selective, sensitive technique capable of multiple detection of these thermally labile compounds in a single analysis.

Sparacino and Hines[1] reported the use of high-performance liquid chromatography (HPLC) for the separation of 25 carbamate pesticides (insecticides, herbicides and fungicides) and five carbamate metabolites on normal- and reversed-phase columns. Twenty of the compounds were separated on a single 10-μm octadecylsilane bonded packing using an acetonitrile–water gradient elution system. Moye *et al.*[2] reported an HPLC post-column fluorometric labeling technique for determination of carbamate insecticides. The carbamates were separated on a reversed-phase column, then hydrolyzed in-line under alkaline conditions to methylamine, followed by a subsequent reaction of the methylamine with *o*-phthalaldehyde and 2-mercaptoethanol and the resulting fluorophore was monitored with a fluorescence detector. The post-column reaction parameters were recently studied and the system was refined[3].

This report describes work undertaken to study the resolution, sensitivity and selectivity obtainable with the HPLC post-column fluorometric labeling technique.

Investigations were conducted to select the reversed-phase column packings which provided good resolution and sensitivity of the carbamate insecticides. The selectivity of this technique was investigated by evaluating the response and retention characteristics of 235 pesticides. The HPLC columns and mobile phase found most acceptable for separation of the carbamates and metabolites of interest are given in the Experimental section.

EXPERIMENTAL

Reagents

Water was purified using a Milli-Q water purification system from Millipore (Bedford, Mass., U.S.A.). Methanol and UV grade acetonitrile were "Distilled in Glass" quality from Burdick and Jackson (Muskegon, Mich., U.S.A.). The purified water, methanol and acetonitrile were degassed just prior to use by applying a vacuum to the solvents for 5 min. A 0.05 *M* sodium hydroxide solution was prepared from reagent-grade sodium hydroxide and degassed purified water. A 0.05 *M* sodium borate solution was prepared by dissolving reagent-grade sodium tetraborate decahydrate in hot degassed purified water. The solution was cooled to room temperature (25°) before diluting to volume. The *o*-phthalaldehyde–2-mercaptoethanol reaction solution was prepared by dissolving 500 mg *o*-phthalaldehyde, "Fluoropa", from Durrum (Palo Alto, Calif., U.S.A.) in 10 ml methanol contained in a 1-l volumetric flask, followed by addition of approximately 500 ml of 0.05 *M* borate buffer and 1.0 ml 2-mercaptoethanol from Aldrich (Milwaukee, Wis., U.S.A.). The solution was diluted to volume with 0.05 *M* borate solution. Pesticide standard solutions (100, 10 and 0.1 μg/ml) were prepared in methanol from reference standards obtained from U.S. Environmental Protection Agency (Washington, D.C., U.S.A.).

Chromatographic system

A schematic diagram of the HPLC fluorometric system used in the study is shown in Fig. 1. The pesticide solutions were injected onto reversed-phase HPLC analytical columns equipped with a guard column using a Valco (Houston, Texas,

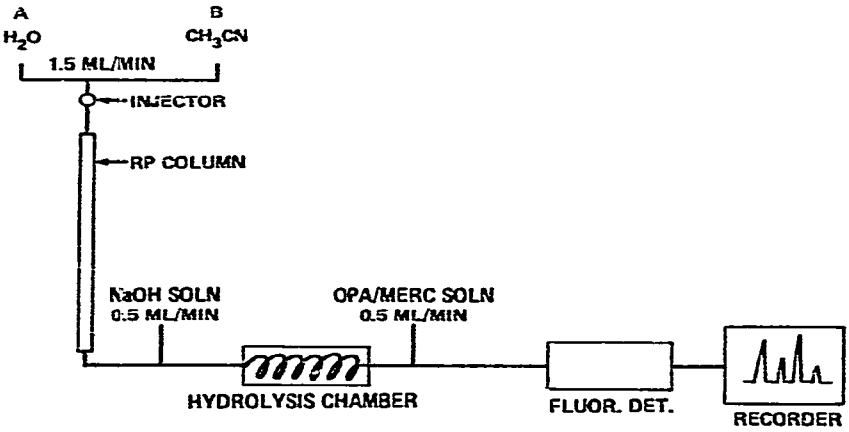

Fig. 1. HPLC post-column fluorometric system. OPA = *o*-Phthalaldehyde; MERC = 2-mercaptoethanol. Hydrolysis chamber: 3-m coil, 100°. Fluorometric detector: excitation wavelength 340 nm, emission wavelength 455 nm.

U.S.A.) Model 16 AS-7000 automatic sampler with 10-μl injection loop. The guard column (7 cm × 2.1 mm I.D.) contained 25–37-μm Co:Pell ODS packing from Whatman (Clifton, N.J., U.S.A.). The analytical column (25 cm × 4.6 mm I.D.) contained 6-μm Zorbax C-8 or CN spherical particles from DuPont (Wilmington, Del., U.S.A.). A 30-min linear gradient from 12 to 70% acetonitrile in water was used to elute the carbamates. The column was maintained at 30° in a custom-built, forced air oven. The gradient was formed and pumped through the column at 1.5 ml/min with an Altex (Berkeley, Calif., U.S.A.) Model 322 MP programmable liquid chromatograph. A 0.05 *M* sodium hydroxide solution was added to the column effluent at 0.5 ml/min through a Valco stainless-steel (SS) tee (0.74 mm I.D.) attached to the exit of the column. The resulting alkaline effluent flows into a 3 m × 0.48 mm I.D. No. 321 SS tubing hydrolysis coil from Tubesales (Englewood, N.J., U.S.A.). This coil was maintained at 100° in an 18 × 18 × 13 cm column oven (obtained from a Model 5360 Barber-Colman gas chromatograph) controlled by a Model 700-115 proportional temperature controller from RFL Industries (Boonton, N.J., U.S.A.). A back pressure of 3.4 bar was applied to the hydrolysis coil to prevent boiling of the alkaline solution by using a restriction coil after the detector. After hydrolysis, 0.5 ml/min *o*-phthalaldehyde–2-mercaptoethanol buffered reaction solution was added through a second 0.74-mm I.D. SS tee. Fluorescence was monitored with a Perkin-Elmer (Norwalk, Conn., U.S.A.) Model 650-10LC detector equipped with a 20-μl cell. Excitation and emission detector wavelengths were set at 340 and 455 nm, respectively; slit widths were set at 15 and 12 nm, respectively. A 1-sec time constant was used. Sensitivity of the detector was adjusted such that 10 ng of carbofuran produced 50% full scale response on a Spectra-Physics Model 4000 microprocessor–printer plotter (Santa Clara, Calif., U.S.A.). (Detector PM gain, low; sensitivity range, 10; sensitivity fine, 6; and printer plotter attenuation, 5.)

The post-column reactants were contained in 60 cm × 25 mm I.D. glass column reservoirs equipped with PTFE fittings from Glenco (Houston, Texas, U.S.A.). The reaction solutions were added to the column effluent through a 6 m × 0.5 mm I.D. PTFE restriction coil by maintaining constant pressure (*ca.* 60 p.s.i.g.) on the reservoirs with nitrogen. No. 321 SS tubing (1.6 mm O.D. × 0.018 mm I.D.) was used to connect injector, column and first tee.

RESULTS AND DISCUSSION

Resolution

Resolution of the carbamate insecticides was studied by comparing acetonitrile and methanol as organic modifiers in the aqueous mobile phase used with reversed-phase HPLC columns. Acetonitrile, which is less viscous, provided superior resolution of the carbamates and doubled peak height sensitivity.

Several commercial alkylsilane bonded reversed-phase columns were investigated as to their ability to separate the carbamate insecticides using the acetonitrile–water mobile phase. The commercial columns varied from 25 to 30 cm in length and 3.9 to 4.6 mm I.D. and contained spherical or irregular shaped silica from 5 to 10 μm diameter bonded with octyl or octyldecylsilane groups. The columns containing the 5- and 6-μm spherical particles produced the best separation efficiencies. Of the alkyl reversed-phase packings investigated, the ten carbamate insecticides that may be used on crops or in food processing facilities and six of their metabolites (see Table I)

TABLE I

RETENTION DATA FOR TEN CARBAMATE INSECTICIDES AND SIX METABOLITES USING C-8 AND CN HPLC COLUMNS

No.	*Carbamate or metabolite*	*Retention time relative to carbofuran*	
		C-8 Column	*CN Column*
1	Aldicarb sulfoxide	0.33	0.29
2	Aldicarb sulfone	0.40	0.39
3	Oxamyl	0.44	0.39
4	Methomyl	0.46	0.43
5	3-Hydroxy carbofuran	0.60	0.55
6	Methiocarb sulfoxide	0.64	0.59
7	Methiocarb sulfone	0.79	0.86
8	Aldicarb	0.83	0.76
9	Propoxur	0.98	0.95
10	Carbofuran	1.00	1.00
11	Bendiocarb	1.00	1.04
12	Carbaryl	1.06	1.21
13	α-Naphthol	1.09	1.30
14	Landrin*	1.15	1.25
15	Methiocarb	1.26	1.45
16	Bufencarb**	1.44***	1.60***

* 3,4,5- and 2,3,5-trimethyl isomers (4:1).

** Mixture of 1-methylbutyl and 1-ethylpropyl phenyl N-methylcarbamates (3:1) with 70% *meta*-, 20% *para*- and 4% *ortho*-isomers.

*** Major peak.

were most effectively separated with a Zorbax C-8 column. Additional separation selectivity was obtained by also using a Zorbax CN column. These packings consist of 6-μm spherical silica particles bonded with monofunctional octyl and cyanopropylsilane reagents, respectively. Fig. 2 shows the separations obtained for 10 ng each of the 16 compounds with the two columns using a 12 to 70% acetonitrile in water 30-min linear gradient. Table I provides peak number identification for Fig. 2 and retention values relative to carbofuran.

The C-8 column completely separated the first 8 compounds; aldicarb sulfoxide, aldicarb sulfone, oxamyl, methomyl, 3-hydroxy carbofuran, methiocarb sulfoxide, methiocarb sulfone and aldicarb. Propoxur was partially resolved from the peak containing carbofuran and bendiocarb. Carbaryl and α-naphthol were almost completely resolved and Landrin, methiocarb and bufencarb were completely separated. (The fluorescent response of carbaryl and α-naphthol will be discussed under *Selectivity*.) Bufencarb is composed of six isomers which were resolved into three peaks. Also the peak for Landrin has a shoulder indicating slight separation of the 2,3,5 and 3,4,5 isomers.

The CN column provided additional separation selectivity in that propoxur, carbofuran, bendiocarb, carbaryl and α-naphthol are completely separated. Aldicarb eluted before methiocarb sulfoxide and Landrin before α-naphthol with this column; however, aldicarb sulfone and oxamyl eluted as one peak. In general the compounds have shorter retention times with the CN column.

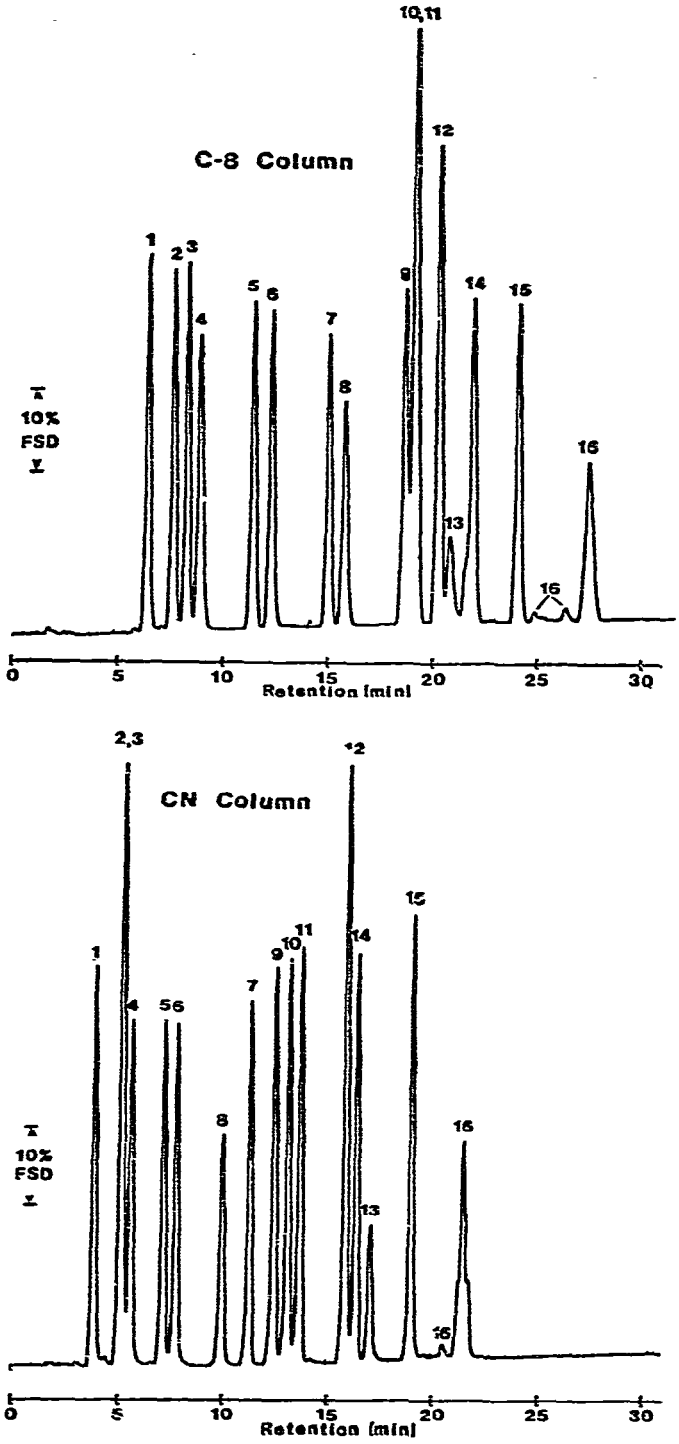

Fig. 2. Chromatograms of ten carbamates and six metabolites at 10-ng level using Zorbax C-8 and CN columns; for identification of peaks see Table I.

Thus, separation efficiency was increased by using the smaller column packing materials and lower viscosity mobile phase, and separation selectivity was increased by using two different reversed-phase columns. These findings agree with Snyder and Kirkland's[4] discussion on HPLC resolution.

Sensitivity

The carbamate sensitivity obtained using the described HPLC system is shown in Figs. 2 and 3. In Fig. 2 the detector sensitivity was adjusted to provide 1/2 full scale deflection (f.s.d.) of printer/plotter to 10 ng of carbofuran as given in Experimental. Baseline noise is less than 1% and drift approximately 2.5% with the C-8 column. The detector sensitivity was increased in Fig. 3 such that 1 ng of the carbamates produced 30–40% f.s.d. This increased sensitivity increased baseline noise to 2% and drift to approximately 25%. The drifting baseline may be due to the change in background fluorescence as mobile phase composition is changed, and appears to be the most limiting factor in increasing sensitivity beyond this. A multicarbamate residue method[5], using this technique at the 10 ng carbofuran 1/2 f.s.d. sensitivity, allows detection of the carbamates at the 10 ppb (10 μg/kg) level with 10% f.s.d. peak heights.

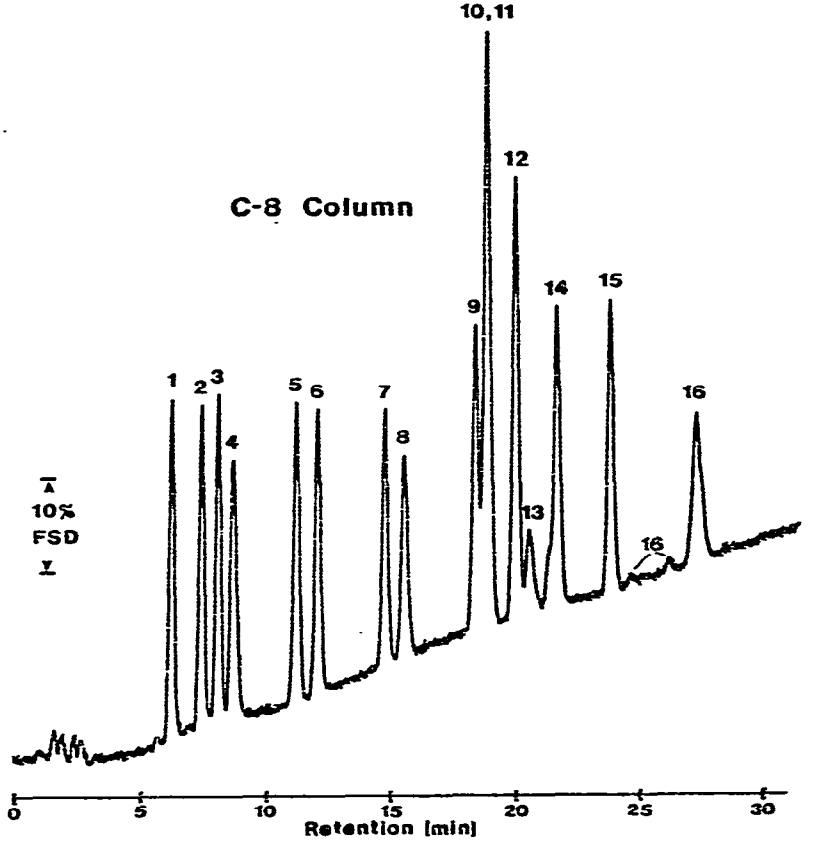

Fig. 3. Chromatogram of ten carbamates and six metabolites at 1-ng level; for identification of peaks see Table I. Detector adjusted to maximum sensitivity.

TABLE II

NON-FLUORESCING PESTICIDES USING PARAMETERS GIVEN IN EXPERIMENTAL

Non-fluorescing pesticides are defined here as those pesticides which produce less than twice baseline noise level response for 1000 ng of pesticide injected unto a C-8 column with detector sensitivity adjusted to provide ½ f.s.d. for 10 ng carbofuran.

Amides
- CDEA
- 2-(3-Chlorophenoxy)α-propionamide
- Desmethyl diphenamide
- Diphenamide
- Naphthalene acetamide
- Naproamid
- Solan

Amines
- Aminobutane hydrochloride
- Diphenylamine

Anilides
- Alachlor
- Carboxin
- Carboxin sulfoxide
- Cypromid
- Delachlor
- Dicryl
- Niclosamide
- Propachlor
- Propanil
- Prynachlor

Anilines
- Butralin
- Dichloran
- Dinitroamine
- Hydroxy pendimethalin
- Isopropalin
- Nitralin
- Pendimethalin
- Pentachloroanaline
- Prodiamine
- Prynachlor hydrolysis product

Benzimidazoles
- 2-Aminobenzimidazole
- BH-584
- Fenazaflor
- Fenazaflor metabolite A
- Fenazaflor metabolite B
- Methyl 2-benzimidazole carbamate (MBC)
- Thiabendazole

Benzothiazoles
- TCMTB

Bipyridinium compounds
- Diquat dibromide
- Paraquat bis methyl sulfate
- Paraquat dichloride

Bis-carbamates
- Thiophanate-methyl

Carbamates, N-dimethyl
- Dimetilan
- Isolan
- Pirimicarb
- Pyrolan

Carbamates, N-phenyl
- Barban
- Chlorpropham
- Desmedipham
- Phenmedipham
- Propham
- Swep

Carboximides
- Captafol
- Captan
- MGK 264
- Norbormide
- Tetramethrin

Dithiocarbamates
- CDEC
- Ferbam
- Maneb
- Metam-sodium
- Metiram
- Nabam
- Zineb
- Ziram

Hydrazines
- β-Hydroxyethylhydrazine

Hydrazones
- Banamite

Naphthylenes (also listed under other pesticide types)
- NPA sodium salt
- NPA
- Naproamid
- Naphthalene acetamide

Nitriles
- Dichlobenil

Organophosphates (containing various nitrogen groups)
- Acephate
- Aminoparathion
- Chlorpyriphos
- Chlorpyriphos oxygen analog
- Cythioate
- Dialifor
- Diamidfos
- Diazinon oxygen analog
- Dicrotophos
- Dimethoate oxygen analog
- Dimefox
- Famphur
- Famphur oxygen analog
- Fenamiphos
- Glyphosate
- Glyphosine
- Methamidophos
- Methidathion
- Phosmet
- Phosmet oxygen analog
- Phosphamidon
- Pirimiphos-ethyl
- Pirimiphos-ethyl oxygen analog
- Schradan
- Thionazin
- Thionazin oxygen analog
- Zytron

Phthalimides
- Emmi
- Folpet

Pyridines
- Avitrol 100

(Continued on p. 622)

TABLE II *(continued)*

Quinones	Metribuzin DA	Benzadox
Dichlone	Prometone	Carbofuran, 7-phenol
	Prometryn	Carbofuran, 3-hydroxy, 7-phenol
Sulfonamides	Propazine	Chloramben
WARF	Secbumeton	Chlordimeform
Ceresan M	Simazine	Chlorphenamidine hydrochloride
	Simetone	Cycloate
Sulfonilamides	Simetryne	Cycloheximide
Oxyzalin	Terbuthylazine	Daminozide
	Terbutryne	Dinoseb triethylamine
Thiocarbamates, N-dimethyl		Dodine
Butylate	*Uracils*	Etridazole
Diallate	Bromacil	ETU
EPTC	Terbacil	Fenaminosulf
Ethiolate		Glyodin
Pebulate	*Ureas*	Glyodin, free base
Triallate	Chlorbromuron	Mercaptobenzothiazole
Vernolate	Chloroxuron	Molinate
	Cisanilide	NPA sodium salt
Toluidines	DCPMU	Nicotine
Benfluralin	DCPU	Nitrapyrin
Chlornidine	Diuron	Norea
Profluralin	Diuron TCA	NPA
Trifluralin	Fluometuron	Oxadiazon
	Fenuron	Panogen
Triazines	Metobromuron	Phenothiazine
Ametryne	Monuron	Picloram methyl ester
Anilazine	Neburon	Pyrazon
Atratone	Siduron	Quinomethionate
Atrazine		Rotenone
Cyanazine	*Miscellaneous*	RP-2929
Cyprazine	Amitrole	Thiram
Cyprazine hydroxy analog	Banamite metabolite	
Dipropetryn	BETU	
Metribuzin		

Thus, the maximum usable sensitivity is determined by detector signal to noise ratio, baseline drift and resolution.

Selectivity

The selectivity of this technique was studied by injecting individually 1000 ng of each of 235 pesticides onto the C-8 column. The detector sensitivity was adjusted to give 1/2 f.s.d. response for 10 ng of carbofuran. The compounds injected included the carbamate insecticides, their metabolites, other insecticides, herbicides and fungicides which were known to fluoresce naturally, had structures indicating natural fluorescence or contained nitrogen groups that could potentially react with the post-column reactants to form fluorescent products.

No fluorescent HPLC peaks were observed for the 182 pesticides listed in Table II. Amides, anilides, analines, carboximides, N-dimethyl carbamates, N-dimethyl thiocarbamates, dithiocarbamates, N-phenyl carbamates, toluidines, triazines and ureas are examples of the types of nitrogen containing pesticides that were

not detected. Naphthylene and quinone compounds are known to fluoresce naturally; however, five compounds of this type were not detected. The phenolic metabolites of carbofuran, which do not contain the carbamate moiety, did not fluoresce.

The response and retention data (relative to carbofuran) on the C-8 column of the remaining 53 pesticides are presented in Table III. Sixteen carbamate insecticides and eight metabolites exhibit fluorescence with peak height response relative to carbofuran of from 0.46 to 1.44. The phenolic moiety of carbaryl, α-naphthol, fluoresces naturally in basic media, thus accounting for the increased fluorescent response of carbaryl and the fluorescence of its metabolite, α-naphthol. The carbamate insecticides, dichlormate, formetanate hydrochloride, karbutilate and thiofanox, are apparently only partially hydrolyzed as their response relative to carbofuran was 0.0003, 0.0059, 0.0089 and 0.022, respectively, much lower than the other carbamates. Bendiocarb, carbofuran and karbutilate have the same retention on the C-8 column; however,

TABLE III

RETENTION AND RESPONSE OF PESTICIDES WHICH FLUORESCE; RELATIVE TO CARBOFURAN ON C-8 COLUMN

Sensitivity of detector adjusted to provide ½ f.s.d. for 10 ng carbofuran. Carbofuran retention approximately 20 min.

Pesticide	*Relative retention*	*Relative response*	*Pesticide*	*Relative retention*	*Relative response*
Picloram	0.12	0.0018	Propoxur*	0.98	1.02
Maleic hyrazide	0.15	0.0012	Bendiocarb*	1.00	0.99
Nitrapyrin metabolite	0.18	0.0003	Carbofuran*	1.00	1.00
Bentazon	0.19	0.0024	Karbutilate*	1.00	0.0089
Asulam	0.24	0.0002	Mobam*	1.02	1.04
Aldicarb sulfoxide*	0.33	1.07	Ethoxyquin	1.04	0.0013
Dimethyl, 2,4-D acetamide	0.35	0.0002	Carbaryl*	1.06	1.44
2,4-D dimethyl amine salt	0.35	0.0002	Thiofanox*	1.08	0.022
β-Naphthoxy acetic acid	0.37	0.0004	α-Naphthol*	1.10	0.47
Aldicarb sulfone*	0.40	1.13	Landrin*	1.14	0.90
Oxamyl*	0.44	1.05	Norflurazon	1.16	0.0009
Methomyl*	0.46	1.01	Chloramben methyl ester	1.18	0.013
Formetanate hydrochloride*	0.54	0.0059	Carbanolate*	1.18	0.95
Perfluidone	0.56	0.0002	Warfarin	1.22	0.0045
Duraset	0.57	0.0004	Dichlormate	1.23	0.0003
Thiofanox sulfoxide*	0.57	0.46	Methiocarb*	1.26	0.97
3-Hydroxy carbofuran*	0.60	0.99	Promecarb*	1.31	0.99
Methiocarb sulfoxide*	0.64	0.86	Azinphos-methyl	1.32	0.014
Dimethoate	0.67	0.0002	Methazole	1.36	0.11
Tranid*	0.67	0.84	Benomyl**	1.23, 1.41	
Thiofanox sulfone*	0.69	0.46	Bufencarb*	1.44	0.53
Methiocarb sulfone*	0.79	0.90	Azinphos-ethyl	1.49	0.0088
AIBA	0.80	0.33	Coumaphos	1.60	0.0002
Naphthylene acetic acid	0.82	0.0002	Phosalone	1.64	0.0012
Aldicarb*	0.83	0.72	Mexacarbate*	2.12	0.60
Azinphos-methyl oxygen analog	0.84	0.0094	Aminocarb*	3.87	0.48
Coumafuryl	0.94	0.0005			

* Carbamate insecticides.

** Multiple peaks from R_c 1.23 to 1.41, major peak at 1.41. Relative response could not be calculated. After 3 days, no peak observed.

bendiocarb and carbofuran separated on the CN column. Of the non-carbamate insecticides injected, AIBA (a metabolite of bentazon) and methazole exhibited the highest fluorescence with responses relative to carbofuran of 0.33 and 0.11, respectively. AIBA partially elutes with methiocarb sulfone on the C-8 column but the two compounds are separated on the CN column. Methazole does not elute with any of the carbamate insecticides or their metabolites on the C-8 column. Duraset, dimethoate and chloramben methyl ester had the same retention time as the carbamate insecticides/metabolites thiofanox sulfoxide, Tranid and carbanolate, respectively. Naphthylene acetic acid and azinphosmethyl oxygen analog, which fluoresce in basic media, both partially elute with the aldicarb peak. Azinphos-methyl also partially elutes with the carbamate, promecarb. The fluorescent intensity of these non-carbamate insecticides is approximately 100 to 1000 times less intense than carbofuran. Other reversed-phase HPLC columns may enable total separation of the carbamate and non-carbamate pesticides. The naturally fluorescent carbamate fungicide, benomyl, produced multiple peaks from R_c 1.23 to 1.41, the major peak being at 1.41. No peak was observed for benomyl, after the standard solution was three days old, indicating rapid degradation.

Selectivity was thus found to be affected by (1) the response of the detection system to various pesticides, and (2) column retention times of the pesticides detected.

CONCLUSIONS

The HPLC post-column fluorometric determinative system has the resolution, sensitivity and selectivity desired for a multicarbamate insecticide residue determinative technique. The carbamate insecticides permitted for use on crops or in food processing facilities are totally separated by use of the Zorbax C-8 and CN columns. The carbamates can be detected at nanogram and/or subnanogram levels. The preferential response of the detection system to the carbamate insecticides and differing retention times of other pesticides which were detected, makes this technique highly selective for the carbamate insecticides and their metabolites. This determinative technique was incorporated into a multicarbonate insecticide residue method.

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