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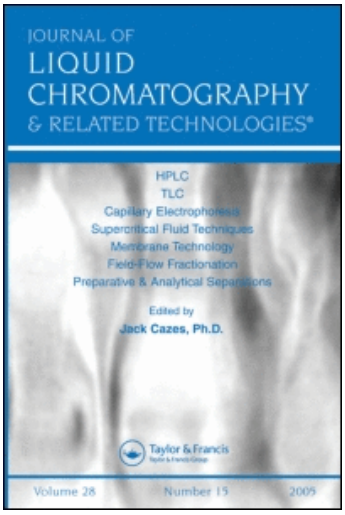

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Fluorometric Determination of Aminocarb and Mexacarbate and Some of Their Metabolites by Liquid Chromatography: Influence of Structural Factors on Fluorescence Intensity

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FLUOROMETRIC DETERMINATION OF AMINOCARB AND MEXACARBATE AND SOME OF THEIR METABOLITES BY LIQUID CHROMATOGRAPHY: INFLUENCE OF STRUCTURAL FACTORS ON FLUORESCENCE INTENSITY

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ABSTRACT

A direct and sensitive high performance liquid chromatographic method with fluorescence detection is reported to identify aminocarb and mexacarbate and some of their metabolites. The observed detection limits were compared by linking the liquid chromatograph to a variable wavelength UV detector. The separation system consisted of an RP-8 OS (10 μm) 20 cm x 4.6 mm I.D. column and acetonitrile-phosphate (pH 7.2) buffer. The excitation and emission wavelengths of the fluorescence detector were set respectively at 200 and 370 nm. The UV detector was set at 242 and 200 nm for aminocarb and mexacarbate, respectively. The sensitivity in fluorescence detection was not superior to UV method because of the influence of substituents on the aryl ring on fluorescence intensity. Both methods were found to be adequate for the determination of most of the analytes from natural water at nanogram levels after necessary extraction and cleanup procedures.

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INTRODUCTION

In previous papers, methods based on high performance liquid chromatography (HPLC) using ultraviolet (UV) absorbance detection (UVD) and post-column derivatization (PCD) to form fluorescent derivatives were reported to quantify two carbamate insecticides, aminocarb (4-dimethylamino-3-methylphenyl N-methylcarbamate) and mexacarbate (4-dimethylamino-3,5-xylyl N-methylcarbamate), used in forest insect control programs in Canada, and some of their common metabolites (1,2). The inherent sensitivity and selectivity of fluorescence detection (FD) in residue analysis has not been explored fully for these compounds although some sporadic attempts have been made earlier (3,4). Aminocarb and mexacarbate and some of their metabolites have intrinsic fluorescence and exhibit measurable fluorometric intensity provided that they are sufficiently excited at a suitable wavelength with a light source, such as a deuterium lamp. The emission wavelength is specific to each molecular species and is measurable using a spectrofluorometric detector. In this paper, we report a simple and direct fluorometric method, without any derivatization, to detect and quantify aminocarb and mexacarbate and some of their metabolites using a fluorescence detector linked to a liquid chromatograph. We have also examined the structural factors of the analytes chromatographed, which influenced, either positively or negatively, the sensitivity and specificity of fluorometric detection. In addition, we have compared the fluorometric sensitivity with the commonly used UVD method. Finally, we used both methods to quantify the analytes in fortified natural water samples to examine the suitability of the FD over UVD method and to evaluate the limits of quantification (LOQ) of the analytes and possible interferences in the chromatographic profiles obtained by both methods.

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MATERIALS AND METHODS

Materials

Analytical grade standards (purity > 98.5 %) of aminocarb and mexacarbate and 14 of their metabolites (7 for each insecticide) used in this study

were supplied respectively by Union Carbide Agricultural Products Co., Inc., Research Triangle Park NC, and Mobay Chemical Corporation, Kansas City, MO. The chemical names of the compounds and abbreviations used for them in this paper are given in Table 1.

Working standards were prepared by diluting 1000 μg/mL stock solutions of the analytes in acetonitrile with aqueous acetonitrile ($CH_3CN:H_2O \doteq 1:2$) to appropriate concentrations. Separate standards were prepared for each analyte to determine its sensitivity and linear range. Mixed standards were prepared consisting of aminocarb and its seven metabolites and mexacarbate and its seven metabolites to study the response of the detectors. The standards were prepared in low actinic stained volumetric flasks and stored at −20°C until use. All standards were thoroughly filtered (Acrodisc® LC13 PVDF, 0.2 μm, Gelman Sciences, Ann Arbor, MI U.S.A.) prior to injection into the HPLC system.

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All solvents and water were HPLC grade supplied by Canlab, Mississauga, Ontario and were tested for their spectral purity prior to use. They were filtered through appropriate Millipore® filters and degassed before use. Alumina (activity 1, type WN-6 neutral), sodium sulphate (anhydrous), potassium dihydrogen phosphate (KH_2PO_4) and sodium hydroxide were of analytical grade and obtained from BDH Chemicals, Canada Ltd., Toronto, Ontario. The buffer solution was prepared by adjusting the pH to 7.2 by adding dropwise 0.10 M sodium hydroxide to 1.0 L of 0.01 M phosphate solution, under magnetic stirring. It was filtered (0.45 μm Millipore filter) and degassed prior to use.

Equipment

A Hewlett Packard (HP)(model 1084B) variable wavelength (190-600 nm UV detector) liquid chromatograph interfaced with a variable volume injector (HP 79842 A) and autosampler (HP 79842) was used. It is also equipped with a microprocessor and an electronic integrator linked to an LC terminal (HP 79850 B) to provide the area, area %, retention time (RT), etc. for each chromatographic peak. The instrument also had automatic degassing system, dual solvent system

TABLE 1

List of Aminocarb and Mexacarbate and Their Carbamate Metabolites Used in the Study

Chemical Name	Abbreviation
4-dimethylamino-3-methylphenyl N-methylcarbamate	A
4-methylformamido-3-methylphenyl N-methylcarbamate	MFA
4-methylamino-3-methylphenyl N-methylcarbamate	MA
4-formamido-3-methylphenyl N-methylcarbamate	FA
4-amino-3-methylphenyl N-methylcarbamate	AA
4-dimethylamino-3-methylphenol	MP
4-methylamino-3-methylphenol	MAP
4-amino-3-methylphenol	AMP
4-dimethylamino-3,5-xylyl N-methylcarbamate	M
4-methylformamido-3,5-xylyl N-methylcarbamate	MFM
4-methylamino-3,5-xylyl N-methylcarbamate	MAM
4-formamido-3,5-xylyl N-methylcarbamate	FM
4-amino-3,5-xylyl N-methylcarbamate	AM
4-dimethylamino-3,5-xylenol	DMAX
4-methylamino-3,5-xylenol	MAX
4-amino-3,5-xylenol	AX

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and dual pump heads with common drive to give stable and reproducible flows. A prepacked HP RP-8 OS (10 μm) stainless steel separation column (20 cm x 4.6 mm I.D.) was used in conjunction with an HP RP-8 guard column (3 cm x 4.6 mm I.D. x 10 μm) throughout this work. The column temperature was kept at 30°C to maintain RT reproducibility and the injection volume used was 100 μL. The fluorescence detector was a Kratos FS 970 LC fluorometer (Kratos Analytical Instruments, Ramsey, NJ) equipped with a 10 μL flow cell and automatic overload reset (FSA 986) with variable excitation wavelengths (GM 970 monochrometer) and fixed wavelength emission filters. Additional operating parameters were as follows:

Mobile system:	Acetonitrile - 0.01 M phosphate buffer (pH 7.2)	
Flow rate:	1.0 mL/min	
Run time:	60 min	
Gradient:	Time(min)	% CH_3CN
	0	0
	25	30
	35	50
	45	50
	55	0
Wavelengths (nm):	Aminocarb	Mexacarbate
UV (sample:reference)	242:430	200:430
FD (excitation:emission)	200:370	200:370

Although the excitation and absorption spectra of many compounds in dilute solutions are nearly identical (5), the use of different wavelengths in absorption and fluorescence for aminocarb was necessary, as a compromise, to enhance the detection sensitivity of some of its metabolites. Also it is observed generally that the use of short excitation wavelength (200 nm) in the fluorometric detection of aminocarb and mexacarbate resulted in higher emission intensities, thus more sensitive detection limits for the analytes studied. In addition, the

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percent composition of the mobile phase constituents in the gradient system was adjusted after repeated trials to give optimum resolution of the peaks.

Method validation

With all system components in place in the UVD system, the column was equilibrated with mobile phase at a flow rate of 1 mL/min until a steady baseline was obtained. The same was repeated for the FD system and intensities are recorded for solvent responses at the chosen wavelengths for necessary corrections. To evaluate sensitivity, individual standard solutions of aminocarb, mexacarbate and their metabolites were injected five times, first with UVD and then with the FD system. Mixed standard solutions were then injected to obtain good resolution of the peaks, reproducible peak area measurements and retention times under the chosen experimental conditions. The coefficient of variation (CV) between injections ranged from 1.7 % to 3.4 % for UVD and 2.7 % to 5.6 % for FD, depending on the type of analyte. Replicate analysis of mixed standards of the insecticides at four day intervals gave good reproducibility (av. CV < 5 %) in both the detector systems. To establish linearity of the detectors, the individual analytes, ranging in concentration from 0.005 μg/mL to 10.0 μg/mL in acetonitrile, were chromatographed, each in triplicate, using both the detectors successively. The linearity of each compound was established by linear regression analysis of peak area responses versus concentration. The correlation coefficients ranged from 0.97 to 1.00 indicating that UVD and FD methods are suitable to analyze the compounds listed in Table 1.

Recovery of analytes from stream water

The procedure used was similar to the one reported recently (2). In brief, water samples (100 mL) fortified with the analytes (0.5 and 5 μg/mL) were serially extracted with dichloromethane, dried with anhydrous sodium sulphate, flash-evaporated to dryness and the residue in acetonitrile was partitioned with hexane. After flash evaporation of the polar layer, the crude residue in ethyl

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acetate was cleaned up over an alumina minicolumn, eluted with ethyl acetate or ethyl acetate/methanol solution depending on the type of analyte, the volume was then adjusted and analyzed by HPLC using the UV and fluorescence detectors. The retention times and peak areas were compared with those of the standard solutions.

Unfortified water samples and reagent blanks were extracted and analyzed following the described method. No interfering peaks corresponding to any of the analytes studied were found.

RESULTS AND DISCUSSION

Optimization of chromatographic response

The LOQ in HPLC depends to a large extent: (a) on the type of detector; (b) the wavelength used for detection (UV) or excitation (FD) and (c) the chromatographic column. Fluorescence is often 10- to 100-fold more sensitive than absorption, provided the analytes have appreciable native fluorescence (6). However due to low quantum efficiency of these molecules, the FD measurements have nearly the same sensitivity as in UV detection. The use of 242 nm for aminocarb and 200 nm for mexacarbate, found by ultraviolet scans, as optimum absorption wavelengths, gave good chromatographic response to the parent materials and their metabolites. Any alteration in the wavelengths resulted in reduced sensitivity (especially for the metabolites) and/or increased background noise due to impurities in the mobile phase. The same is true for the selection of 200 nm, as the excitation wavelength for FD, which resulted in reasonably high emission intensities for the fluorescent analytes and low background fluorescence.

Based on our earlier studies (1,2), the HP RP-8 10 μm - 20 cm column gave better resolution of chromatographic peaks, which are symmetrical, compared to the shorter columns with 5 μm column packing. The use of acetonitrile - phosphate buffer as the mobile phase increased chromatographic efficiency (better resolution of peaks) compared to either methanol - water or acetonitrile - water

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used previously (1,2). The buffer system was also necessary to reduce tailing of the phenol metabolites which were not previously studied (1,2). The system chosen was also sufficiently selective to resolve, within the chromatographic run time of 60 minutes, most of the analytes (except FA/AA and AM/MAX) belonging to each insecticide.

Chromatograms of the analytes

Figures 1 and 2 illustrate the separation achieved for aminocarb and its metabolites using the UV and fluorescence detectors respectively. Figures 3 and 4 illustrate the same for mexacarbate and its metabolites. The concentration shown in the figures is 1.0 μg/mL for all the analytes. This is below the LOQ in FD for AMP, MAP, AX and MAX. Their retention times only, without their chromatographic response, are depicted in figures 2 and 4. Mean RT (min) obtained for each individual compound after replicate determinations (n>5) using the UVD are given in the caption of figures 1 and 3. The variation in RT was less than 3 % for all the analytes.

The elution patterns of both insecticides show that more hydrophilic analytes, especially the phenols with some exceptions, and aldehydes, eluted sooner (low RT) compared to the hydrophobic moieties, such as the parent insecticides. The elution region of FA and AA in the UVD are nearly similar, therefore it is not possible to detect and quantify them from the mixed standards unless the analytes were injected separately. However FA is non-fluorescent, therefore the analysis of AA in the mixed standard by fluorescent detection is feasible with direct sample injection. A similar situation is also encountered between AM and MAX. MAX does fluoresce, but its LOQ is 1000 ng, compared to 20 ng for AM, so the interference from MAX is minimal. The aldehyde metabolites, MFA, FA, MFM and FM are quantified only by using the UV absorbance detector, because fluorescence detection is not possible for them, due to lack of sufficient quantum efficiency. The metabolites MAP, AMP, MAX and AX all have low fluorescent signals and the use of FD to quantify them is not

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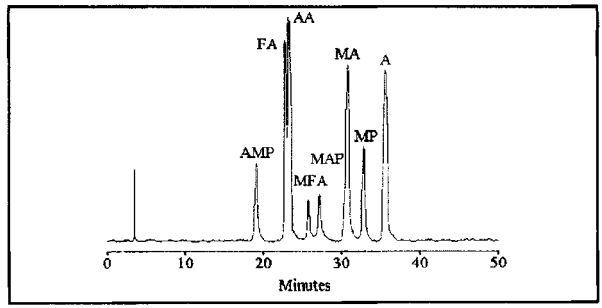

Figure 1: Chromatogram of aminocarb and metabolites by UVD with RT (min): A=35.94; MFA=25.83; MA=30.90; FA=23.80; AA=24.22; MP=32.44; MAP=27.12; AMP=19.06.

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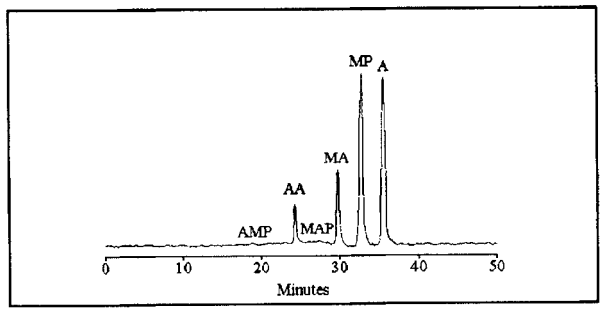

Figure 2: Chromatogram of aminocarb and its metabolites by FD. Retention times set to match with UVD values.

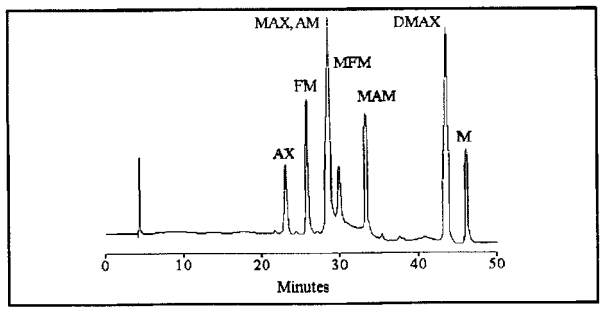

Figure 3: Chromatogram of mexacarbate and metabolites by UVD with RT (min): M=46.11; MFM=30.02; MAM=33.11; FM=25.85; AM,MAX=28.86; DMAX=43.43; AX=23.02.

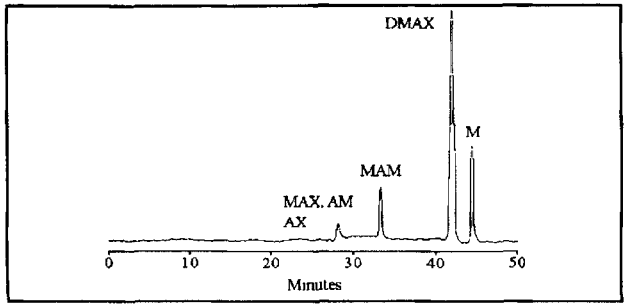

Figure 4: Chromatogram of mexacarbate and its metabolites by FD. Retention times set to match with UVD values.

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desirable. Among the analytes studied, DMAX was highly sensitive to the HPLC-fluorescence technique, giving a sharp symmetrical peak even at the 1 ng level.

The LOQ (taken arbitrarily as five times signal/noise ratio) for aminocarb and its metabolites for ultraviolet detection, in the standard solutions, ranged from 5 to 50 ng, whereas for mexacarbate the values ranged from 5 to 20 ng. However, in the fluorometric method, the LOQ for the fluorescent moieties ranged from 5 to 1000 ng for aminocarb and 1 to 1000 ng for mexacarbate. Therefore, it is apparent that fluorometric quantification for these series of compounds is less sensitive than UV detection, unless a post-column derivatization technique (2) is adapted to improve the situation.

Structural factors affecting fluorescence

Aminocarb and mexacarbate and some of their metabolites listed in Table 1 exhibit weak native fluorescence due to the interaction among delocalized π orbital electrons of the aryl (Ar) ring, lone electron pairs on the N atom of NMe_2 group and O atom of the C_{Ar}-O-C group. In addition, the methyl (Me) and NMe_2 groups attached to the Ar ring are electron donors, thus increasing the electron density on the Ar ring, which facilitates fluorescence. On the other hand, the

presence of Me groups (one in aminocarb and two in mexacarbate) in the *o*-position to the NMe_2, interfere sterically and inhibit the overlap of lone pair electrons on the NMe_2 group with the π electrons of the Ar ring, reducing fluorescence intensity (7).

The four compounds, MFA, FA, MFM and FM, did not show any measurable fluorescence, because of the presence of the -CHO group, which is a strong electron withdrawer from the Ar ring. Thus fluorescence detection is not feasible for these molecules with direct sample injection. Successive demethylation to form NHMe and NH_2 from NMe_2 also diminished the electron density on the AR ring, leading to higher LOQ levels, increasing progressively from MAM (5 ng) to MA (10 ng) and finally to AA, AM (20 ng).

Among the six phenols, DMAX exhibited maximum fluorescence, probably due to the formation of highly conjugated phenoxide ion by the loss of proton accelerated by the presence of electron donating Me and NMe_2 groups on the Ar ring. However, the fluorescence intensity diminished (or the fluorometric LOQ increased) from DMAX to MP, due to the absence of electron donating Me groups in the latter. On the other hand, the other four phenols, MAX, AX, MAP and AMP, all show low quantum efficiency or low fluorescence intensity (LOQ 1000 ng), although the phenolic group is an electron donor. The reasons for this are not clear, however we can speculate the formation of a protonated structure, such as $Me_2H\text{-}N^+\text{-}Ar\text{-}O^-$ established by the interaction between the non-bonding electron pairs on the N atom and the proton from phenolic OH, thus preventing lone pair - π electron interactions, eventually reducing fluorescence intensity (6). This aspect requires further investigation, but it is apparent that the molecular environment of these molecules has profound effect on their fluorescence intensity.

Recoveries of aminocarb and mexacarbate and their metabolites from water

Percent recoveries of aminocarb, mexacarbate and their metabolites from stream water are given in Table 2 along with their standard deviation (SD) and CV. Corresponding chromatograms are given in Figures 5A and B (A - UV

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TABLE 2

Average Percent Recoveries (n=3) of Aminocarb and Mexacarbate and Their Metabolites from Natural Water by UV Absorbance and Fluorometric Methods

Analyte	Percent Recovery ± SD (CV)			
	Fortification level			
	0.5 μg/mL		5.0 μg/mL	
	UVD	FD	UVD	FD
A	98.1 ± 4.8 (4.9)	99.8 ± 7.9 (7.9)	99.4 ± 5.2 (5.2)	101.3 ± 9.1 (9.0)
MFA	81.1 ± 7.1 (8.8)	-	83.9 ± 7.3 (8.7)	-
MA	101.3 ± 6.1 (6.0)	98.8 ± 8.4 (8.5)	97.7 ± 3.8 (3.9)	102.1 ± 8.4 (8.2)
FA	80.6 ± 7.8 (9.7)	-	81.1 ± 9.2 (11.3)	-
AA	93.9 ± 3.3 (3.5)	94.9 ± 6.4 (6.7)	96.2 ± 4.9 (5.1)	96.8 ± 9.1 (9.4)
MP	78.2 ± 6.6 (8.4)	81.0 ± 7.7 (9.5)	73.9 ± 7.2 (9.7)	74.3 ± 8.8 (11.8)
MAP	77.7 ± 8.1 (10.4)	80.2 ± 8.7 (10.8)	78.9 ± 8.1 (10.3)	82.2 ± 9.3 (11.3)
AMP	79.3 ± 7.7 (9.7)	76.8 ± 9.3 (12.1)	81.2 ± 3.9 (4.8)	80.8 ± 8.1 (10.0)
M	98.9 ± 2.9 (2.9)	103.2 ± 7.2 (7.0)	98.3 ± 4.4 (4.5)	102.6 ± 7.3 (7.1)
MFM	80.0 ± 6.2 (7.8)	-	83.1 ± 7.1 (8.5)	-
MAM	92.4 ± 5.1 (5.5)	98.8 ± 8.3 (8.4)	97.3 ± 6.1 (6.3)	103.2 ± 10.1 (9.8)
FM	80.8 ± 7.2 (8.9)	-	77.6 ± 8.1 (10.4)	-
AM	87.1 ± 8.2 (9.4)	86.0 ± 6.9 (8.0)	86.7 ± 7.0 (8.1)	86.2 ± 8.2 (9.5)
DMAX	79.9 ± 6.6 (8.3)	84.2 ± 9.1 (10.8)	83.3 ± 6.1 (7.3)	84.1 ± 9.0 (10.7)
MAX	74.7 ± 7.4 (9.9)	78.1 ± 9.9 (12.7)	77.4 ± 5.9 (7.6)	81.2 ± 8.8 (10.8)
AX	71.7 ± 6.9 (9.6)	73.2 ± 8.4 (11.5)	76.6 ± 7.2 (9.4)	80.0 ± 9.4 (11.8)

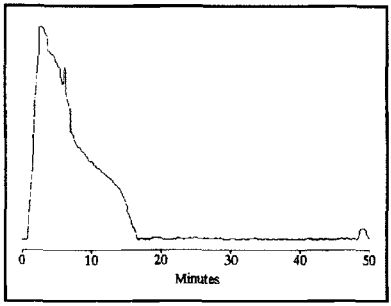

Figure 5A: Chromatogram of stream water blank by UVD, after column cleanup.

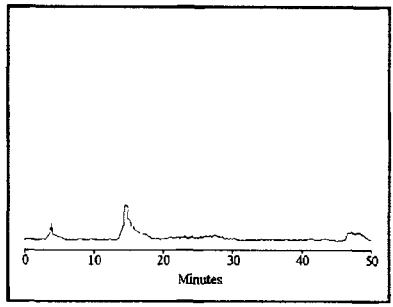

Figure 5B: Chromatogram of stream water blank by FD, after column cleanup.

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absorbance, B - fluorometric)(water blank after cleanup), 6A and B (aminocarb and its metabolites in water at 0.5 μg/mL fortification level) and 7A and B (mexacarbate and its metabolites in water at 0.5 μg/mL fortification level). Recoveries of both insecticides at the two fortification levels were quantitative (> 98 %) indicating that the parent materials are amenable to analysis by both UVD and FD methods. Although the recoveries by fluorometric method seem to be higher, the CV is rather high (range 6.7 to 12.7 %) compared to UVD (range 2.9 to 11.3 %). Nevertheless, both techniques are suitable to analyze the parent materials from natural water.

The recoveries of MA, AA, MAM and AM are reasonably good (range 86.0 to 103.2 %) at both fortification levels by the UVD and FD methods. However, we have to point out that, because of the high LOQ (about 10 to 20 ng, except MAM), the FD method is not suitable if the analyte concentrations are very low in water. Compared to the parent materials, the recoveries of the four aldehydes (MFA, FA, MFM and FM) by the UVD method averaged only about 81.0 ± 1.9 %. As mentioned earlier, no quantification by FD was possible for them due to their poor quantum efficiency. Similarly the recoveries of six phenols (MP, MAP, AMP, DMAX, MAX and AX) by both UVD and FD methods

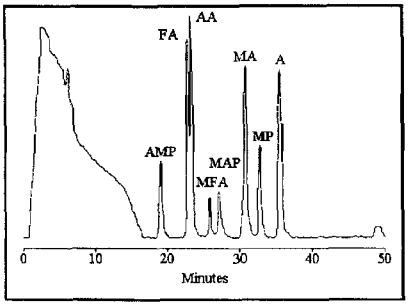

Figure 6A: Chromatogram of water by UVD, fortified with aminocarb and its metabolites each at 0.5 µg/mL.

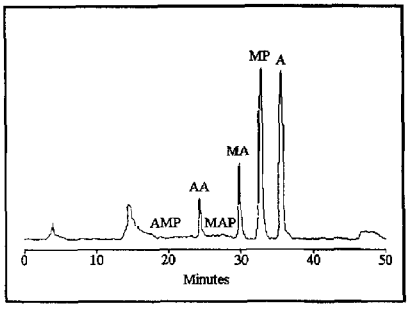

Figure 6B: Chromatogram of water by FD, fortified with aminocarb and its metabolites each at 0.5 µg/mL.

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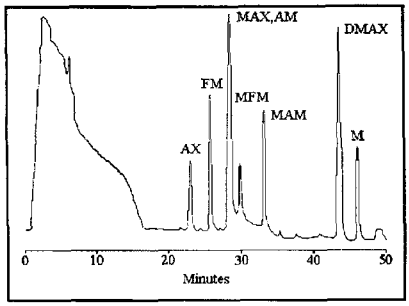

Figure 7A: Chromatogram of water by UVD, fortified with mexacarbate and its metabolites each at 0.5 µg/mL.

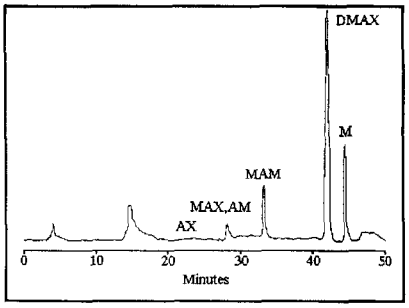

Figure 7B: Chromatogram of water by FD, fortified with mexacarbate and its metabolites each at 0.5 µg/mL.

averaged only about 78.7 ± 3.4 %. The low recoveries of aldehydes and phenols could be due to their high polarity and enhanced water solubility, preventing quantitative partition from aqueous to organic phase. The LOD for each analyte from water calculated as three times the SD of the blank response (8) was in the range of 1 to 10 ng in UVD and 0.2 to 200 ng in FD, depending on the type of analyte. The LOQ was five times the SD and ranged from 5 to 50 ng in UVD

and 1 to 1000 ng in FD. The LOQ values found here agreed with the values reported earlier for the standards using the two techniques.

CONCLUSION

The results presented in this study indicate that UV and fluorometric methods are suitable for isolating aminocarb and mexacarbate and some of their metabolites from natural waters. Contrary to the high sensitivity observed in other compounds with strong native fluorescence, most of the analytes in the present study have marginal quantum efficiency. Consequently, their sensitivities in FD are not high. In addition, the FD method has some inherent limitations such as: (a) the fluorescence intensity varied among the analytes studied and (b) the four aldehydes did not fluoresce at all. In such cases, the fluorescence detection is not feasible with direct injection unless a post-column treatment, such as derivatization is introduced (2) to improve the situation. The UVD is straight-forward and sensitive enough to quantify the analytes. It is applicable to all compounds, except for those solutions containing the AM and MAX or FA and AA together, because the elution regions of AM and FA overlap with the elution regions of MAX and AA.

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