

# Quantitative Determination of Ultratrace Level *N*-Methyl Carbamates in Rice Samples by Accelerated Solvent Extraction (ASE) and Ultrahigh Performance Liquid Chromatography Tandem Mass Spectrometry (UHPLC-MS/MS)

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## Introduction

*N*-Methyl carbamates (NMCs) are widely used as pesticides and have been reported at various levels in the environment and foods.<sup>1-4</sup> They vary in their spectrum of activity and persistence, and are suspected endocrine disruptors<sup>5</sup> that exert an anticholinesterase action on the nervous system similar to organophosphates.<sup>6</sup> NMCs are relatively unstable compounds that subsequently break down in the environment fairly rapidly.<sup>7</sup> Several carbamates have systemic use in plants because they have high water solubility which allows them to be taken up by the roots and into the leaves of plants. Although the cumulative risk assessment from U.S. EPA concluded that exposure to NMC is not of concern,<sup>1</sup> a recent report from the National Marine Fisheries Service reveals carbaryl, carbofuran, and methomyl jeopardize the continued existence of protected species of salmon and steelhead.<sup>2</sup> This report re-emphasizes concern over applications of NMCs and their ongoing effect on the environment. Most recently, U.S. EPA announced the final rule for carbofuran and banned its usage on food crops.<sup>8</sup>

Previously reported methods for NMC quantification include gas chromatography (GC) with nitrogen- or sulfur-specific detection, or mass spectrometry (MS) detection.<sup>9,10</sup> High performance liquid chromatographic (HPLC) methods were developed for NMC determination because some analytes are polar and/or thermally labile and not suitable for GC analysis.<sup>11-14</sup> Currently, carbamates in environmental water samples are quantified according to U.S. EPA Method 531.2, using HPLC for separation followed by postcolumn derivatization and fluorescence detection.<sup>11</sup> Recently developed HPLC methods increase method specificity and sensitivity with the use of MS detection.<sup>4,10,15,16</sup> Reported methods have detection limits at low parts-per-billion (ppb) levels. For trace-level determination of NMC residues at sub-ppb levels, sample enrichment and/or large-volume injection were usually applied.<sup>11,16</sup>

This study demonstrates a UHPLC-MS/MS method for quantitative analysis of NMCs at parts-per-trillion (ppt) levels in rice samples with automated sample extraction and improved chromatography in a 12 min run. This method has been successfully applied to the determination of NMCs in various types of rice samples, and single-lab method validation was performed with respect to linearity, specificity, quantitation limit, carryover, precision and accuracy, and recovery.

## Experimental

### Chemicals and Reagents

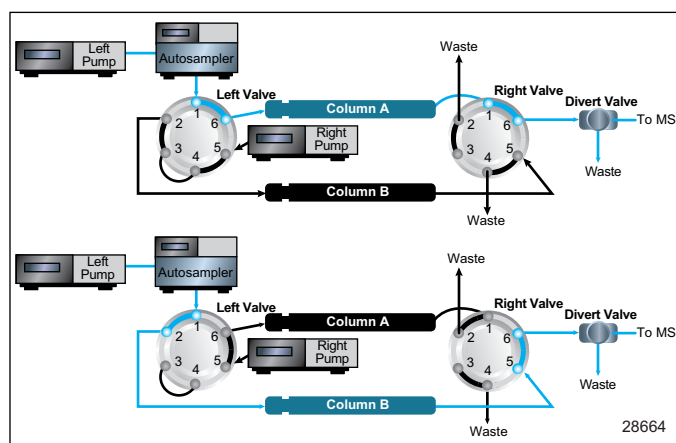
A standard pesticide mixture containing the 11 carbamates in EPA Method 531.2, was purchased from AccuStandard (M-531M, AccuStandard, New Haven, CT). An isotope-labeled internal standard (IStd), carbaryl-*d*<sub>7</sub>, for MS detection was purchased from C/D/N isotopes (D-5468, C/D/N Isotopes Inc, Pointe-Claire, Quebec, Canada). Methanol was purchased from Burdick & Jackson (HPLC/UV grade, Honeywell, Muskegon, MI ). Deionized water (DI water) was obtained from a Millipore water station (Billerica, MA) with 18.2 MΩ-cm resistivity.

The standard mixture solution was diluted in DI water to the concentrations of 10 ppb and 1 ppb as working solutions to prepare calibration standards. IStd stock solution was prepared by dissolving the appropriate amount of carbaryl-*d*<sub>7</sub> in methanol and diluted to 1 ppm (or µg/mL) as stock solution, then diluted in DI water to 10 ppb and 1 ppb as working solution to prepare calibration standards and spike unknown samples. Calibration standards were prepared in DI water by a series of dilutions of the working solution to 1 ppb, 500 ppt, 200 ppt, 100 ppt, 50 ppt, 20 ppt, and 10 ppt with IStd spiked at 100 ppt at each level. Calibration standards and samples were prepared daily due to the known instability of some analytes in aqueous solution.

## Instrumentation

A Thermo Scientific Dionex ASE™ 350 Accelerated Solvent Extractor was used here to perform automated extraction of target analytes from rice samples. The 5 mL (small) extraction cell was selected to minimize solvent usage, solvent evaporation time, and generated waste. After evaluation of the temperature to achieve the best extraction efficiency, the temperature was set at 40 °C on the ASE 350. The composition of the reconstitution solution showed significant effect on the peak shapes of earlier eluters and was shown to have minimal impact when made to match the initial mobile phase composition (20/80, organic/aqueous, v/v).

**FIGURE 1. Flow diagram of sequential analysis using two-column switching. Active components are colored blue.**



A Thermo Scientific Dionex UltiMate™ 3000 UHPLC system equipped with a dual-gradient pump module, a thermostatted autosampler, a column oven, and a six-channel on-line degasser were used to achieve chromatographic separation on two Thermo Scientific Dionex Acclaim™ Carbamate columns (2.1 × 150 mm, 3 μm). The dual-pump system was configured with column-switching ability to utilize sequential analysis on two analytical columns; one column was used as separator, while the other was washed and equilibrated for the next run. As shown in Figure 1, the left pump was used as the elution pump and the right pump was used to deliver solvent for column cleaning and equilibration. Gradient programs (Table 1) were applied on both pumps consisting of: A) 100% methanol; B) DI water; C) 1 mM ammonium formate in DI water. Ammonium formate (C) was maintained at 5% throughout the run to provide a total of 0.05 mM ammonium formate in the mobile phase. The gradient for the left pump started with 20% methanol for 0.1 min, ramped up to 55% in 7.1 min, was increased to 95% in 0.1 min and held for 2.7 min to elute strongly retained analytes, then returned to 20% initial condition. The right pump started with 90% methanol and held for 4 min, was decreased to 20% in 1 min and held for 5 min, then returned to initial condition and held for 2 min. The detailed mobile phase gradient profiles are shown in Table 1. Flow rate was set at a constant 0.6 mL/min for both pumps and 20 μL of sample was injected for each analysis.

**Table 1. Detailed Mobile Phase Profiles for Dual Pumps Shown in Figure 1**

Time	Pump Left			Pump Right			Valve Left	Valve Right	Divert Valve
	% A	% B	% C	% A	% B	% C			
0.00	20	75	5	90	5	5	6-1	6-1	To Waste
0.10	20	75	5						
1.20									To MS
4.00				90	5	5			
5.00				20	75	5			
7.20	55	40	5						
7.30	95	0	5						
9.99				20	75	5			
10.00	95	0	5	90	5	5	1-2	1-2	To Waste
10.01	20	75	5						
11.99				90	5	5			
12.00	20	75	5						

An MS/MS instrument was used as the detector operated in Product Ion-Monitoring mode. Electrospray was used as the interface, and the source parameters were optimized to achieve maximum system sensitivity and stability. The scan events are shown in Table 2, and ionization source and collision parameters are instrument specific.

## Sample Preparation

The nine rice samples used here were purchased from local stores with domestic origins or imported from Thailand, Argentina, Italy, India, and Bhutan. For each sample, 20 g ( $\pm 1$  g) was ground using a coffee grinder (DCG-12BC, Cuisinart, East Windsor, NJ) for 1 min into fine flour. Then 3 g of each rice flour was weighed in a 5 mL ASE Extraction Cell (P/N 068096, Thermo Scientific Dionex) and spiked with 0.3 ng of IStd (100 pg IStd/g of rice sample). Two 0.2  $\mu$ m cellulose filters (P/N 068093, Thermo Scientific Dionex) were inserted into each

extraction cell and precleaned on the ASE 350 at 100 °C with methanol prior to sample loading (Solvent-Saver Pressure mode, 100 °C using a 5 min static time and 0% rinse volume). Samples were added to the extraction cells and extracted in Solvent-Saver Pressure mode at 40 °C using a 5 min static time and 60% rinse volume. Each extract was collected in a 60 mL vial (P/N 048784, Thermo Scientific Dionex), filtered through a 0.20  $\mu$ m cellulose syringe filter (28145-477, VWR International, Brisbane, CA), collected in a 12 mL glass sample tube (53283-802, VWR), and evaporated to dryness under gentle nitrogen gas in a water-heated bath ( $45 \pm 5$  °C). The sample was reconstituted using 0.6 mL methanol, vortex-mixed for 30 sec, and centrifuged for 20 min at 5000 rpm. Then 0.2 mL of the clear solution was pipetted into a 1.5 mL autosampler vial and mixed with 0.7 mL DI water and 100  $\mu$ L of 1% formic acid. Finally, 20  $\mu$ L of prepared sample was injected for UHPLC-MS/MS analysis.

**Table 2. Scan Events for *N*-Methyl Carbamates**

Name	$t_R$ (min)	Scan Times (min)	Precursor ( $m/z$ )	Product ( $m/z$ )	Scan Time (ms)
Aldicarb sulfoxide	1.6	0.0–3.5	207.2	131.8	100
		0.0–3.5	207.2	88.9	50
Aldicarb sulfone	1.8	0.0–3.5	240.1	85.9	100
		0.0–3.5	240.1	148.1	50
Oxamyl	2.1	0.0–3.5	237.2	72.0	100
		0.0–3.5	237.2	90.0	50
Methomyl	2.4	0.0–3.5	163.1	87.9	100
		0.0–3.5	163.1	105.8	50
3-Hydroxycarbofuran	4.6	3.5–5.0	238.1	162.9	300
		3.5–5.0	238.1	220.0	300
Aldicarb	6.0	5.0–7.0	208.2	115.9	300
		5.0–7.0	208.2	88.9	300
Propoxur	7.5	7.0–8.25	210.2	111.0	100
		7.0–8.25	210.2	168.0	50
Carbofuran	7.7	7.0–8.25	222.2	165.0	100
		7.0–8.25	222.2	123.0	50
Carbaryl	8.1	7.0–8.25	202.1	144.9	100
		7.0–8.25	202.1	127.0	50
IS: Carbaryl- $d_7$	8.0	7.0–8.25	209.2	151.9	100
1-Naphthol	8.4	8.28–9.18	-143.0	-114.5	300
		8.28–9.18	-143.0	-40.9	300
Methiocarb	9.6	9.20–10.0	226.2	168.9	150
		9.20–10.0	226.2	121.0	150

## Results and Discussion

### Chromatography

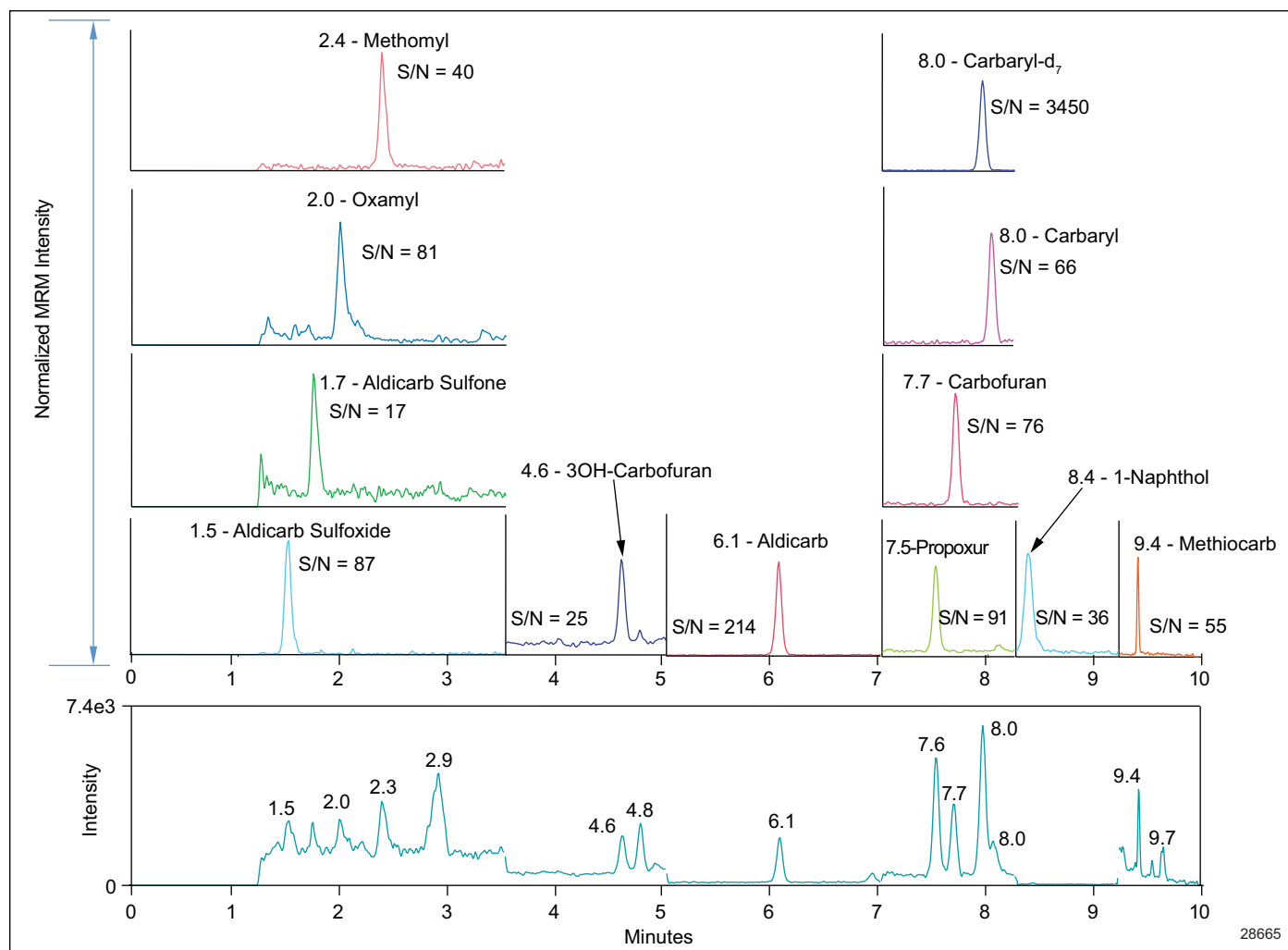
Most LC methods use different formats of reversed-phase columns to separate NMCs with a methanol/water mobile phase.<sup>1,7</sup> Reported methods were usually a compromise between throughput and total resolution. This study demonstrates a balanced approach with improved throughput and total chromatographic resolution of all target analytes using an Acclaim Carbamate column with small particle size (3  $\mu\text{m}$ ) and high-pressure pumps. To further improve method throughput, a dual-pump module was used here for simultaneous analytical separation and column flush/equilibration. Ammonium formate was added to mobile phase to facilitate the formation of analyte-ammonium adducts to improve sensitivity and minimize the formation of other adducts (See Mass Spectrometry below). Formate and acetate at various concentrations did not exhibit a noticeable effect on the chromatographic retention and resolution of the

studied analytes. Changes in column operating temperature also showed negligible effect on chromatographic performance. Mobile phase strength was the most significant factor effecting retention and resolution. Under optimum conditions, all NMCs were separated to baseline, including the critical pairs aldicarb sulfone/oxamyl and propoxur/carbofuran.

### Mass Spectrometry

MS instrumentation capable of MS/MS scan functionality was selected in this study and operated in reaction-monitoring mode to provide optimal sensitivity and selectivity. Compared with the reported best results on the limit of detection (LOD) with fluorescence detection at 0.2 to 0.6 ppb, the MS/MS detection in this study extends the lower limit of quantitation (LLOQ) down to 10 ppt with the signal-to-noise (S/N) ratio of most analytes greater than 20 (S/N >20). As shown in Figure 2, quantification can be routinely performed at 20 ppt in complex matrices.

FIGURE 2. Ultratrace level *N*-methyl carbamate spiked in rice sample A.



When no added ammonium ion was present in the mobile phase, NMCs were observed as protonated molecular ion ( $[M+H]^+$ ) and/or adducts ( $[M+NH_4]^+$  and  $[M+Na]^+$ ). To promote the ammonium-analyte adduct and suppress the formation of other adducts, ammonium formate was added into the mobile phase. To decide whether to use  $[M+H]^+$  or  $[M+NH_4]^+$  as the quantitative precursor ion, experiments were performed to compare the intensities of observed MS/MS transitions of protonated molecular ions or ammonium adducts. The intensities of MS/MS candidates were collected at optimized conditions for each. As a result, ammonium-analyte adducts were used as the quantitative precursor ion for aldicarb sulfone, oxamyl, and aldicarb, and protonated/deprotonated molecular ions were used for the remaining analytes in this study. Mobile phases with different ammonium concentrations were evaluated for their effects on method sensitivity.

Similar MS responses were observed when using 0.05 mM or 0.25 mM ammonium formate in mobile phase, which indicated 0.05 mM ammonium ion in the mobile phase was sufficient for adduct formation. The lower concentration of 0.05 mM was chosen to minimize extraneous source contamination, thus improving long-term instrument stability and reproducibility. As shown in Table 2, two transitions were selected for each target analyte with the more intense transition used for quantitation and the other for confirmation.

## Method Validation

Single-lab validation of this method was performed with respect to linearity and calibration, specificity, limit of quantitation, precision and accuracy, recovery, and matrix effect.

Linear response was achieved for each analyte with coefficients of correlation ( $r$ ) greater than 0.99 throughout the calibration range of over two orders of magnitude (LLOQ to 1 ppb) as shown in Table 3 along with the presentation of the calibration equations. A  $1/x$  weighting factor was applied to each calibration to achieve better quantitation accuracy at low levels. Due to the known instability of NMCs, working standards and calibration standards were prepared daily in DI water and kept in the refrigerated autosampler at 4 °C. Because two columns were used in this method, linearity and calibration were compared with results from each single column. No significant difference was observed with respect to linearity/calibration or quantitation.

**Table 3. Linearity, Calibration, and LLOQ**

Name	$r$	Equation*	LLOQ	S/N at LLOQ (Quantitative)	S/N at LLOQ (Confirmative)
Aldicarb sulfoxide	0.9992	$y = 0.00688x - 0.00291$	10	172	4
Aldicarb sulfone	0.9989	$y = 0.00538x + 0.00602$	10	17	26
Oxamyl	0.9998	$y = 0.00971x - 0.000882$	10	47	15
Methomyl	0.9998	$y = 0.00609x - 0.00858$	10	57	6
3-Hydroxycarbofuran	0.9995	$y = 0.00671x + 0.00244$	10	46	20
Aldicarb	0.9995	$y = 0.0113x - 0.00783$	10	132	26
Propoxur	0.9995	$y = 0.00121x - 0.00949$	10	41	57
Carbofuran	0.9993	$y = 0.00142x - 0.00884$	10	87	46
Carbaryl	0.9995	$y = 0.0086x + 0.00118$	10	58	19
1-Naphthol	0.9970	$y = 0.000166x - 0.00233$	100	16	7
Methiocarb	0.9998	$y = 0.00938x - 0.00302$	10	275	129

\* Calibration range from LLOQ to 1000 ppt

The method specificity was evaluated by comparing expected responses from blank and spiked samples. Assays of blank samples spiked with IStd showed no peaks at the specific retention times of all the target analytes. These blank matrices were then spiked with carbamate standards and specificity was confirmed with positively detected peaks at the specific retention times. The effect of using an IStd for the quantitation of a low-level native analyte was also evaluated because the impurity, i.e., unlabeled analyte, in IStd (99.8% atom % D) could artificially elevate the natural analyte level. A blank sample was spiked with IStd at a high concentration (20 ppb), and no detectable peak was observed for the native form.

LLOQ was determined as the lowest concentration in the calibration standards with the S/N of a quantitative peak greater than 10 and confirmative peak greater than 3. The LLOQ for this method was 10 ppt for each analyte except for 1-naphthol (0.10 ppb). Refer to Table 3 for details.

Precision and accuracy were evaluated by seven repeated assays of standards of 5 pg (equivalent to 25 ppt) and 10 pg (equivalent to 500 ppt) injections. Accuracy was calculated by  $\text{Observed Amount (mean of seven replicates)} / \text{Specified Amount} \times 100\%$  and shown as % Accuracy in Table 4, and precision was addressed by % RSD. Excellent accuracy was observed in the range from 94.1% (oxamyl at 500 ppt) to 117% (methomyl at 500 ppt). Excellent precision was also achieved with the maximum %RSD observed at 6.57% (aldicarb sulfoxide at 500 ppt).

Method recovery for target analytes was evaluated by assays of spiked rice samples free of target analytes. Two rice samples—white rice from USA and red rice imported from Bhutan—were analyzed using this method and no quantifiable amounts of target analytes were found. These samples were used for the evaluation

**Table 4. Precision and Accuracy**

Analyte	25 ppt (n = 7)			500 ppt (n = 7)		
	Mean	% Accuracy	% RSD	Mean	% Accuracy	% RSD
Aldicarb sulfoxide	24.4	97.5	5.79	495	99.0	6.57
Aldicarb sulfone	26.4	106	5.18	575	115	4.60
3-Hydroxycarbofuran	25.4	102	3.67	529	106	3.47
Oxamyl	24.5	97.9	3.93	470	94.1	3.40
Methomyl	27.0	108	5.92	584	117	3.04
Aldicarb	27.0	108	6.20	576	115	4.38
Propoxur	26.6	106	4.02	567	113	3.13
Carbofuran	26.3	105	3.19	568	114	4.98
Carbaryl	25.6	102	5.15	527	105	3.68
1-Naphthol	N/A	N/A	N/A	542	108	5.93
Methiocarb	26.3	105	3.11	544	109	3.11

of method recovery and matrix effects. For each rice sample, NMCs were spiked at 20 ppt and 500 ppt (n = 3), and the observed amounts were used for recovery calculation using the equation:  $\% Recovery = \text{Observed Amount} / \text{Spiked Amount} \times 100\%$ . The result is shown in Table 5.

Observed recovery ranged from 78.5% to 102% for white rice and 41.8% to 133% for red rice except for a high recovery for the low-level propoxur and a low recovery for methiocarb. The low recovery of methiocarb may be explained by the observation that the matrix posed significantly greater signal suppression for methiocarb than IStd (matrix index observed at 0.57 for matrix I, and 0.17 for matrix II). The higher recovery for propoxur at low levels can be explained by a nonlinear matrix effect observed for that compound.

### Analysis of Carbamates in Rice Samples

Samples were randomly selected with the intention to evaluate method performance on different types of rice; a complete survey of all rice samples on the market was beyond the scope of this study. Among the nine samples tested, four were domestically grown with one labeled as organic; and five were imported with one labeled as organic. Two rice samples (both imported) showed quantifiable levels of NMCs: 29.9 ppt propoxur and 11.5 ppt carbaryl in the rice imported from Italy; and 10.8 ppt carbofuran in the rice imported from India labeled as organic. No quantifiable levels of NMC were found in the other rice samples.

Table 5. Recovery								
%Recovery	Matrix I (White Rice)				Matrix II (Red Rice)			
	20 ppt	% RSD	500 ppt	% RSD	20 ppt	% RSD	500 ppt	%RSD
Aldicarb sulfoxide	88.3	2.29	78.5	6.20	42.5	2.15	55.3	3.57
Aldicarb sulfone	83.2	4.40	81.7	3.90	41.8	20.4	73.5	3.55
Oxamyl	102	2.86	95.6	5.78	54.9	15.2	61.1	6.38
Methomyl	102	3.27	89.5	4.25	97.5	6.22	117	1.85
3-Hydroxycarbofuran	102	1.50	98.6	1.76	79.5	6.07	120	3.16
Aldicarb	96.8	1.81	87.7	0.95	81.3	7.69	106	1.99
Propoxur	223	16.2	101	3.87	223	10.8	133	2.87
Carbofuran	109	1.74	99.5	3.25	115	2.42	124	1.75
Carbaryl	99.2	7.14	98.1	2.75	96.5	4.61	99.4	1.94
1-Naphthol	N/A	N/A	90.0	11.1	N/A	N/A	74.3	6.65
Methiocarb	64	6.94	51.5	9.74	23.3	9.04	17.7	5.58

## Conclusion

The work shown here describes a high-throughput UHPLC-MS/MS method for ultratrace level analysis of 11 U.S. EPA specified *N*-methyl carbamates in rice samples. Accelerated solvent extraction was used for sample preparation and MS/MS instrument operated in product ion-monitoring mode was selected to provide sensitive and selective quantification. As demonstrated here, NMCs can be routinely quantified at extremely low levels (LLOQs at 10 ppt; 100 ppt for 1-naphthol). Excellent linearity and correlations of determination (*r*) were observed through a two orders-of-magnitude calibration range. Method specificity was validated and confirmed. Method quality parameters such as precision and accuracy, recovery, and matrix effect were also evaluated and presented. This method was then successfully applied to the quantification of NMCs in rice samples, and three NMCs were found in two imported rice samples.

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