Improvements to EPA Method 531.1 for the Analysis of Carbamates that Resulted in the Development of U.S. EPA Method 531.2

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Abstract

This project is undertaken to fully optimize the U.S. Environmental Protection Agency Method 531.1 post-column chemistries and to incorporate recent advances in liquid chromatographic separation, post-column derivatization, and detection techniques. Sample preservation and storage stability studies establish citric acid as a suitable replacement for the caustic monochloroacetic acid in the current method and confirm its antimicrobial effectiveness. Performance of an alternate set of commercially available post-column reagents is also investigated. This research has resulted in the publication of Method 531.2, a high-performance liquid chromatographic direct injection method for the analysis of *N*-methylcarbamoyloximes and *N*-methylcarbamates using post-column derivatization and fluorescence detection.

Introduction

Carbamates, introduced in the 1950s, are widely used as insecticides to control pests on agricultural crops including potatoes, peanuts, citrus, and sugar beets. They are also commonly used to control lawn and garden insects (1). Carbamates are effective because of their ability to cause cholinesterase inhibition at low exposure levels (1). Human exposure to these toxic compounds through drinking water is of concern because many of them are relatively soluble in water and mobile in a wide variety of soil types (1). For example, aldicarb, which is considered highly toxic (Toxicity Category I) (1) has been found in drinking water sources in 13 states at concentrations in excess of the U.S. Environmental Protection Agency (EPA) advisory level of 10 µg/L (2). Carbofuran has been found in groundwater in Wisconsin and New York (3). Concerns about the groundwater contamination even led one aldicarb manufacturer to voluntarily discontinue its sale for use on potatoes (4). Although gas chromatographic methods using conditions designed to reduce thermal degradation (such as cold on-column injection) have been reported (5), methods for carbamate analysis are generally based on high-performance liquid chromatography (HPLC). This is primarily caused by two factors: (*a*) the thermal instability of these compounds and (*b*) the development of post-column chemistries compatible with HPLC that have sufficient sensitivity to permit direct injection of water samples.

EPA Method 531.1 was published in 1985 for the analysis of 10 *N*-methylcarbamoyloximes and *N*-methylcarbamates, hereafter referred to as carbamates (6). It is based on the post-column chemistries originally reported by Moye et al. (7).

As seen in Figure 1, each method analyte is separated chromatographically and is then hydrolyzed at an elevated temperature and high pH in a post-column reactor into a common product, *n*-methylamine. The methylamine is mixed with a second solution at ambient temperature containing *o*-phthaldehyde (OPA) and 2-mercaptoethanol (MCE), which react with the methylamine to form a fluorescent isoindole. These factors result in a method with high selectivity and sufficient sensitivity to allow direct injection of samples without a preconcentration step.

Although Method 531.1 has been well-suited to support compliance monitoring for over a decade, the EPA decided it needed to be updated for several reasons. First, the EPA is considering a new maximum contaminant level (MCL) for the sum of aldicarb, aldicarb sulfoxide, and aldicarb sulfone (8,9). At this stage it is unknown whether new levels may ultimately be promulgated. However, in anticipation of the need to measure relatively low levels, research began assuming the need to accurately measure a concentration for each compound of just over 2 µg/L. In past aldicarb MCL proposals, concern about establishing the practical quantitation limit (PQL) was the focus of some discussion (9). This led to a goal of establishing a minimum reporting level (MRL) of 10 times lower than 2 (0.2 µg/L) for the revised method. As written, it was uncertain whether Method 531.1 had adequate sensitivity to allow quantitation at 0.2 µg/L. Because of the potential monitoring levels,

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method performance needed to be adequate at this low level, which included ensuring that method conditions (e.g., reagent pH and concentration) were fully optimized. Second, the current Method 531.1 preservative [monochloroacetic acid (MCAA)] needed to be eliminated. MCAA is corrosive, a suspected carcinogen, and regulated as one of the haloacetic acids (HAAs) in the National Primary Drinking Water Regulations (10). A substitute was needed that would meet the EPA objectives of safe, effective, and low-cost preservation (11). Finally, alternative reagents that became commercially available subsequent to publication of the original method were evaluated. In this paper, research resulting in the revised method (published as Method 531.2) is presented. This includes the evaluation of sample preservation and storage stability that led to the selection of an alternate preservative, post-column chemistry optimization, and reagent stability studies with two sets of reagents. Method performance is summarized briefly as well.

Experimental

Reagents and standards

Methanol, acetonitrile, sodium thiosulfate, hydrochloric acid, and sodium hydroxide solution (50%, w/w) were purchased from Fisher Scientific (Pittsburgh, PA). Reagent water from a Millipore (Bedford, MA) MilliQ Plus TOC system was used. Sodium borate decahydrate, citric acid, MCE, and OPA were purchased from Aldrich (Milwaukee, WI). Potassium dihydrogen citrate and naphthalene-2,3-dicarboxaldehyde (NDA) were obtained from Fluka (Milwaukee, WI). Premade monochloroacetic acid buffer; post-column reagents (OPA dilutent and hydrolysis reagent); and *N*,*N*-dimethyl-2-mercaptoethylamine (Thiofluor) were purchased from Pickering Labs (Mountain View, CA). Solvents were HPLC grade and chemicals were American Chemical Society reagent grade or better. All post-column reagents were prepared as described in EPA Method 531.2 and stored in clear, 1-L bottles unless otherwise noted.

Analytes were obtained as neat materials (> 95%) from ChemService (West Chester, PA) and Reidel-de-Haen (Seelze, Germany) or as commercially prepared stock standards from Supelco (Bellefonte, PA), Accustandard (New Haven, CT), and Ultra Scientific (North Kingstown, RI). The analytes were the pesticides aldicarb, aldicarb sulfone, carbaryl, carbofuran, methiocarb, methomyl, oxamyl, and propoxur, as well as the metabolites aldicarb sulfoxide and 3-hydroxycarbofuran. 1-Naphthol, a fluorescent metabolite of carbaryl that is stable under the hydrolysis conditions, was added to the analyte list. The surrogate compound used was 4-bromo-3,5-dimethylphenyl *N*-methylcarbamate (BDMC).

Instrumentation

A Waters (Milford, MA) Model 2690 HPLC system equipped with a Waters 3.9- × 150-mm C18 carbamate column was used with a Waters post-column carbamate system for most of the method refinement. A Pickering Model 5200 post-column carbamate system was also evaluated. A Waters Model 474 fluorescence detector was used unless otherwise specified. A Waters Model 2475 fluorescence detector also became available and its sensitivity was evaluated towards the end of the method development research. Sample and reagent pHs were determined using a Corning (New York, NY) Model 440 pH meter equipped with a 3-in-1 pH electrode.

Chromatographic conditions described in EPA Method 531.2 (12) were used throughout this study unless otherwise noted. Analyte recovery data were reported as a percent of the original fortified concentration (% recovery).

Sample preparation

Target compounds were fortified into reagent water and finished (tap) water (from both surface and ground water sources) as specified in the "Results and Discussion" section. Sample hardness and residual chlorine were determined using Hach (Loveland, CO) test kits. Samples were fortified with the surrogate compound BDMC just prior to filtration and loading into the autosampler. All samples were filtered with Millipore Millex 0.22-µm polyvinylidene fluoride filters.

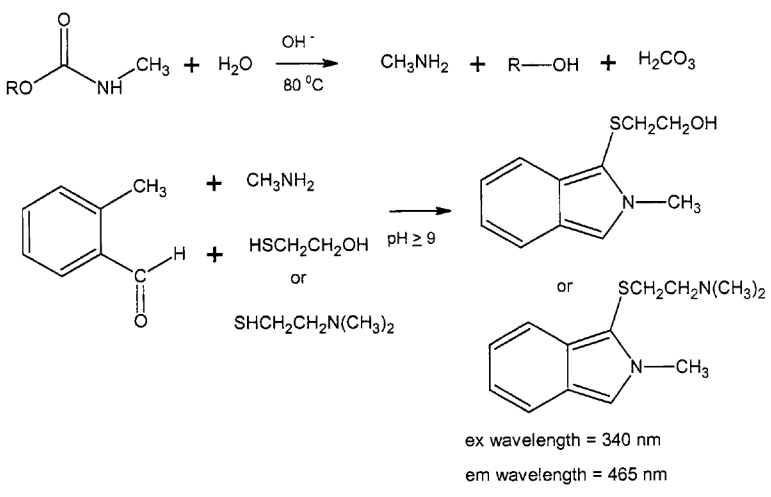

Figure 1. Hydrolysis and derivatization reactions.

Heterotrophic plate counts

In order to determine the biocidal efficacy of the antimicrobial preservation schemes, heterotrophic plate counts were conducted using R2A agar and the pour plate technique (13). For the 28-day storage stability study reported in the Results and Discussion section, each triplicate sample was plated in triplicate, which resulted in the inoculation of nine plates per experiment on each analysis day.

Results and Discussion

Sample dechlorination and pH adjustment

Foerst et al. were first to report that dechlorination of finished drinking water

samples is necessary to prevent the oxidation of aldicarb to aldicarb sulfoxide (14). The detrimental effect of residual chlorine was confirmed by fortifying drinking water (pH adjusted to 3.9 and containing chlorine at approximately 0.7 mg/L) with target compounds at 2.0 µg/L. No recovery for aldicarb or methiocarb was seen. Residual chlorine had been successfully eliminated using sodium thiosulfate in the previous method. It was also evaluated for this method at sample concentrations of 80 and 320 mg/L in pH adjusted (pH 3.9) finished ground water. At both levels, analyte recoveries were acceptable for samples stored for 28 days as specified in Method 531.2.

Previous research indicated that carbamates are subject to hydrolysis at neutral-to-elevated pH levels (14,15). A storage stability study was conducted over 28 days to confirm the need for pH adjustment. Concurrently, experiments were conducted to find a replacement for the MCAA, which is presently being used to adjust sample pH. Citric acid, a commonly used HPLC mobile phase buffer, was chosen as an alternate preservative. It is safe, inexpensive, and available in dry form, all of which make it an improvement over MCAA.

Two citric acid formulations at pHs near 3 and 4 were added at concentrations that had a buffering capacity similar to that of MCAA in Method 531.1. The pH 4 experiment was included because preliminary data indicated that aldicarb recovery might be better at a slightly higher pH, and it added the convenience of weighing out one reagent rather than two. A set of samples preserved with MCAA, which is used to acidify the samples at pH 3, was included to benchmark Method 531.1 performance. Finally, a set of neutral pH samples was included to track carbamate degradation over the length of the study. The quantities of preservatives and final sample pH are detailed in Table I.

Triplicate samples for each day's analysis were prepared from finished (tap) ground water (hardness of 310 mg/L as $CaCO_3$, residual chlorine of 0.7 mg/L). Samples were dechlorinated with 80 mg/L sodium thiosulfate and fortified at 2.0 µg/L with target compounds and 4 mL of Ohio River water (discussed later) on day 0. All samples were stored at 10ºC for 48 h and then at 6ºC for the remaining 26 days. The first 48-h temperature reflects the temperature at which samples are typically received when packed with cold packs for shipment. The temperature for the remaining storage

Table I. Storage Stability Preservation Experiments*

Experiment	MCAA	Citric acid	Citric acid, monopotassium salt
Citric (pH 3.2)	–	3.8 g	4.6 g
Citric (pH 3.9)	–	–	9.2 g
MCAA (pH 3.0)	30 mL	–	–
Neutral pH (pH ~ 7.9)	–	–	–

* The quantity of chemicals is stated in amount per liter of sample. All samples were dechlorinated using 80 mg/L sodium thiosulfate and fortified with 4 mL/L of river water. Samples were split into 40-mL vials for storage.

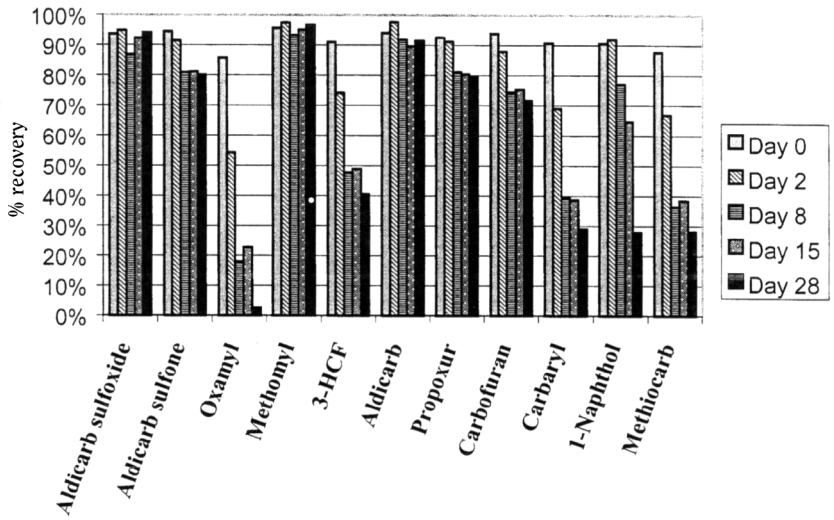

Figure 2. Storage stability results for finished ground water with neutral pH (7.80–8.01).

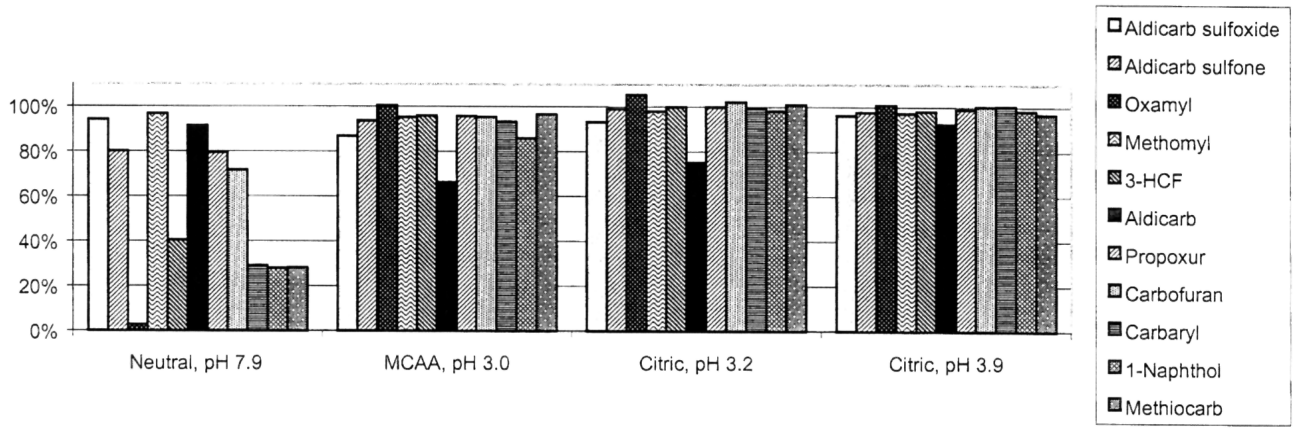

Figure 3. Day 28 of storage stability studies.

time is the maximum refrigerated storage temperature allowed in recent EPA methods.

Recovery data from the neutral (pH 7.9) samples are presented in Figure 2. These data confirm the need to control pH. Two other sets of neutral pH samples that were not fortified with river water and showed no microbe growth when plated had almost identical results, which suggested that in this case the degradation was chemical in nature. After two days at 10ºC, oxamyl recovery was down to 54%, and carbaryl and methiocarb exhibited recoveries of less than 70%. The previous storage stability work, conducted at 5ºC by Foerst et al., led the authors to conclude that acidification could be accomplished after the samples reached the laboratory (14). These data indicated that not only is pH adjustment required, but it is also necessary to acidify samples in the field prior to shipment.

Day-28 data for all four preservation experiments are contrasted in Figure 3. With the exception of aldicarb, analyte recoveries for all samples dechlorinated and preserved at pH 4 or below had recoveries within 15% of the spiked value. Aldicarb recoveries for the samples preserved with citric acid at pH 3.2 were comparable to the MCAA (pH 3.0) preserved samples at 75% and 66%, respectively. Samples preserved with citric acid at pH 3.9 had an increase of aldicarb recovery to 93%. The aldicarb recovery in these experiments confirmed that acidification at pH 3.9 is preferred over the pH used in the current method.

Microbe inhibition studies

Several of the target compounds are known to biodegrade in water, whereas others have been reported to biodegrade in soils (1,3). Acidification of samples protects analytes from biodegradation. Although storage at pH 4 promoted chemical stability and improved aldicarb recovery, there was concern that pH 4 was not low enough to inhibit microbe growth. In order to test the ability of the preservation schemes to inhibit bacterial growth, samples were fortified with 4 mL of Ohio River water per liter of sample on day 0 of the stability study. This was enough to challenge the preservative with a relatively large population of microbes.

After a portion of the sample was taken for liquid chromatographic analysis, biocidal efficacy was determined each analysis day by using the remaining portion of each sample to conduct heterotrophic plate counts. These data are reported in Figure 4 as colony forming units (CFU) per mL of sample.

Exponential bacterial growth was noted for the neutral pH samples by day 8 (off-scale in Figure 4) and the growth increased to approximately 300,000 CFU by day 28. All three preservation schemes adequately inhibited bacterial growth. Based on analyte recovery and bacterial count results, preservation at pH of approximately 3.9 with potassium dihydrogen citrate was chosen as the alternative preservative to MCAA.

Post-column reagent optimization

The post-column reaction for Method 531.1 is shown in the top portion of Figure 1. As method analytes elute from the separation column they are mixed with sodium hydroxide (NaOH) and heated to 85–90ºC as they enter a post-column reactor. In the reactor, each carbamate hydrolyzes to form methylamine, which is then carried into the second, ambient temperature reaction region where it is mixed with OPA and MCE to form the fluorescent isoindole.

The purpose of these studies was to fully optimize post-column conditions (e.g., pH, temperature, and reagent concentration) and to evaluate other commercially available post-column reagents. This included the evaluation of the reagent *N*,*N*-dimethyl-2-mercaptoethylamine (Thiofluor) as an alternate nucleophile to MCE (bottom of Figure 1), as well as a cursory evaluation of naphthalene-2,3-dicarboxaldehyde (NDA) as a potential replacement for OPA. Thiofluor has been reported to form an isoindole with similar sensitivity and enhanced stability (16), as well as enhance the stability of the post-column reagent (17). NDA has been reported to react with primary amines in the presence of the cyanide ion (CN^-) in a manner similar to OPA, which yields a fluorescent product with up to 50-times more sensitivity for detection of some amino acids (18,19).

The sample matrix for reagent optimization experiments was reagent water unless otherwise noted. Samples were preserved as specified in Method 531.2 and fortified with target compounds at 2.0 µg/L.

Evaluation of NDA as a substitute for OPA

With the aim of improved analyte sensitivity, a cursory examination of NDA as a potential substitute for OPA in the reaction sequence was undertaken. In the literature, attempts to use NDA with MCE produced unstable isoindoles (19). Therefore, we attempted to use NDA with Thiofluor instead of MCE. Although CN^- is reported to be the preferred reagent for this reaction, this was not considered because of safety and waste

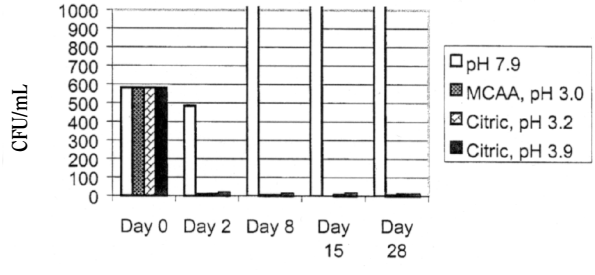

Figure 4. Bacterial growth during 28-day storage stability study. The CFU/mL count for pH 7.9 sample is shown as off-scale on this chart by day 8; on day 28 the count was approximately 300,000.

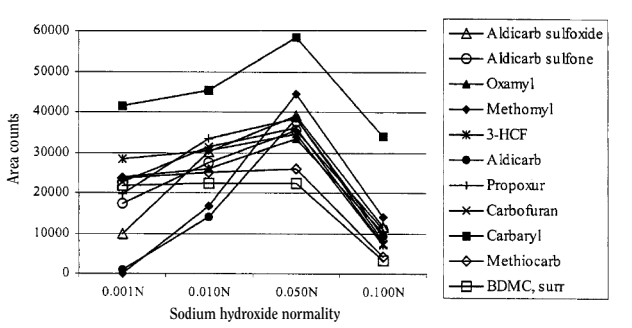

Figure 5. Analyte response against NaOH concentrations.

concerns. Problems with precipitation were encountered when substituting the NDA at the same concentration as the OPA in the sodium borate solution. The reagent was diluted 1:1 with methanol, which dissolved the precipitate. The Thiofluor was then added. This effort did not result in detectable peaks from the carbamates even at relatively high concentrations. Attempts to optimize detection by scanning the detector wavelengths were also unsuccessful. These experiments were not pursued further.

Hydrolysis reagent concentration

Using OPA and MCE in the derivatization reagent, the affect of the hydrolysis reagent concentration on analyte response was tested. Response of the analytes was investigated at NaOH concentrations of 0.001-, 0.010-, 0.050-, and 0.100N as seen in Figure 5. Results reported for these experiments were from fortified finished (tap) ground water samples. Almost identical results were seen with fortified reagent water. These data indicate that optimum analyte response on the Waters post-column system with a reactor temperature of 80°C was achieved with 0.05 N NaOH. A drop-off in response for many of the analytes was noted on either side of this concentration. Other researchers who conducted similar studies using a homemade reactor coil heated at 100ºC reported an optimal response using 0.004 N NaOH for most of the analytes (20). Separate experiments indicated that 1-naphthol response increased in the more basic reagent. The conversion to the naphtholate ion is likely accompanied by an increase in molar absorptivity or fluorescence quantum yield (or both).

Hydrolysis reactor temperature

Using the Waters carbamate system, hydrolysis reaction temperature was tested between 60 and 90ºC in 10ºC increments using 0.05 N NaOH. Triplicate 2.0 µg/L samples analyzed at 80ºC, the temperature recommended by Waters, were used to calibrate the instrument. Target compound responses were reported as percent responses relative to the 80ºC samples. The compounds most affected by the hydrolysis temperature were aldicarb and methomyl. At temperatures lower than 80ºC there was a steep drop in response for aldicarb and methomyl (58% and 64% at 70ºC, and 18% and 23% at 60ºC, respectively). At 90ºC responses for these compounds were 118% and 112%, respectively. Increased baseline noise was also observed at 90ºC. Responses for all other compounds at 90ºC were within 10% of the 80ºC value.

The response using a Pickering carbamate system, which is configured with a restrictor to prevent the boiling of post-column reagents, was tested at 80 and 100ºC. At 100ºC aldicarb and methomyl responses jumped to 216% and 179% relative to the 80ºC injections. The remaining analyte responses were only slightly elevated (within 6% of the spiked value) except for a drop in 1-naphthol response, which decreased to 89%. Additionally, the baseline noise appeared unchanged. Although Pickering recommends a 100ºC reactor temperature, enough sensitivity to quantitate analytes at 0.2 µg/L was achieved for both post-column systems using the 80ºC reactor temperature. This eliminates the need for a restrictor after the detector to prevent reagent boiling.

MCE and Thiofluor concentrations

The concentration of 2-mercaptoethanol in the second post-column reagent was evaluated at three levels: 50 µL, 1 mL, and 2 mL of MCE per liter of solution. Method 531.1 utilizes an MCE concentration of 50 µL per liter of reagent. Response for the carbamates increased approximately 25% in the 1-mL solutions except for 1-napthol, which had a higher response at the lower MCE concentration. The response did not show further increase at the higher (2-mL) concentration. These data parallel findings by Krause, et al. (20). A four-day reagent storage stability study conducted at room temperature (discussed later)

Table II. Single Analyst Precision and Accuracy Data of Low- and High-Level Fortified Waters

	Reagent water				Drinking water, surface water				Drinking water, ground water			
Fortified concentration	0.20 µg/L		10.0 µg/L		0.20 µg/L		10.0 µg/L		0.20 µg/L		10.0 µg/L	
Compound	MR%*	%RSD†	MR%	%RSD	MR%	%RSD	MR%	%RSD	MR%	%RSD	MR%	%RSD
Aldicarb sulfoxide	112	6.2	106	1.8	113	7.0	104	2.8	111	7.3	106	1.1
Aldicarb sulfone	92	9.5	106	2.6	104	5.5	106	1.4	98	9.2	106	0.9
Oxamyl	101	8.6	106	2.2	107	6.4	104	2.2	99	8.4	105	1.2
Methomyl	101	6.5	106	2.9	110	9.8	104	1.6	99	10.2	105	1.4
3-Hydroxycarbofuran	105	6.8	108	1.2	128	3.9	107	1.1	107	3.0	108	0.4
Aldicarb	95	7.4	106	1.3	123	2.7	105	1.5	100	6.3	105	0.6
Propoxur	109	5.9	109	2.0	128	6.0	106	2.1	112	6.1	107	0.8
Carbofuran	112	6.7	110	2.2	140	5.6	105	2.5	112	4.1	107	1.6
Carbaryl	112	7.0	107	2.1	112	9.7	106	0.9	119	5.1	108	1.3
1-Naphthol	113	12.6	108	3.1	113	12.1	101	1.3	109	8.2	109	1.2
Methiocarb	105	5.9	107	1.5	104	13.3	107	1.1	105	3.9	107	1.0
BDMC (SUR)‡	108	4.3	101	2.3	108	2.1	96	3.9	109	2.0	97	4.3

* MR%, mean recovery expressed as % recovery.
† %RSD, percent relative standard deviation.
‡ The surrogate concentration in all samples was 2.0 µg/L; all data from $n = 7$ replicates using a 1000-µL injection volume.

also indicated that the 1-mL concentration had improved stability. These data justified increasing the MCE concentration to 1 mL/L in the revised method.

The Thiofluor concentration was evaluated at 1, 2, and 4 g/L. The concentration recommended by the manufacturer is 2 g/L (21). Response at 1 and 2 g/L was very similar. At 4 g/L, the average response for the target compounds decreased by approximately 8%.

OPA concentration

Analyte response was tested next with OPA concentrations of 50, 100, and 200 mg/L in the second post-column reagent. By calibrating the instrument with the 100-mg/L concentration, the response dropped 15% for all compounds using the 50 mg/L solution. The response increased by only 5% for the samples analyzed with 200 mg/L OPA. This increase did not warrant the additional reagent cost. As a result, the OPA concentration was kept at 100 mg/L in the method.

pH of derivatization reagent

The pH of the OPA derivatization reagent was tested by preparing borate buffer solutions in increments of approximately 0.5 pH (between pH 8.5 and 10) for both MCE and Thiofluor nucleophiles. Triplicate injections at each pH yielded a fairly flat plot of fluorescent response as a function of pH for both reagents, with an optimal pH of 9.2. As expected, 1-naphthol behaved differently from the other method analytes. Its highest response was at pH 10.0, which was a 46% increase in response. These results indicated that no modification of the pH in the method was necessary.

Derivatization reagent stability

Reagent stability of the OPA derivatization reagent was tested over a 42-h period using the final conditions specified in Method 531.2. The reagents were stored in 4-L amber bottles that were open to air. Samples were loaded into the autosampler at room temperature and injected at the rate of 1 sample per hour. Using either MCE or Thiofluor, all recoveries were within ± 10% of true value at the end of the 42-h experiment. Similar results were obtained when the experiment was repeated using 50 µL of MCE per liter of reagent. Both MCE and Thiofluor have distinctive, pungent odors and should be opened under a fume hood.

A 4-day extended stability study of the derivatization reagent using 100 mg/L OPA and 1 mL/L MCE was conducted. Freshly prepared OPA solution was made approximately 2 h prior to the analysis of the calibration curve and samples. The solution was open to air during sample analysis for approximately 7 h and then capped and stored at room temperature. The reagent bottle was opened on days 2 and 3 for 2 h each day and used for analysis on day 4. Lower responses were expected, but results using the 4-day-old OPA were almost identical to results using freshly made OPA reagent (analyte recoveries were within 3% of true value). A similar, but longer 6-day reagent stability experiment was conducted to compare reagents made with 50 µL MCE (as originally specified in Method 531.1) with the proposed 1 mL MCE. Reagent degradation in the form of diminished recoveries was seen by day 6 for both MCE concentrations. Day 6 analyte recoveries for the 1-mL MCE reagent averaged 4–16% lower than on day 0, with recoveries even lower for the 50-µL MCE reagent; they averaged 22–39% lower. Thus, the OPA reagent made with 1 mL MCE appears to be stable when stored up to 4 days under normal laboratory conditions, and the larger amount of MCE helps to ensure reagent stability.

System optimization

During method development, data were collected using two manufacturers' post-column reagent delivery systems, two fluorescence detectors, and a variety of injection volumes. These data are reported in Section 17 of EPA Method 531.2 (12). Although both post-column systems had adequate performance, the Pickering post-column unit offered a signal-to-noise improvement of approximately four-fold. The new Waters fluorescence detector (Model 2475) offered nearly five times more sensitivity when compared with its precursor (Model 474), thereby achieving similar detection limits with one-fifth of the injection volume. This should offer similar method performance to that reported in Table II with a 200-uL injection volume. A smaller injection volume could, in turn, prolong analytical column life.

Conclusion

Method 531.2 is an updated direct-injection HPLC method for the analysis of 11 carbamates and carbamate derivatives. The revised method fully optimizes reaction conditions and reagent concentrations. An alternate nucleophile (*N*,*N*-dimethyl-2-mercaptoethylamine hydrochloride) that became available subsequent to publication of the original method was evaluated and found to perform almost identically to the previously used 2-mercaptoethanol. Citric acid (monopotassium salt) was substituted as a safe replacement for the preservative MCAA and should also improve data reliability for aldicarb. Data demonstrating single analyst precision and accuracy are presented in Table II. These data indicate that method performance is more than adequate to quantitate target compounds, specifically aldicarb and its metabolites, down to 0.2 µg/L.

Acknowledgments

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