High Throughput Determination of VX in Drinking Water by Immunomagnetic Separation and Isotope Dilution High Performance Liquid Chromatography Tandem Mass Spectrometry (HPLC/MS/MS)
HIGH THROUGHPUT DETERMINATION OF VX IN DRINKING WATER BY IMMUNOMAGNETIC SEPARATION AND ISOTOPE DILUTION HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (HPLC/MS/MS)

Version 1.0

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Disclaimer

The U.S. Environmental Protection Agency (EPA) through its Office of Research and Development funded and managed the research described herein under EPA Interagency Agreement (IA) # DW75-92259701 with the Centers for Disease Control and Prevention (CDC). The VX standard provided for this research was provided by Lawrence Livermore National Laboratory (LLNL) under EPA IA#DW89-92261601 and a material transfer agreement between LLNL and CDC. This content has been peer and administratively reviewed and has been approved for publication as a joint EPA and CDC document. Approval does not signify that the contents necessarily reflect the views of the EPA, LLNL, the CDC, the Public Health Service, or the U.S. Department of Health and Human Services. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not constitute or imply its endorsement, recommendation, or favoring by the United States government. The views and opinions expressed herein do not necessarily state or reflect those of the United States government and shall not be used for advertising or product endorsement purposes.

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Acronyms

BuChE ................................................................. butyrylcholinesterase
CAS ................................................................. chemical abstract service
CCC ................................................................. continuing calibration check
CDC ................................................................. Centers for Disease Control and Prevention
CR ................................................................. confirmation ratio
CTMDL ......................................................... Chemical Terrorism Methods Development Laboratory
DMP ............................................................. dimethylpimelimidate dihydrochloride
DL ................................................................. detection limit
eI ................................................................. electron ionization
EPA ............................................................. U.S. Environmental Protection Agency
FD ................................................................. field duplicate
LC ................................................................. liquid chromatography
LCt50 .......................................................... median lethal concentration in time
HR
pir. .............................................................. half range for the predicted interval of results
HPLC .......................................................... high performance liquid chromatography
i.d. .............................................................. inside diameter
IDC ............................................................. initial demonstration of capability
IMS ............................................................... immunomagnetic separation
IS ............................................................... internal standard
LD
50 ................................................................. median lethal dose
LFB ............................................................... laboratory fortified blank
LFSM .......................................................... laboratory fortified sample matrix
LFSMD ........................................................ laboratory fortified sample matrix duplicate
LLNL ............................................................ Lawrence Livermore National Laboratory
LRB ............................................................... laboratory reagent blank
MRL ............................................................. minimum reporting level
MRM ............................................................. multiple reaction monitoring
MS/MS ......................................................... tandem mass spectrometer
MSDS ........................................................ Material Safety Data Sheet
OSHA ........................................................ Occupational Safety and Health Administration
PAL .............................................................. provisional advisory level
PBS ............................................................ phosphate buffered saline
PBST ........................................................... phosphate buffered saline with Tween-20®
PJR .............. mean prediction interval of result ± half range for the predicted interval of results
QC ................................................................. quality control
QCS ............................................................. quality control sample
RBC ............................................................. risk based criteria
RPD ............................................................. relative percent difference
Sect. ................................................................ section
TBS ............................................................. tris buffered saline
TOC ............................................................. total organic carbon
VX ............................................................. O-Ethyl S-2-Diisopropylamino-Ethyl Methylphosphonothioate
Executive Summary

This document provides the standard operating procedure for determination of the chemical warfare agent VX (O-Ethyl S-2-Diisopropylamino-Ethyl Methylphosphonothioate) in drinking water by isotope dilution liquid chromatography tandem mass spectrometer (LC/MS/MS). This method was adapted from one that was initially developed by the Centers for Disease Control and Prevention, in the National Center for Environmental Health for the determination and quantitation of VX in aqueous matrices. This method is designed to support site characterization and to inform site-specific cleanup goals of environmental remediation activities following a homeland security incident involving this analyte.

In this method, magnetic beads coated with butyrylcholinesterase (BuChE), a serum protein target of VX, form stable covalent adducts with VX that can be digested into peptides and analyzed for VX. First, a 50-mL water sample is collected, and preserved with sodium thiosulfate (80 mg/L) (a dechlorinating agent) and sodium omadine (64 mg/L) (an antimicrobial preservative). The sample is buffered and aliquotted into a 96-well plate containing magnetic beads that have been conjugated to antibodies against BuChE and further conjugated to BuChE from human serum. The sample is incubated with the magnetic beads for two hours allowing VX-BuChE adducts to form. After washing the beads to remove residual sample, the extracted proteins are enzymatically digested to convert the protein adducts into smaller peptide adducts. Following digestion, the beads are removed from digest solution, isotopically-labeled peptide internal standard is added, and the sample is filtered to remove residual beads and undigested proteins. Filtered samples are then separated by high performance liquid chromatography and analyzed by tandem mass spectrometry (HPLC/MS/MS) for VX-BuChE peptide adducts using multiple reaction monitoring. Analyte identification is accomplished by comparing the acquired mass spectra, including ion ratios, and retention times to reference spectra and retention times for calibration standards acquired under identical LC/MS/MS conditions. Quantitation is performed using the internal standard technique. Utilization of an isotopically labeled internal standard VX-BuChE adducts provides a high degree of accuracy and precision for sample quantitation by accounting for analyte recovery from sample filtration and analytical efficiency.

Accuracy and precision data have been generated in reagent water, and in finished ground and surface waters containing residual chlorine or chloramine that have been used as disinfectants.
HIGH THROUGHPUT DETERMINATION OF VX IN DRINKING WATER BY IMMUNOMAGNETIC SEPARATION AND ISOTOPE DILUTION HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (HPLC/MS/MS)

1. SCOPE AND APPLICATION

1.1 This report describes an isotope dilution liquid chromatography tandem mass spectrometer (LC/MS/MS) method for the determination of the organophosphorous nerve agent, VX (O-Ethyl S-2-Diisopropylamino-Ethyl Methylphosphonothioate; Chemical Abstract Services [CAS] Registry Number® 50782-69-9) in the drinking water matrix. This method, including quality control (QC) requirements, is designed to support site characterization and to inform site-specific cleanup goals of environmental remediation activities following a homeland security incident involving this analyte. VX is not a chemical regulated in drinking water under the Safe Drinking Water Act (as amended), so there is no maximum contaminant level for VX in drinking water that has been set by federal regulation [1].

1.2 Significance: VX is a highly toxic synthetic organophosphorous nerve agent, which is used solely as a chemical weapon. The estimated oral LD$_{50}$ (median lethal dose) for liquid VX in rats has been reported at 100 μg/kg and 66 μg/kg [2]. Risk-based criteria (RBC) have been identified from existing health benchmarks to serve as analytical targets when developing analytical methods for various chemicals. RBCs reflect a lifetime exposure and are expected to generally be lower than minimum reporting levels (MRLs) required for most site-specific objectives. The RBC for VX in water is 0.021 μg/L for the general public for 30 days [3]. VX is persistent in the environment and less volatile than other nerve agents such as sarin [2,4]. Subchronic exposure to VX has been shown to induce behavioral effects in rats [5] so monitoring for low levels of VX is necessary to protect the environment and public health.

1.3 Samples are extracted by immunomagnetic separation (IMS), which provides increased selectivity and sensitivity for VX in aqueous matrices. Magnetic beads coated with butyrylcholinesterase (BuChE), a serum protein target of VX, form stable covalent adducts with VX that can be digested into peptides and analyzed for VX.

1.4 Whether performed manually or with automation, the use of 96-well plates for the IMS procedure provides a key benefit to sample extraction throughput. The 96-well plates allow for extensive automation of the method, thereby enabling high throughput of samples, as might be required during environmental remediation.

1.5 Isotopically labeled peptides corresponding to VX adducts to butyrylcholinesterase (BuChE) serve as an internal standard. The internal standard
is added equally to all unknowns, quality controls, and calibration standards after sample extraction and before sample filtration. In addition to enabling accurate quantitation of samples, calibrators, and QC samples by tandem mass spectrometry (MS/MS), internal standards also account for and resolve some of the issues surrounding analysis including analysis efficiency and sample loss during filtration. The overall QC approach utilizing quantitation and confirmation ions, as well as an isotopically labeled internal standard, greatly increases confidence that VX, and not another molecule with similar fragmentation patterns, is being quantitated during analysis.

1.6 This method was adapted from one that was initially developed by the Centers for Disease Control and Prevention (CDC), in the National Center for Environmental Health, Division of Laboratory Sciences, Emergency Response Branch, in the Chemical Terrorism Methods Development Laboratory (CTMDL) for the determination and quantitation of VX in aqueous matrices [6]. For the adapted method, accuracy and precision data have been generated in reagent water, and in finished ground and surface waters that contain residual chlorine or chloramine that have been used as disinfectants.

1.7 The QC approach in this method conforms to CTMDL standards for clinical samples, and is presented here in terms more familiar to drinking water laboratories. Methods developed by CTMDL are distributed to the CDC’s laboratory network, and the QC approach included in these methods, while single lab verified by the CTMDL lab, is designed to be sufficiently rigorous that network labs can successfully perform the method.

1.8 The minimum reporting level (MRL) is the lowest analyte concentration that meets data quality objectives for the intended use of the method, e.g., to meet site-specific remediation goals. Laboratories will need to demonstrate that their laboratory MRL meets the requirements described in Section 9.2.4.

1.9 Determining the detection limit (DL) is optional (Sect. 9.2.6). Detection limit is defined as the statistically calculated minimum concentration that can be measured with 99% confidence that the reported value is greater than zero.

1.10 This method is intended for use by analysts skilled in the performance of IMS extractions, the operation of high performance liquid chromatography tandem mass spectrometer (HPLC/MS/MS) instruments, and the interpretation of the associated data.

1.11 This method has been verified using only the conditions and equipment specified in the method. Alteration of this method is not recommended.
2. SUMMARY OF METHOD

2.1 A 50-mL water sample is collected, and preserved with sodium thiosulfate (80 mg/L) (a dechlorinating agent) and sodium omadine (64 mg/L) (an antimicrobial preservative). Magnetic beads are conjugated to antibodies against BuChE and further conjugated to BuChE from human serum. The sample is buffered and dispensed as aliquots into a 96-well plate containing the prepared magnetic beads. The sample is incubated with the magnetic beads for two hours in order to allow VX-BuChE adducts to form. After washing the beads to remove residual sample, the proteins are enzymatically digested to convert the protein adducts into smaller peptide adducts. Following digestion, the beads are removed from digest solution, isotopically-labeled peptide internal standard is added, and the sample is filtered to remove residual beads and undigested proteins. Filtered samples are then separated by high performance liquid chromatography and analyzed by tandem mass spectrometry (HPLC/MS/MS) for VX-BuChE peptide adducts using multiple reaction monitoring. Analyte identification is accomplished by comparing the acquired mass spectra, including ion ratios, and retention times to reference spectra and retention times for calibration standards acquired under identical LC/MS/MS conditions. Quantitation is performed using the internal standard technique. Utilization of an isotopically-labeled internal standard VX-BuChE adduct peptide provides a high degree of accuracy and precision for sample quantitation by accounting for analyte recovery from sample filtration and analytical efficiency.

2.2 Compared to some drinking water methods (e.g., certain EPA 500 series methods), the initial laboratory demonstration of capability (IDC) is lengthier than some drinking water methods, the frequency of the on-going calibration is shorter, and the number of continuing calibration checks (CCC) is higher. Based on experience in the developer’s lab, this QC approach ensures successful long-term implementation of the method in other labs, particularly when these methods are used infrequently (e.g., in emergency situations). Due to site-specific circumstances during an environmental remediation activity, changes to the on-going calibration frequency and number of CCCs may be necessary and appropriate. However, initial and ongoing QC requirements and acceptance criteria (see Section 9) should not be changed. Adopting steps, such as a replacing on-going recalibration with a calibration check only, to save time may result in higher QC failure rates and perhaps less accurate quantitation. Labs should discuss these increased risks with sample submitters before taking such steps.

3. DEFINITIONS

3.1 ANALYSIS BATCH – a sequence of samples, analyzed within a 24-hour period, including no more than 20 field samples in addition to all of the required QC samples (Sect. 9.3).
3.2 CALIBRATION STANDARD STOCK SOLUTION – a solution prepared from the primary dilution standard solution(s) and/or stock standard solution(s) and the internal standard(s). The calibration standard stock solutions are used to calibrate the instrument response with respect to analyte concentration.

3.3 CONFIRMATION ION TRANSITION – the second most abundant ion transition for the VX-BuChE peptide adduct analyte (see Confirmation Ratio, Sect. 3.4, below). The confirmation ion transition for this peptide is listed in Table 3-1. The confirmation ion transition is used to calculate the confirmation ratio (Sect. 3.4).

3.4 CONFIRMATION RATIO (CR) - peak area produced by the confirmation ion transition divided by the peak area produced by the quantitation ion transition which serves as an additional QC measure of analyte selectivity.

3.5 CONTINUING CALIBRATION CHECK (CCC) SOLUTION – a calibration solution containing the method analyte(s), which is extracted in the same manner as the samples and analyzed periodically to verify the accuracy of the existing calibration for those analyte(s).

3.6 DETECTION LIMIT (DL) – the minimum concentration of an analyte that can be identified, measured, and reported to be greater than zero with 99% confidence.

3.7 FIELD DUPLICATES (FD1 and FD2) – two separate samples collected at the same time and place under identical circumstances, and treated exactly the same throughout field and laboratory procedures to provide check the precision associated with sample collection, preservation, storage, and laboratory procedures.

3.8 ISOTOPICALLY-LABELED INTERNAL STANDARD – a pure chemical added to an extract or to a standard solution in a known amount(s) and used to measure the relative response of other method analytes and surrogates that are components of the same solution. The internal standard ion transition monitored in this method is listed in Table 3-1.

3.9 LABORATORY FORTIFIED BLANK (LFB) – a volume of reagent water or other blank matrix to which known quantities of the method analytes and all the preservation reagents are added in the laboratory (Sect. 7.3.5.2). The LFB is analyzed exactly like a sample and its purpose is to determine whether the methodology is in control and whether the laboratory is capable of making accurate and precise measurements.

3.10 LABORATORY REAGENT BLANK (LRB) – an aliquot of reagent water that is treated exactly as a sample and used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus (Sect. 7.3.5.3).
3.11 MATERIAL SAFETY DATA SHEET (MSDS) – written information provided by vendors detailing a chemical’s toxicity, health hazards, physical properties, fire and reactivity data, and including precautions for storage, spill, and handling.

3.12 MINIMUM REPORTING LEVEL (MRL) – the minimum concentration qualified to be reported as a quantitated value for a method analyte in a sample following analysis (Sect. 9.2.4. for MRL verification procedure).

3.13 PRIMARY DILUTION STANDARD SOLUTION – a solution containing the analytes prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

3.14 QUANTITATION ION TRANSITION – the most abundant ion transition for each analyte and internal standard as shown below (See Confirmation Ratio, Sect. 3.4, above). Only quantitation ion transitions are monitored for internal standards. The quantitation ion for this method is listed in Table 3-1.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>VX-BuChE Peptide Analyte</td>
<td>902</td>
<td>778</td>
<td>673</td>
</tr>
<tr>
<td>Internal Standard</td>
<td>913</td>
<td>785</td>
<td>Not Monitored</td>
</tr>
</tbody>
</table>

3.15 SECOND SOURCE QUALITY CONTROL SAMPLES – materials obtained from a source different than the original and used to verify the accuracy of the existing calibration for those analytes

4. INTERFERENCES

4.1 Method interferences that can lead to discrete artifacts and/or elevated baselines in the chromatograms can be caused by contaminants in solvents, reagents (including reagent water), sample bottles and caps, and other sample processing hardware. All such items must be routinely demonstrated to be free from interferences under the conditions of the analysis by analyzing laboratory reagent blanks. Subtracting blank values from sample results is not permitted.

4.2 Matrix interferences may be caused by contaminants that are co-extracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature of the water.

4.3 Relatively high concentrations, in the mg/L range, of preservatives, antimicrobial agents, or dechlorinating agents might be added to sample collection vessels (Section 8.1.2). The potential exists for trace-level organic contaminants in these
reagents. Interferences from these sources should be monitored by analysis of laboratory reagent blanks particularly when new lots of reagents are acquired.

4.4 Due to the nature of the matrix analyzed in this procedure, occasional interferences from unknown substances might be encountered. Interfering compounds can be recognized by deviations in the sample quantitation/conformation ratios from the calibration standard ratios and can also be monitored using appropriate LRBs. Any interference that results in QC failure (Sect. 9) results in rejection of the entire analysis batch. If repeating the analysis does not remove the interference with the reference standard, the results for that analyte are not reportable.

4.5 All glassware should be chemically cleaned before running this method. Wash glassware thoroughly with bleach and reagent-grade water followed by acetonitrile. Allow glass to dry completely before use. It is important that all residual bleach is removed from glassware that will be used in this method as bleach will degrade VX. An oven can be used to dry glassware thoroughly, but should not be used for decontamination purposes.

4.6 Care should be taken at all times to prevent contamination of QC materials, standards, and samples.

4.7 Chromatographic separation of the analyte should be carefully monitored for unknown interferences. See Section 11.3.5 for analyte confirmation.

5. SAFETY

5.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined. Each chemical should be treated as a potential health hazard, and exposure to these chemicals should be minimized. Each laboratory is responsible for complying with OSHA regulations regarding safe handling of chemicals used in this method. A reference file of MSDSs must be made available to all personnel involved in sampling handling or chemical analyses. Additional references to laboratory safety are available [5, 7, 8].

5.2 VX is highly toxic and all routes of exposure (dermal, inhalation, ocular, etc.) should be avoided. The estimated LD$_{50}$ value for VX is 10 mg for dermal exposure and the estimated LC$_{t0}$ (median lethal concentration in time) value for VX exposure to vapors is 10 mg • min/m$^3$ [7]. The oral LD$_{50}$ value is estimated between 66-100 µg/kg [2] with a provisional advisory level (PAL) value for ingestion of 0.22 µg/L [3]. The concentration of solutions of VX used in this method should never exceed 10 µg/mL. Universal safety precautions should be used including the use of a lab coat, safety glasses, appropriate gloves, and a high quality-ventilated chemical fume hood and/or biological safety cabinet. In addition, all work areas should be thoroughly cleaned with a 5% hypochlorite
solution before and immediately after work with VX is performed in these areas. All materials that come in contact with VX should be decontaminated in a container of 5% solution of hypochlorite. An additional container of same solution should be available during any work with VX and used to decontaminate spills, equipment, and other work areas.

5.3 Avoid inhalation or dermal exposure to acetonitrile, methanol, and formic acid, which are used in the sample preparation and analysis steps.

5.4 Mechanical hazards when performing this procedure using standard safety practices are minimal. Read and follow the manufacturers’ information regarding safe operation of the equipment. Avoid direct contact with the mechanical and electronic components of the liquid chromatograph and mass spectrometer, unless all power to the instrument is off. Generally, maintenance and repair of mechanical and electronic components should be performed only by qualified technicians.

6. EQUIPMENT AND SUPPLIES (It is important to note that specific brands or catalog numbers included in this section are examples only and do not imply endorsement of these particular products. These specific products were used during the verification of this method.)

6.1 MICRODISPENSERS – with adjustable volume (5-100 μL, 100-1000 μL) (Eppendorf Co., Westbury, NY) or equivalent

6.2 REPEATER PIPETTE – Model 4780 (Eppendorf Co., Westbury, NY) or equivalent

6.3 ANALYTICAL BALANCE – Capable of weighing to the nearest 0.0001 g

6.4 IMMUNOMAGNETIC SEPARATION EQUIPMENT 96-WELL MAGNET OR MAGNETIC BEAD AUTOMATION EQUIPMENT WITH 96 WELL PLATES

6.4.1 IMS can be performed manually or using automated equipment.

6.4.2 Manual extractions require magnets compatible with 96-well formatting. Equipment required for manual extractions:

6.4.2.1 DynaMag® 2 magnetic bead separator (PN# 12321D, available from Invitrogen, Grand Island, NY) or equivalent

6.4.2.2 Dynal® sample mixer (PN# 947-01, available from Invitrogen, Grand Island, NY) or equivalent
6.4.3 Automated extraction can be performed using automation instrumentation such as the KingFisher® Flex Magnetic Particle Processor with deep-well head (Thermo Fisher Scientific, Waltham, MA; PN# 95041-912 available from VWR, St. Louis, MO). Consumables for use with the KingFisher instrument include:

6.4.3.1 Eppendorf® MixMate® plate mixer (Eppendorf AG, Hamburg, Germany; PN#14900-548 available from VWR, St. Louis, MO) or equivalent

6.4.3.2 96-well deep V-bottom KingFisher microplates, 2mL (PN#11388-566 available from VWR, St. Louis, MO) or equivalent

6.4.3.3 KingFisher 96-well plates, 200 µL (PN# 83007-596 available from VWR, St. Louis, MO) or equivalent

6.4.3.4 KingFisher tip comb for 96-well deep well magnets (PN# 83007-594 available from VWR, St. Louis, MO) or equivalent

6.4.4 All extractions require the following additional instrumentation:

6.4.4.1 A water bath capable of maintaining 37°C such as the Precision water bath (Winchester, VA) or equivalent

6.4.4.2 Heat sealer (PN# AB0384/110, available from ABgene House, Surrey, UK) or equivalent

6.4.4.3 Easy Pierce 20 µm heat sealing foil (Thermo Fisher Scientific, Waltham, MA; PN# AB1720, available from ABgene House, Surrey, UK) or equivalent

6.4.4.4 Eppendorf adhesive foils (PN# 0030127820, available from Fisher Scientific, Fair Lawn, NJ) or equivalent

6.4.4.5 96-well PCR plate (ABgene product, Thermo Fisher Scientific, Waltham, MA; sold by Advion, Inc., Ithaca, NY, PN# 1002611) or equivalent

6.4.4.6 Multiscreen Ultracel®-10 filter plates (PN# MAUF01010 available from Fisher Scientific, Pittsburgh, PA) or equivalent

6.4.4.7 MixMate plate mixer (PN# 53532G807598 available from Eppendorf, Hamburg, Germany) or equivalent
6.5 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ELECTRON IONIZATION TANDEM MASS SPECTROMETRY SYSTEM (HPLC/MS/MS)

6.5.1 HPLC COLUMN – Aquasil® C18 column, 50 x 1 mm inside diameter (i.d.), 3 μm particle size (Aquachemi Inc., Missouri, TX; PN# 77503-051030 available from Fisher Scientific, Pittsburgh, PA) or equivalent

6.5.2 HPLC SYSTEM – The LC system (Waters nanoAcquity HPLC, Waters Technology Inc., Milford, MA; or equivalent) should be equipped with an autosampler and injector and should provide consistent sample injection volumes. Mobile phases should be connected to an inline degasser that runs consistently during sample analysis. The HPLC should be capable of being configured exactly as stated in Table 6-1.

Table 6-1. High Performance Liquid Chromatograph (HPLC) Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC Method</td>
<td>Gradient:</td>
</tr>
<tr>
<td></td>
<td>Reservoir A = 0.1% Formic Acid in HPLC-Grade Water</td>
</tr>
<tr>
<td></td>
<td>Reservoir B = 0.1% Formic Acid in Acetonitrile</td>
</tr>
<tr>
<td>Time (min)</td>
<td>%A</td>
</tr>
<tr>
<td>0</td>
<td>98</td>
</tr>
<tr>
<td>0.1</td>
<td>98</td>
</tr>
<tr>
<td>1.4</td>
<td>65</td>
</tr>
<tr>
<td>2.2</td>
<td>65</td>
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<tr>
<td>2.5</td>
<td>55</td>
</tr>
<tr>
<td>4.5</td>
<td>55</td>
</tr>
<tr>
<td>4.51</td>
<td>98</td>
</tr>
<tr>
<td>5.0</td>
<td>98</td>
</tr>
<tr>
<td>Column type</td>
<td>Aquasil C18 3μm column, 1.0x50 mm, 3 μm particle size</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>10 μL</td>
</tr>
<tr>
<td>Autosampler Tray Temperature</td>
<td>4°C</td>
</tr>
<tr>
<td>Column Temperature</td>
<td>25±5°C</td>
</tr>
<tr>
<td>Injection Settings</td>
<td>Draw Speed: 200 μL/min</td>
</tr>
<tr>
<td></td>
<td>Eject Speed: 200 μL/min</td>
</tr>
<tr>
<td></td>
<td>Injection Mode: Standard</td>
</tr>
<tr>
<td>Needle Rinse Settings</td>
<td>Weak Solvent Wash: Water, 600 μL</td>
</tr>
<tr>
<td></td>
<td>Strong Solvent Wash: 50% Methanol in HPLC-Grade Water (200 μL)</td>
</tr>
<tr>
<td>Sample Loop Option</td>
<td>Partial Loop (Loop Offline Disabled)</td>
</tr>
<tr>
<td>Typical retention time for VX-BuChE</td>
<td>1.87 minutes</td>
</tr>
</tbody>
</table>
6.5.3 MASS SPECTROMETER (MS) – The MS/MS (Applied Biosystems API 4000 quadrupole ion trap mass spectrometer, Foster City, CA; or equivalent) should be capable of performing electrospray ionization with both positive and negative ion detection and must be configured for multiple reaction monitoring (MRM) with a dwell time of 100 msec per ion. Tandem mass spectrometer (MS/MS) parameters used during method verification are shown in Table 6-2.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS Scan Mode</td>
<td>Multiple Reaction Monitoring</td>
</tr>
<tr>
<td>Ionization Type</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>Dwell Time</td>
<td>35 msec per channel</td>
</tr>
<tr>
<td>Curtain Gas</td>
<td>5</td>
</tr>
<tr>
<td>Source Temperature</td>
<td>250 (interface heater ON)</td>
</tr>
<tr>
<td>Ion Source Gas 1</td>
<td>40</td>
</tr>
<tr>
<td>Ion Source Gas 2</td>
<td>50</td>
</tr>
<tr>
<td>Collision Gas</td>
<td>11</td>
</tr>
<tr>
<td>Ion Spray Voltage</td>
<td>5500</td>
</tr>
<tr>
<td>Entrance Potential</td>
<td>12</td>
</tr>
<tr>
<td>Collision Energy</td>
<td>41</td>
</tr>
<tr>
<td>Cell Exit Potential</td>
<td>20</td>
</tr>
</tbody>
</table>

7. REAGENTS AND STANDARDS (These reagents were used during the verification of the method, and only these or their equivalent are acceptable for use. No endorsement of any supplier or organization should be inferred.)

7.1 GASES, REAGENTS, AND SOLVENTS – Reagent grade or better chemicals should be used. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first determined that the reagent is of sufficiently high purity to permit its use without lessening the quality of the determination.

7.1.1 NITROGEN - 99.9999% pure or better, MS/MS collision cell gas

7.1.2 REAGENT WATER – purified, deionized water which does not contain any measurable quantities of the method analyte or interfering compounds, HPLC or equivalent grade water (available from Tedia, Fairfield, OH and other commercial sources)
7.1.3 METHANOL - (CH$_3$OH, CAS#: 67-56-1) – high purity, demonstrated to be free of analytes and interferences (Tedia HPLC or equivalent)

7.1.4 ACETONITRILE - (CH$_3$CN, CAS#: 75-05-8) – high purity, demonstrated to be free of analytes and interferences (available from Tedia HPLC and other commercial sources)

7.1.5 FORMIC ACID - (HCOOH, CAS#: 64-18-6) – reagent grade >95% purity, demonstrated to be free of analytes and interferences (available from Sigma-Aldrich, St. Louis, MO and other commercial sources)

7.1.6 10x PHOSPHATE BUFFERED SALINE WITH TWEEN-20®, pH 7.4 (PBST) – demonstrated to be free of analytes and interferences (available from Sigma-Aldrich and other commercial sources)

7.1.7 TRIETHANOLAMINE BUFFER SOLUTION – demonstrated to be free of analytes and interferences (PN# T0449-120ML from Sigma-Aldrich, or equivalent)

7.1.8 PHOSPHATE BUFFERED SALINE 10x - Phosphate buffered saline 10x concentrate demonstrated to be free of analytes and interferences (available from Sigma-Aldrich and other commercial sources)

7.1.9 DIMETHYLPIMELIMIDATE DIHYDROCHLORIDE (DMP) - demonstrated to be free of analytes and interferences (available from Sigma-Aldrich and other commercial sources)

7.1.10 0.2M TRIS BUFFERED SALINE, 10x solution - demonstrated to be free of analytes and interferences (available from Sigma-Aldrich and other commercial sources)

7.1.11 PEPSIN FROM PORCINE GASTRIC MUCOSA - demonstrated to be free of analytes and interferences (available from Sigma-Aldrich and other commercial sources)

7.1.12 BuChE ANTIBODIES FROM CLONE 3EA – Product number HAH0020102 (Fisher Scientific, Fair Lawn, NJ) or equivalent

7.1.13 PROTEIN G DYNABEADS® magnetic beads - Product number 10003D (from Invitrogen, Grand Island, NY) or equivalent

7.1.14 POOLED HUMAN SERUM – Pooled human serum preserved with potassium EDTA (ethylenediaminetetraacetic acid) from Tennessee Blood Services (Memphis, Tennessee) or equivalent. Serum should be verified as
free from interferences by extracting blank samples as described in Sect. 11.

7.1.15 SYNTHETIC UNLABELED AND ISOTOPICALLY-LABELED PEPTIDES CORRESPONDING TO VX-BuChE ADDUCTS –custom synthesized at Los Alamos National Laboratory (Los Alamos, NM)

7.1.16 VX IN ISOPROPANOL, 10 ppm – Obtained from Lawrence Livermore National Laboratory through an agreement with the EPA (Interagency Agreement #DW89-92261601)

7.1.17 SAMPLE PRESERVATION REAGENTS – the following sample preservation reagents are required for this method:

7.1.17.1 SODIUM THIOSULFATE (Na₂S₂O₃, CAS#: 7772-98-7) – an additive used in sample collection (Sigma-Aldrich ACS grade or equivalent)

7.1.17.2 SODIUM OMADINE (C₅H₄NNaOS, CAS#: 3811-73-2) – an additive used for sample collection (Sigma-Aldrich > 96% pure or equivalent)

7.2 REAGENT PREPARATION

7.2.1 PHOSPHATE BUFFERED SALINE 1X (PBS) – Dilute 100 mL of 10x phosphate buffered saline with 900 mL of HPLC-grade water and mix well.

7.2.2 PHOSPHATE BUFFERED SALINE WITH TWEEN-20 1X (PBST) – Dilute 100 mL of 10x phosphate buffered saline with 900 mL of HPLC-grade water and mix well.

7.2.3 TRIS BUFFERED SALINE 1X (TBS) - Dilute 100 mL of 10x tris buffered saline with 900 mL of HPLC-grade water and mix well.

7.2.4 0.1% FORMIC ACID IN WATER (MOBILE PHASE A) – Prepare a 0.1% solution of formic acid through dilution with HPLC-grade water. For example, add 500 µL formic acid to 500 mL HPLC-grade water in a volumetric container.

7.2.5 0.1% FORMIC ACID IN ACETONITRILE (MOBILE PHASE B) – Prepare a 0.1% solution of formic acid through dilution with acetonitrile. For example, add 500 µL formic acid to 500 mL HPLC-grade water in a volumetric container.
7.2.6 0.6% FORMIC ACID IN WATER – Prepare a 0.6% solution of formic acid through dilution with HPLC-grade water. For example add 600 μL formic acid to 100 mL HPLC-grade water in a volumetric container.

7.2.7 DMP SOLUTION – Prepare a 5.4 mg/mL solution of DMP in triethanolamine buffer. For example, weigh out 27 mg DMP and dissolve in 4 mL triethanolamine buffer.

7.2.8 PEPsin SOLUTION – Prepare a 2 mg/mL solution of pepsin in 5% formic acid in water. For example, weigh out 20 mg of pepsin and dissolve in 9.5 mL HPLC-grade water with 0.5 mL formic acid and mix thoroughly. This solution should be prepared 30 minutes prior to use.

7.3 STANDARDS SOLUTIONS – When a compound purity is assayed to be 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard. Solution concentrations listed in this section were used to develop this method and are included as an example. Standards for sample fortification generally should be prepared in the smallest volume that can be accurately measured to minimize the addition of excess organic solvent to aqueous samples. Store all calibration and control materials at either -20±5°C when not in use. Even though stability times for standard solutions are suggested in the following sections, laboratories should use standard QC practices to determine when their standards need to be replaced.

7.3.1 ISOTOPICALLY-LABELLED INTERNAL STANDARD SOLUTIONS – The internal standard for VX-BuChE is $^{13}$C₄D₆$^{15}$N-labeled and is custom-synthesized by Los Alamos National Laboratory (Los Alamos, NM). The internal standard can be custom-synthesized by other companies including Battelle Memorial Institute (Columbus, OH) or other appropriate vendors. Note that in this method, the isotopically-labeled internal standard is structurally identical to the method analyte, but substituted with $^{13}$C₄, D₆, and $^{15}$N. Isotopically-labeled internal standards have no potential to be present in water samples, and are not method analytes. These internal standards are added to all samples, standards, and QC solutions as described in Section 11.1.3.

7.3.2 Prepare or purchase the internal standard at a concentration of 550 ng/mL. Steps for the preparation of the mixture are described below:

7.3.2.1 RECONSTITUTE INTERNAL STANDARD – Weigh out 19.2 mg of VX-BuChE internal standard and dilute to 2 mL with 0.6% formic acid in water using a 2 mL volumetric flask. The concentration of this solution is 9.6 mg/mL.

7.3.2.2 INTERNAL STANDARD STOCK SOLUTION – Dilute the reconstituted internal standard solution to 550 ng/mL. To make 10
mL of this solution, dilute 573 μL of the reconstituted internal standard (9.6 mg/mL) to 10 mL with 0.6% formic acid in water using a 10 mL volumetric flask.

7.3.3 ANALYTE STOCK STANDARD SOLUTIONS – Obtain a stock solution of 10 ppm VX in isopropanol from an appropriate source. These stock solutions are stable for at least one year when stored at -20±5°C.

7.3.3.1 ANALYTE STOCK STANDARD SOLUTION 1 – Make 50 μL of a 1000 ng/mL solution of VX in HPLC-grade water. To make this solution, dilute 5 μL of 10 ppm VX with 45 μL HPLC-grade water and mix well. This solution should be prepared fresh for each analysis.

7.3.3.2 ANALYTE STOCK STANDARD SOLUTION 2 – Make 200 μL of a 100 ng/mL solution of VX in HPLC-grade water. To make this solution, dilute 20 μL of analyte stock standard solution 1 (1000 ng/mL) with 180 μL HPLC-grade water and mix well.

7.3.3.3 ANALYTE STOCK STANDARD SOLUTION 3 – Make 1000 μL of a 1 ng/mL solution of VX in HPLC-grade water. To make this solution, dilute 10 μL of analyte stock standard solution 2 (100 ng/mL) with 990 μL HPLC-grade water and mix well.

7.3.4 CALIBRATION STANDARD STOCK SOLUTIONS – Prepare the calibration standard stock solutions from dilutions of the analyte stock solutions in reagent water containing any preservatives required by site-specific circumstances (See Sections 2.2 and 8.1.3). The calibration curve is composed of at least five concentrations. These calibration standard solutions are stable for at least one year when stored at -20±5°C.

7.3.4.1 PREPARATION OF CALIBRATION STANDARD STOCK SOLUTIONS – Calibrations standard stock solutions may be prepared using the volumes listed in Table 7-1 below. The concentrations, along with the numbers of solutions, are for illustration purposes only. Other concentrations may be required in practice to meet performance and QC goal. (See Sect. 10.3 for the number of calibration solutions required for calibration.) All standards should be diluted into HPLC-grade water.
Table 7-1. Calibration Standard Stock Solution Volumes

<table>
<thead>
<tr>
<th>Concentration VX (ng/mL)</th>
<th>Total Volume (µL)</th>
<th>Analyte Stock Solution 2 (µL)</th>
<th>Analyte Stock Solution 3 (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>700</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>700</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>1.13</td>
<td>700</td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td>0.31</td>
<td>700</td>
<td></td>
<td>217</td>
</tr>
<tr>
<td>0.25</td>
<td>700</td>
<td></td>
<td>175</td>
</tr>
<tr>
<td>0.09</td>
<td>700</td>
<td></td>
<td>63</td>
</tr>
<tr>
<td>0.025</td>
<td>700</td>
<td></td>
<td>17.5</td>
</tr>
<tr>
<td>0</td>
<td>700</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

7.3.5 QUALITY CONTROL SOLUTIONS – There are several types of quality control solutions, some of which are identical in composition but serve different QC functions and hence may be referred to by different names in Section 9.

7.3.5.1 SECOND SOURCE QUALITY CONTROL SAMPLE – These samples are used to verify the accuracy of the calibration standard solutions (7.3.4) and are prepared the same way as the calibration standards. They are prepared from an analyte source different than the calibration standard solutions as described more completely in Section 9.3.7.

7.3.5.2 LABORATORY FORTIFIED BLANKS (LFBs) - LFBs are used throughout this method for various purposes. The LFB is analyzed exactly like a sample, and its purpose is to verify that the methodology is competently replicated, and that the laboratory has the capability to make accurate and precise measurements. The two specific LFBs required in this method are referred to as LFB-low and LFB-high, which relate to initial and ongoing QC. For the demonstration of the method in the developer’s laboratory, the LFB-low is 0.25 ng/mL VX. The LFB-high for this demonstration is 2.0 ng/mL VX. LFB-low and LFB-high can be prepared as indicated in Table 7-1, in Section 7.3.4. In a particular lab, the LFBs should be selected from similar points in their calibration range (e.g., LFB-low should be around 10 times the MRL (Sect. 9.2.4) and LFB-high should be around 150 times the MRL.

The LFBs are inherently calibration standards and can be used to construct the calibration curve. However, the LFBs are specifically used to develop QC criteria during the initial demonstration of capability (Sect. 9.2) and serve as an additional QC function during...
each analysis batch. The LFBs serve a similar, but generally more stringent, QC function as continuous calibration checks (Sect. 10.3).

7.3.5.3 LABORATORY REAGENT BLANK. This blank is prepared as a LFB with no analyte added (i.e., the 0 ng/mL in Table 7-1).

8. SAMPLE COLLECTION, PRESERVATION, AND STORAGE

8.1 SAMPLE VESSEL PREPARATION COLLECTION

8.1.1 Samples can be collected in a 50-mL polypropylene vessel fitted with a flat-top polyethylene screw-cap (e.g., BD Falcon™ 50 mL centrifuge tube [BD, Franklin Lakes, NJ] or equivalent).

8.1.2 VX is not stable in some finished tap water samples without preservatives and will rapidly degrade over several days. All samples used in development of this method, including QC samples, were preserved via addition of sodium thiosulfate (80 mg/L) (a dechlorinating agent) and sodium omadine (64 mg/L) (an antimicrobial preservative). The choice of these preservative is based on the stability of VX in the presence of chlorine and monochloramine with preservatives tested, as described in Knaack et al. [8], which suggests VX degradation rate is slowed to the theoretical minimum by this combination of preservatives. Studies of VX indicate that hydrolysis can only be slowed and not stopped. Therefore, any data quality objectives for site-specific remediation plans must take this degradation into account. Minimizing the time between sample collection and analysis will maximize analyte signal.

8.1.3 SAMPLE STORAGE STABILITY STUDIES - Stability data were collected up to 91 days preserved with sodium omadine and sodium thiosulfate and stored at 4°C. This corresponds to approximately one half-life of VX degradation in the presence of these preservatives.

8.1.4 Vessels should be prepared before sample collection with sodium thiosulfate and sodium omadine according to Table 8-1. Preservation through binding free chlorine or dechlorination is necessary for all samples that will be analyzed for VX. All initial and on-going QC requirements should be demonstrated for the preservatives added to the sample.
Table 8-1. Preservative Concentrations and Purposes of Preservatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass added to sample (mg)</th>
<th>Concentration in sample (g/L)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium thiosulfate</td>
<td>4</td>
<td>0.08</td>
<td>Dechlorinates free chlorine and chloramine</td>
</tr>
<tr>
<td>Sodium Omadine</td>
<td>3.2</td>
<td>0.064</td>
<td>Microbial inhibitor</td>
</tr>
</tbody>
</table>

8.2 SAMPLE COLLECTION - When sampling from a water tap, samplers should request guidance about how long to flush the tap, if at all. Depending on site-specific goals, incident managers may request that the tap not be flushed to minimize loss of contaminant. If incident managers do not specify a shorter time, flush until the water temperature has stabilized (approximately 3-5 minutes). Collect samples from the flowing stream. It may be convenient to collect a bulk sample in a polypropylene vessel from which to generate individual 50 mL samples. Keep samples sealed from collection time until analysis. When sampling from an open body of water, fill the sample container with water from a representative area. Sampling equipment, including automatic samplers, should be free of tubing, gaskets, and other parts that could leach interfering analytes into the water sample.

8.3 SAMPLE SHIPMENT AND STORAGE – Sample stability was tested at 4°C and stability measurements are only valid at this temperature. As a matter of practice, ensure that samples do not experience excessive heat above this temperature. It is recommended that all samples be iced, frozen (-20±5°C), or refrigerated (4±2°C) from the time of collection until extraction. During method development, no significant differences were observed between standards that were frozen or refrigerated.

8.4 SAMPLE AND EXTRACT HOLDING TIMES – Results of the sample storage stability study (Table 8-1) suggest that VX stability is best preserved when samples are collected, preserved, shipped, and stored as described in Sections 8.1, 8.2, and 8.3. VX will hydrolyze in aqueous matrices although adequate detection is possible after holding samples for 91 days. As matter of practice, water samples should be extracted as soon as possible but must be extracted within 91 days for this method. Data generated during this study indicates that extracts are stable for at least 28 days when preserved and stored at 0°C or lower. As matter of practice, analysis should occur as soon as possible.
9. **QUALITY CONTROL**

9.1 QC requirements include the initial demonstration of capability (IDC) and ongoing QC requirements that must be met when preparing and analyzing field samples. This section describes the QC parameters, their required frequencies, and the performance criteria that must be met in order to meet typical EPA quality objectives for drinking water analysis, although these objectives will be site specific during a remediation activity. These QC requirements are considered the minimum acceptable QC criteria in particular for this method which utilizes an isotopically labeled internal standard. Laboratories are encouraged to institute additional QC practices to meet specific needs [9].

9.2 **INITIAL DEMONSTRATION OF CAPABILITY (IDC)** – The IDC must be successfully performed prior to analyzing any field samples. Prior to conducting the IDC, the analyst must first generate an acceptable initial calibration following the procedure outlined in Section 10.2. It should be noted that the IDC is lengthier than some drinking water methods, but based on experience in the developer’s lab, the IDC helps to ensure successful long-term implementation of the method in a variety of other labs. Due to site-specific conditions during an environmental remediation activity, a shorter IDC may be necessary and appropriate. For example, a more minimal IDC could consist of: (a) demonstration of low system background (Sect. 9.2.1); (b) 4-7 same-day replicates fortified near the midrange of the initial calibration curve for precision and accuracy demonstration, combined with (c) the MRL estimation described in Section 9.2.4. However, QC acceptance requirements, both initial (Sect. 9.2.1-9.2.4) and ongoing (Sect. 9.3) should not be changed, and a shorter IDC might result in higher QC failure rates and less accurate quantitation in some concentration ranges. Labs should consider these risks before choosing a shorter IDC.

9.2.1 **INITIAL DEMONSTRATION OF LOW SYSTEM BACKGROUND** – Any time a new lot of solvents, reagents, magnetic beads, or autosampler vials/plates are used, it must be demonstrated that an LRB is reasonably free of contamination and that the criteria in Section 9.3.1 are met.

9.2.2 **INITIAL DEMONSTRATION OF PRECISION** – Prepare and analyze at least seven replicates of both laboratory fortified blanks (LFB-high and LFB-low, see Sect. 7.3.5.2) over the course of at least 10 days. Any sample preservative, as described in Section 8.1.2, must be added to these samples. For the initial demonstration of precision, the relative standard deviation for the concentrations of the replicate analyses should be less than 20%.

9.2.3 **INITIAL DEMONSTRATION OF ACCURACY** – Using the same set of replicate data generated for Section 9.2.2, calculate the mean recovery. For the initial demonstration of accuracy, the mean recovery of the replicate values should be within ± 30% of the true value.
9.2.4 MINIMUM REPORTING LEVEL (MRL) ESTIMATION – Because cleanup goals will be site specific, laboratories need to estimate a minimum reporting level so that incident managers can understand a specific laboratory’s capabilities and can distribute samples to appropriate laboratories. Establishing the MRL concentration too low may cause repeated failure of ongoing QC requirements. If the IDC procedure (Sect. 9.2.1-9.2.3) is followed explicitly, establishing the MRL as the lowest standard is expected to ensure compliance with QC requirements. This is a result of the rigor of the QC requirements in the lengthy IDC (Sect. 9.2.1-9.2.3), especially those associated with the LFBs (see Sect. 10.3.3). If a shorter IDC is required by site specific conditions (see Sect. 2.2), the MRL should be confirmed with the procedure below.

9.2.4.1 Fortify and analyze seven replicate LFBs at the proposed MRL concentration. These LFBs must contain all method preservatives described in Section 8.1.2. Calculate the mean measured concentration ($\text{Mean}$) and standard deviation for the method analytes in these replicates. Determine the half range for the prediction interval of results ($\text{HR}_{\text{PIR}}$) for each analyte using the equation below:

$$\text{HR}_{\text{PIR}} = 3.963s$$

where $s$ is the standard deviation and 3.963 is a constant value for at least seven replicates.

9.2.4.2 Confirm that the upper and lower limits for the prediction interval of result ($\text{PIR} = \text{Mean} \pm \text{HR}_{\text{PIR}}$) meet the upper and lower recovery limits as shown below:

The Upper PIR Limit should be $\leq 150\%$ recovery.

$$\frac{\text{Mean} + \text{HR}_{\text{PIR}}}{\text{FortifiedConcentration}} \times 100\% \leq 150\%$$

The Lower PIR Limit should be $\geq 50\%$ recovery.

$$\frac{\text{Mean} - \text{HR}_{\text{PIR}}}{\text{FortifiedConcentration}} \times 100\% \geq 50\%$$

9.2.4.3 The MRL is validated if both the upper and lower PIR limits meet the criteria described above (Sect. 9.2.4.2). If these criteria are not met, the MRL has been set too low and should be confirmed again at a higher concentration.
9.2.5 CALIBRATION CONFIRMATION – The calibration is confirmed by analysis of a second source quality control sample as described in Section 9.3.5

9.2.6 DETECTION LIMIT (DL). This is a statistical determination of precision and accurate quantitation is not expected at this level. Replicate analyses for this procedure should be done over at least three days (i.e., both the sample preparation and the LC/MS/MS analyses should be done over at least three days). At least seven replicate LFBs should be analyzed during this time period. The concentration can be estimated by selecting a concentration at two to five times the noise level. The appropriate fortification concentrations will be dependent upon the sensitivity of the LC/MS/MS system used. Any preservation reagents added in Section 8.1.2 must also be added to these samples. Note that the concentration for some IDC steps may be appropriate for DL determination, in which case the IDC data may be used to calculate the DL. (For example, for the results presented in Section 13, eight replicate LFBs were analyzed over 10 days, with two LFBs individually fortified on day one, two LFBs individually fortified on day two, and two LFBs individually fortified on day three, etc.) Analyze the replicates through all steps of Section 11. Calculate the DL from the equation: 

\[ DL = s \times t_{(n-1)} \]

where:
- \( s \) = standard deviation of replicate analysis, without blank subtraction
- \( t \) = Student’s \( t \) value for the 99% confidence level with \( n-1 \) degrees of freedom
- \( n \) = number of replicates

9.3 ONGOING QC REQUIREMENTS – This section summarizes the ongoing QC criteria when processing and analyzing field samples. The required QC samples for an analysis batch include the laboratory reagent blank (LRB) and four continuing calibration check (CCC) solutions.

9.3.1 LABORATORY REAGENT BLANK (LRB) – An LRB is required with each analysis batch (Sect. 3.1) to confirm that potential background contaminants are not interfering with the identification or quantitation of the method analyte. Running the LRB first could prevent unnecessary analysis if the LRB is invalid. Preparation of the LRB is described in Section 7.3.5. If the LRB produces a peak within the retention time window of the analyte, accurate determination of the analyte will not be possible. Determine the source of contamination and eliminate the interference before processing samples. Background contamination must be reduced to an acceptable level before proceeding. Background from method analyte or other contaminants that interfere with the measurement
of method analyte should be below 1/3 of the MRL. Blank contamination is estimated by extrapolation, if the concentration is below the lowest calibration standard. This extrapolation procedure is not allowed for sample results as it may not meet data quality objectives. If the method analyte is detected in the LRB at concentrations greater than 1/3 the MRL, then all data for VX are considered invalid for all samples in the analysis batch.

9.3.2 ONGOING CALIBRATION – The analytical system is recalibrated at the beginning of each analysis batch using the same analyte concentrations determined during the initial calibration. The acceptance criteria for the ongoing calibration are described in Section 10.2.5, except that removal of calibration points could result in too few calibration points and therefore an invalid calibration. The ongoing calibration is performed after the first two continuing calibration check (CCC) samples (Sec. 9.3.3) to allow for corrective action if the calibration fails. As mentioned in Sect. 2.2, in some well-considered circumstances and in consultation with the sample submitter about increased QC and quantitation risk, it may be desirable to not perform the ongoing calibration (Sect. 9.3.2) and instead rely on CCC samples (as described in Sect. 9.3.3) to verify ongoing calibration. If so, the beginning CCC of each analysis batch should be at or below the MRL in order to verify instrument sensitivity prior to any analyses. Subsequent CCCs should alternate between a medium and high concentration calibration standard.

9.3.3 CONTINUING CALIBRATION CHECK (CCC) – CCC standards, containing the preservatives, if any, are analyzed at the beginning of each analysis batch, after every 20 field samples. Note that there are up to four CCCs depending on the IDC appropriate for the site-specific circumstance. In the lengthier IDC described in Sect. 9.2, there are four CCCs: LFB-low and LFB-high, which are analyzed before the batch, and the lowest and highest calibration standards from the ongoing calibration (Sect 9.3.2), which are analyzed after the field samples. If this IDC approach is not appropriate, then there are at most two CCC standards, i.e. the calibration standards. Depending on site-specific goals and tolerance of QC and quantitation risk, it may acceptable to only run one of these calibration standards as the CCC before and after the batch. If so, the beginning CCC of each analysis batch should be at or below the MRL in order to verify instrument sensitivity prior to any analyses. Subsequent CCCs should alternate between a medium and high concentration calibration standard. See Section 10.3 for acceptance criteria for the various CCCs. Preparation of the CCC is described in Section 7.3.5.

9.3.4 LABORATORY FORTIFIED BLANK (LFB) – Since this method utilizes procedural calibration standards, which are fortified reagent waters, there is no difference between the LFB and the CCC, except for the order in
which they are run as part of an analysis batch and the corresponding QC acceptance criteria. The acronym LFB is used for clarity in the IDC.

9.3.5 SECOND SOURCE QUALITY CONTROL SAMPLES (QCS) – As part of the IDC (Sect. 9.2), each time a new VX analyte stock standard solution 1 (Sect. 7.3.3.1) is prepared, at least quarterly, analyze a QCS sample from a source different from the source of the calibration standards. If a second vendor is not available, then a different lot of the standard should be used. The QCS should be prepared near the midpoint of the calibration range and analyzed as a CCC. Acceptance criteria for the QCS are identical to the CCCs; the calculated amount for each analyte should be ± 30% of the expected value. If measured analyte concentrations are not of acceptable accuracy, check the entire analytical procedure to locate and correct the problem.

9.3.6 INTERNAL STANDARD (IS) – The analyst should monitor the peak area of the IS in all injections during each analysis day. The IS peak area must meet the criteria in the following two subsections (9.3.6.1 and 9.3.6.2).

9.3.6.1 The internal standard should produce a peak area at least five times higher than the peak area of the quantitation ion transition of the corresponding analyte in the lowest concentration calibration solution. If it does not, the concentration of IS may not be as predicted. Prepare new calibrations solutions, QC samples, and field samples with an appropriately increased concentration of IS.

9.3.6.2 The IS response (peak area) in any chromatographic run must not deviate from the response in the most recent CCC by more than 30%, and must not deviate by more than 50% from the area measured during initial analyte calibration. If the IS area in a chromatographic run does not meet these criteria, inject a second aliquot of that extract.

- If the reinjected aliquot produces an acceptable IS response, report results for that aliquot.

- If the reinjected aliquot fails the IS criterion, the analyst should check the calibration by reanalyzing the most recently acceptable calibration standard. If the calibration standard fails the criteria of Section 10.3, recalibration is in order per Section 10.2. If the calibration standard is acceptable, report results obtained from the reinjected aliquot, but annotate as “suspect/IS recovery.” Alternatively, prepare another aliquot of the sample or collect a new sample and re-analyze.
9.3.7 LABORATORY FORTIFIED SAMPLE MATRIX (LFSM) and LFSM Duplicates (LFMSD) – The isotopically-labeled internal standard in this method also serves the role of the LFSM, which is used to determine that the sample matrix does not adversely affect method accuracy. In the context of application of this method for environmental remediation, it is not expected that there would be a VX background concentration. Also, it is likely that the water samples will come from the same drinking water system, and hence the sample matrices from a single collection time will be very similar. Further, experience with the automated extraction equipment used suggests that most failures in IS QC requirements result from failure of the automation equipment. This would correspond to LFSM failure, as well. Accordingly, neither LFSMs nor duplicate LFSMs would be expected to yield additional information about influence of sample matrix on method accuracy, except for the unlikely case of a feature of the sampling/remediation plan that produces a co-eluting peak with identical chromatographic and mass spectral properties as VX. In this case, the lab should discuss the irregularities with the submitter.

9.3.7.1 If an LFSM and LFSMD are deemed necessary, calculate the relative percent difference (RPD) for duplicate LFSMs (LFSM and LFSMD) using the equation

\[
RPD = \frac{|LFSM - LFSMD|}{(LFSM + LFSMD)/2} \times 100
\]

9.3.7.2 Relative percent difference (RPD) for duplicate LFSMs should be \(\leq 30\%\) for samples fortified at or above their native concentration. Greater variability could be observed when LFSMs are fortified at analyte concentrations that are within a factor of two of the MRL. LFSMs fortified at these concentrations should have RPDs that are \(\leq 50\%\). If the RPD of any analyte falls outside the designated range, and the laboratory performance for that analyte is shown to be in control in the CCC, the recovery is judged to be matrix-biased. The result for that analyte in the unfortified sample is labeled “suspect/matrix” to inform the data user that the results are suspect due to matrix effects.

9.3.8 FIELD DUPLICATE (FD) – Field duplicates are used to check the precision associated with sample collection, preservation, storage, and laboratory procedures. Some of these factors are out of the control of the laboratory, and the rest are covered by other QC checks. Accordingly, results of any field duplicates requested should be discussed with the sample submitter if the results do not meet the following criteria.
9.3.8.1 Calculate the relative percent difference (RPD) for duplicate samples (FD1 and FD2) using the equation

\[ RPD = \frac{|FD1 - FD2|}{(FD1 + FD2)/2} \times 100 \]

9.3.8.2 RPDs for FDs should be ≤30%. Greater variability could be observed when FDs have analyte concentrations that are within a factor of two of the MRL. At these concentrations, FDs should have RPDs that are ≤50%. If the RPD of any analyte falls outside the designated range, and the laboratory performance for that analyte is shown to be in control in the CCC, the recovery is judged to be biased. The result for that analyte in the unfortified sample is labeled “suspect/field duplicate bias” to inform the data user that the results are suspect due to field bias. (Note some other sources of lab bias may also be present.)

10. CALIBRATION AND STANDARDIZATION

10.1 All laboratory equipment should be calibrated according to manufacturer’s protocols and equipment with expired calibrations should not be used. Demonstration and documentation of acceptable mass spectrometer tune and initial calibration is required before any samples are analyzed. After the initial calibration is successful, the instrument is recalibrated using the same conditions as the initial calibration before each analysis batch. After the batch, the lowest and highest calibration solutions are run as continuing calibration checks (CCC). Verification of mass spectrometer tune should be repeated each time a major instrument modification is made or maintenance is performed, and prior to analyte calibration.

10.2 INITIAL CALIBRATION

10.2.1 MS/MS TUNE – Calibrate the mass and abundance scales of the MS/MS with calibration compounds and procedures prescribed by the manufacturer with any modifications necessary to meet tuning requirements. For an Applied Biosystems® API 4000 tandem mass spectrometer, some labs have experienced better results if following the automatic tune, they perform a manual tune to fine-tune declustering potential, collision energy, and collision cell exit potential settings. For other instruments, follow manufacturer’s protocols to tune the instrument.

10.2.2 INSTRUMENT CONDITIONS – Operational conditions for the instrument used in this verification are tabulated in Section 6.8.3 and Table 6-2. Alteration of the conditions is not recommended and would require redevelopment of QC criteria. Frequently reported problems can
be avoided by: (1) checking that needle wash solutions are adequately filled and the injection needle is functioning properly and (2) changing the analytical column as needed.

10.2.3 CALIBRATION STANDARDS- Prepare six calibration standards as described in Section 7.3.4. Note that as procedural calibration standards, they are processed through the procedure in Section 11, in which isotopically-labeled internal standard is added after sample extraction and prior to filtration. In practice, the lowest concentration of the calibration standard should be at or below the MRL (Sect. 9.2.4), which will depend on system sensitivity. The lowest point on the calibration curve is close to the reported detection limit and the highest point is above the expected range of results. The remainder of the points is distributed between these two extremes, with the majority of points in the concentration range where contaminated samples are expected to fall.

10.2.4 HPLC/MS/MS CALIBRATION- The HPLC/MS/MS system is calibrated using the internal standard technique, as implemented by the data system software. Construct a calibration curve using at least a six-point curve of response ratios (i.e., ratio of calibration standard peak area to internal standard peak area). As the internal standard concentration is consistent among samples and calibrators, some labs have found it convenient to set it to a value of one instead of the actual concentration.

10.2.5 CALIBRATION ACCEPTANCE – Calculate the slope and intercept of the calibration curve with 1/x weighting (or other appropriate weighting) by a linear least squares fit (or other appropriate calibration function). Evaluate the $r^2$ value for the curve, which must be greater than 0.980. Linearity of the standard curve should extend over the entire standard range. Each calibration point for the analyte should calculate to be 70 to 130 percent of its true value. If these criteria cannot be met, the analyst will have difficulty meeting ongoing QC criteria. If any standard is in error and does not fit the standard curve (i.e., the $r^2$ value for the curve is < 0.980), it can be removed from the calibration. No more than one standard may be discarded in any given calibration curve. If either the high or low standard is dropped, the reporting limits should be adjusted accordingly. The resulting $r^2$ value should be greater than 0.980.

10.3 CONTINUING CALIBRATION CHECKS (CCCs) – As described in Sect 9.3.3, up to four CCCs are used in conjunction with each analysis batch. If applicable, LFB-low and LFB-high are run at the beginning of the batch, and the calibration solutions are run at the end. The LFBs serve to verify the initial IDC, and the calibration solutions verify the calibration generated at the start of the analysis. The LRBs, LFBs, and CCCs are not counted as the 20 samples that constitute an analysis batch.
10.3.1 Inject an aliquot of calibration salutation at the appropriate concentration and analyze with the same conditions used during the initial calibration.

10.3.2 Acceptance of the calibration solutions is based on the same criteria as described in Section 10.2.5. Failure to meet these criteria is a rare occurrence, and suggests maintenance of the HPLC/MS/MS system is required.

10.3.3 Acceptance of the results of the LFB-Low and LFB-High is based on the Quality Control Limits (Sect. 10.3.3) established via the IDC. Acceptance of results for that entire analytical batch is dependent upon the agreement of the results from these control materials within established ranges. Quality Control Limits for the CCCs are based primarily on the standard deviation ($\sigma_{n-1}$, sigma) of the replicate analysis in the IDC (Sect. 9.2.2). Section 13.3 presents sample values for these parameters obtained in the developer’s laboratory, in which 20 replicate analyses performed over no less than 10 days are used to establish the LFB-Low and -High limits (Sect. 9.2.2). If the CCC results do not meet the following criteria, it is “out-of-control,” and the cause of the failure should be determined and corrected. No results from the associated analytical batch may be reported. These criteria apply to non-zero analyte concentrations used to make the quality control solutions in section 7.3.5.1

10.3.3.1 If both of the LFB-Low and LFB-High results are within 2$\sigma_{n-1}$ of the mean determined during the IDC, then accept the entire analytical batch. Otherwise, reject the entire analytical batch.

10.3.4 Common remedial actions if the CCCs fails to meet acceptable criteria:

10.3.4.1 LOW ANALYTE RESPONSE – If the signal-to-noise of the low standard confirmation ion falls below 10, this indicates that the instrumental sensitivity, or solid phase extraction recovery, has fallen below acceptable limits. The following steps should be taken and the instrument sensitivity rechecked after each corrective action is performed. Once sensitivity has been reestablished, further steps are not necessary.

i. Re-extract the samples or re-inject standards.

ii. If peak tailing or fronting is a significant issue, replace the HPLC column.

iii. Ensure the source of the MS/MS is clean.

iv. Clean the mass spectrometer source plate.

v. Flush all tubing on the HPLC/MS/MS instrument with 95% / 5% acetonitrile/water for 15 minutes followed by 5 minutes of equilibration with 5% / 95% acetonitrile/water.
10.3.4.2 Analyte in standards – If an inordinately large amount of analyte is measured in one of the calibration standards, but this is not seen in the remainder of the samples, this indicates a contamination of this particular sample. The source of this incident should be investigated to prevent repeat occurrences, but no further action is required. The contaminated calibration standard should be excluded when developing the calibration curve.

10.3.4.3 Analyte in all samples – If an inordinately large amount of analyte is present in all measurements for a particular day, it is likely that one or more of the spiking solutions are contaminated. If necessary, prepare new solutions.

11. PROCEDURE

11.1 SAMPLE PREPARATION

11.1.1 Samples are preserved, collected and stored as presented in Section 8.

Note: Steps 11.1.4 through 11.1.7 can be performed using an automated magnetic bead processor or with stationary magnets. Data presented in this document was collected using an automated magnetic bead processor.

11.1.2 Prepare Magnetic Beads (enough for 20 samples)

11.1.2.1 Resuspend Protein G Dynabeads® magnetic beads and transfer 2 mL to a 15-mL Falcon tube. Apply a DynaMag-2 magnet to the sample for 30 seconds and then remove the supernatant without disturbing the beads.

11.1.2.2 Add 4 mL PBS to the beads, mix by vortexing, apply a DynaMag-2 magnet to sample, and then remove supernatant. Repeat this step two more times.

11.1.2.3 Dilute 400 ug antibody (400 µL of a 1 mg/mL solution) in 8 mL PBST. Add this antibody solution to the bead solution and vortex the beads.

11.1.2.4 Incubate overnight at room temperature with rotation on the Dynal sample mixer at a setting of 21.
11.1.2.5  Apply a DynaMag-2 magnet to the sample, wait 30 seconds, and then remove the supernatant.

11.1.2.6  Add 4 mL triethanolamine buffer to the sample, vortex, apply magnet, and then remove the supernatant. Repeat.

11.1.2.7  Add 4 mL DMP/triethanolamine buffer (refer to section 7.2.7) to sample. Vortex.

11.1.2.8  Incubate for 30 minutes at room temperature using a Dynal sample mixer at a setting of 21.

11.1.2.9  Apply a DynaMag-2 magnet to the sample, wait 30 seconds, and then remove the supernatant.

11.1.2.10 Add 4 mL TBS to sample. Vortex. Incubate at room temperature for 15 minutes using a Dynal sample mixer at a setting of 21.

11.1.2.11 Apply a DynaMag-2 magnet to the sample, wait 30 seconds, and then remove the supernatant.

11.1.2.12 Add 2 mL PBST to sample, vortex, apply magnet, and then remove supernatant. Repeat twice.

11.1.2.13 Add 10 mL pooled human serum free from interferences (refer to section 7.1.14) to the beads.

11.1.2.14 Incubate for 2 hours at room temperature using a Dynal sample mixer at a setting of 21.

11.1.2.15 Apply a DynaMag-2 magnet to the sample, wait 30 seconds, and then remove the supernatant.

11.1.2.16 Add 2 mL PBST to sample, vortex, apply magnet, and then remove supernatant. Repeat.

11.1.2.17 Add 2 mL PBST to sample and vortex. This is the final prepared bead solution. Store at 4°C for up to 8 months.

11.1.3 Prepare 96-wells plates

11.1.3.1 Sample Plate – Label a 96-well deep V-bottom KingFisher microplate (VWR, St. Louis, MO) as the “Sample Plate” and add 500 μL of each calibrator, blank, quality control and sample into individual wells (refer to section 7.3.3).
• Add 55 μL 10x PBST to each well containing a calibrator, blank, quality control, or sample.

11.1.3.2 Wash Plates – Label three 96-well deep V-bottom KingFisher microplates (VWR, St. Louis, MO) as “Wash Plate 1,” “Wash Plate 2,” and “Wash Plate 3.” Add 500 μL of 1x PBST (refer to section 7.2.2) to each well corresponding to a calibrator, blank, quality control or sample.

11.1.3.3 Digestion Plate – Label a 200 μL 96-well Kingfisher plate (VWR, St. Louis, MO) as “Digestion Plate” and set aside.

11.1.3.4 Bead Plate – Label a 200 μL 96-well Kingfisher plate (VWR, St. Louis, MO) as “Bead Plate” and add 100 μL of the prepared magnetic beads (refer to section 11.1.2) to each well that corresponds to a calibrator, blank, quality control or sample.

11.1.3.5 Tip Plate – Place a tip comb into a 200 μL 96-well Kingfisher plate (VWR, St. Louis, MO) and label the plate “Tip Comb Plate.”

11.1.4 Extract Samples

11.1.4.1 Run KingFisher Flex "Step 1 - Add Beads" Program (See "KingFisher Flex Magnetic Particle Processor Methods", Section 11.2.1). The KingFisher Flex particle processor will transfer the antibody-conjugated beads from the "Bead Plate" to the "Sample Plate".

11.1.4.2 Seal the "Sample Plate" (now containing magnetic beads) with an Eppendorf adhesive foil.

11.1.4.3 Incubate “Sample Plate” on a MixMate plate mixer for 2 hours at 1400 rpm.

11.1.4.4 Prepare pepsin solution. Make a concentrated pepsin solution 30 minutes before samples are finished the 2-hour incubation period in the magnetic bead solution. It is important to make this solution exactly 30 minutes before the samples are finished the 2-hour incubation. Mix thoroughly and then set aside.

11.1.4.5 When the sample plate has finished mixing for 2 hours, dilute 207 μL of the pepsin solution with 1440 μL of HPLC-grade water and mix thoroughly.
11.1.4.6 Dispense a 75 μL aliquot of the diluted pepsin solution into the wells corresponding to calibrators, blanks, quality controls, and samples in the "Digestion Plate".

11.1.5 Wash Beads using the KingFisher Flex.

11.1.5.1 Remove adhesive PCR foil from "Sample Plate."

11.1.5.2 Run KingFisher Flex: "Step 2 - Bead Washes" program (see "KingFisher Flex Magnetic Particle Processor Methods", Section 11.2.2).

11.1.5.3 Add "Digestion Plate" plate to the KingFisher processor when prompted.

11.1.6 Digest Extracts in Pepsin

11.1.6.1 Remove "Digestion Plate" from the KingFisher processor and cover with an adhesive PCR foil. Carefully place "Digestion Plate" into a 37°C water bath while being careful to not get water on top of the plate. The plate will float. Incubate "Digestion Plate" plate in 37°C water bath for 1.5 hours.

11.1.6.2 Remove "Digestion Plate" plate from 37°C water bath after the 1.5-hour incubation period and flash centrifuge for 3-5 seconds to collect condensation. Remove PCR foil from the "Digestion Plate".

11.1.6.3 Run KingFisher Flex: "Step 3 - Remove Beads from Digestion Plate" program (see "KingFisher Flex Magnetic Particle Processor Methods", Section 11.2.3) to Remove Beads from the “Digestion Plate.” The “Digestion Plate” contains the extracted samples.

11.1.7 Add Internal Standard Solution to Extracts and Filter Extracts

11.1.7.1 Add 10 μL internal standard stock solution (refer to section 7.3.2.2) to each calibrator, blank, quality control, and sample using a single- or multi-channel pipettor, and mix samples with the pipettor or on a plate shaker.

11.1.7.2 Transfer internal standard-containing extracts from "Digestion Plate" to a 96-well, 10 kDa filter plate using a single- or multi-channel pipettor.
11.1.7.3 Place the filter plate directly on top of 96-Well ABgene PCR Plate and seal with tape around the four sides for maximum sample recovery.

11.1.7.4 Centrifuge at 3700 rpm for 1.5 hours.

11.1.7.5 Remove the plate assembly from centrifuge and discard the filter plate. Seal the collection plate with the heat sealer. The plate is now ready for analysis.

11.2 KingFisher Flex Magnetic Particle Processor Methods. Program the following methods into the processor.

11.2.1 “Step 1 - Add Beads”
11.2.1.1 Pick up plate “Tip Plate”
11.2.1.2 Collect Beads from “Bead Plate”
   • Collect count 1
   • Collect time [s] 30
11.2.1.3 Release beads in “Sample Plate”
   • Release time [hh:mm:ss] 00:00:05
   • Release speed Medium

11.2.2 “Step 2 - Bead Washes”
11.2.2.1 Pick up plate “Tip Plate”
11.2.2.2 Collect beads from “Sample Plate”
   • Collect count 1
   • Collect time [s] 30
11.2.2.3 Wash 1
   • Release beads into “Wash Plate 1”
   • Mixing time [hh:mm:ss] 00:00:30
   • Mixing speed Slow
   • Collect count 3
   • Collect time [s] 1
11.2.2.4 Wash 2
11.2.2.5 Release beads into “Wash Plate 2”
   • Mixing time [hh:mm:ss] 00:00:30
   • Mixing speed Slow
   • Collect count 3
   • Collect time [s] 1
11.2.2.6 Wash 3
11.2.2.7 Release beads into “Wash Plate 3”
   • Mixing time [hh:mm:ss] 00:01:00
   • Mixing speed Slow
   • Pause with the message “Add Digestion Plate”
   • Collect count 1
   • Collect time [s] 30
11.2.2.8 Begin Digestion
- Release beads into “Digestion Plate”
- Release time [hh:mm:ss] 00:00:05
- Release speed Fast

11.2.3 “Step 3 - Remove Beads from Digestion Plate”
11.2.3.1 Pick up plate “Tip Plate”
11.2.3.2 Collect beads from “Digestion Plate”
   - Collect count 1
   - Collect time [s] 30
11.2.3.3 Release beads into “Wash Plate 1”
   - Release time [hh:mm:ss] 00:00:05
   - Release speed Fast

11.3 ANALYSIS OF SAMPLE EXTRACTS

11.3.1 Establish operating conditions for the instrument as described in Section 10.2.2.

11.3.2 Establish a valid initial calibration following the procedures outlined in Section 10.2 or confirm that the calibration is still valid by running both CCCs as described in Section 10.3. If establishing an initial calibration for the first time, complete the IDC as described in Section 9.2.

11.3.3 Set up the available automation equipment and software as specified by the manufacturer for batch analysis, paying particular attention to the following:

11.3.3.1 On the instrument computer, edit the automation software:
   (a) Select the sample type.
   (b) Identify the correct vial position.
   (c) Name the sample. Due to large number of samples analyzable with the automation equipment, it is important that appropriate record keeping (e.g., database, notebooks, data files) should be used to track specimens.
   (d) Enter information related to particular specimens into the software manually or by electronic transfer.
   (e) Select the instrument control method.
   (f) Identify the target path where the data will be stored.

11.3.3.2 Check to be sure that the number and positions of samples entered on the sequence set-up page correspond to the samples in the autosampler.

11.3.4 Run the automation sequence to analyze the batch of aliquots of field and QC samples at appropriate frequencies (Sect. 9, 10.3). All field, QC, and calibration standards should be run using the same HPLC/MS/MS
conditions. At the conclusion of data acquisition, use the same software that was used in the calibration procedure to identify the peaks in predetermined retention time windows of interest. Use the data system software to examine the ion abundances of components of the chromatogram.

11.3.5 COMPOUND IDENTIFICATION – The presumed peak in the sample must appear in the same retention time window as the internal standard corresponding to VX (around 2.2 min in the developer’s lab) and have similar chromatographic characteristics such as peak shape. Relative to the analyte retention time, the internal standard retention time should be + or - 0.02 minutes. This relies on expert judgment of the analyst since the retention times reported by the software are not always reliable. Identification of the peak as VX (corresponding to VX-BuChE adducts) is then confirmed through calculating the confirmation ratio (CR), i.e., by dividing the response for confirmation transition by the response for quantitation transition of the presumed analyte peak. Using the manufacturer’s software or manually, compare the confirmation ratio of the peak from the sample with the mean of the CRs measured for the six calibration standards associated with that batch. The mean CR is the average CR from the calibration standards only and is batch dependent. The CR value for each sample should be within 30% of the mean. (CR value was approximately 0.9 in the developer’s lab.)

12. DATA ANALYSIS AND CALCULATIONS

12.1 Concentrations are calculated using the ions listed in Table 3-1. Use of other ions is not advised. If a particular instrument cannot produce the fragments listed in Table 3-1, this instrument should not be used to run this method.

12.2 Calculate analyte concentrations using the ongoing multipoint calibration established in Section 9.3.2. Do not perform calibration using just the CCC or LFB-low and -high data to quantitate analytes in samples, although these samples might be part of the ongoing calibration curve.

12.3 All raw data files are quantified using the quantitation capabilities of the instrument software. The peaks are automatically integrated using the software-associated integration program, and the integration of each peak is reviewed and manually corrected as appropriate. This is particularly important for the calibration standards. The quality control samples (e.g., CCCs and LFBs) are quantified and evaluated against the calibration curve, and each field sample is then quantified against that calibration curve. The run data can be processed within instrument data analysis software and exported to external spreadsheets, per laboratory policy, generating files containing the unknown and QC concentrations, retention times, standard curves, and other run information.
12.3.1 Results are generally reported to two significant digits. In addition to analytical measurements of unknowns, statistical results of measurement of blanks should accompany all results.

12.3.2 Check all sample and analytical data for transcription errors and overall validity after being entered into the instrument software database. Back up onto external media both the instrument and data storage databases according to individual laboratory guidelines.

13. METHOD PERFORMANCE

13.1 ANALYTICAL IDENTIFICATION–Analyte identification using the approach described in Section 11.3.5 resulted in no false positives or negatives for the samples reported below. There was very low background noise according to the signal-to-noise ratios for the ion transitions monitored.

13.2 SINGLE LABORATORY MINIMUM REPORTING LEVELS and DETECTION LIMIT– The reportable range of results for VX are summarized below, along with the DL determined from the IDC procedure described previously. The lowest standard is used as the minimum reporting level, and the DL calculated from the standard deviation of replicate measurements of that standard (in the case of Table 13-1, 5.6 ng/mL). The highest reportable limit is based on the highest linear standard.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Minimum Reporting Level (µg/L)</th>
<th>Highest Reportable Limit (µg/L)</th>
<th>Method DL (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VX</td>
<td>0.025</td>
<td>4.00</td>
<td>0.006</td>
</tr>
</tbody>
</table>

13.3 SINGLE LABORATORY ACCURACY AND PRECISION for LFBs – Single lab precision and accuracy data is represented in Table 13-2. Accuracy is defined as the mean of the measured concentration in the fortified samples divided by the fortification concentration, expressed as a percentage. Method accuracy was determined by analyzing LFBs at the two non-zero levels in Section 7.3 (i.e., LBF-low and –high) and at least seven replicates for each of the two concentration levels over a period of 91 days. The means, standard deviations, and relative standard deviations for the two LFBs are shown in Table 13-2. The means are less than one standard deviation from the known concentration.
Table 13-2. Single Lab Precision and Accuracy Data

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Sample</th>
<th>Fortified Concentration (μg/L)</th>
<th>Number of Replicates</th>
<th>Mean of IDC Replicates (μg/L)</th>
<th>Standard Deviation (μg/L)</th>
<th>RSD (%)</th>
<th>Accuracy of Mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFB-low</td>
<td>0.25</td>
<td>8</td>
<td>0.25</td>
<td>0.01</td>
<td>3</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>LFB-high</td>
<td>2.0</td>
<td>8</td>
<td>2.05</td>
<td>0.12</td>
<td>6</td>
<td>103</td>
<td></td>
</tr>
</tbody>
</table>

13.4 SINGLE LABORATORY RECOVERY AND PRECISION FOR TAP WATER MATRICES. Table 13-3 expresses percent mean recoveries for VX in several different chlorinated and chloraminated tap waters derived from the types of sources (i.e., ground or surface water) indicated. Samples were extracted immediately after preparation. Water quality parameters describing these sources are indicated in the footnotes. Percent recoveries were determined by dividing the measured concentration by the spiked concentration (n=3). No analytes or interferences were detected in the unspiked samples.

Table 13-3. Percent Recovery of VX from Several Tap Water Matrices with Sodium Omadine and Sodium Thiosulfate (n=3)

<table>
<thead>
<tr>
<th>Water Type</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground Water 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81 ± 1</td>
</tr>
<tr>
<td>Surface Water 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>85 ± 2</td>
</tr>
<tr>
<td>Surface Water 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>92 ± 2</td>
</tr>
<tr>
<td>Surface Water 4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>85 ± 3</td>
</tr>
<tr>
<td>Surface Water 5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>92 ± 4</td>
</tr>
<tr>
<td>HPLC-Grade Water</td>
<td>101 ± 2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Total organic carbon (TOC) not detected in well-field; pH 7.6; hardness 500 mg/L; Chlorine 0.2-0.4 mg/L (monthly averages)

<sup>b</sup>TOC 1.0 mg/L; pH 8.5; hardness 130 mg/L; Chlorine 0.8 mg/L (monthly averages)

<sup>c</sup>TOC 2.3 mg/L; pH 7.4; hardness 190 mg/L; Monochloramine 3.4 mg/L (monthly averages)

<sup>d</sup>TOC 7.6 mg/L; pH 9.2; hardness 65 mg/L; Monochloramine 2.4 mg/L (monthly averages)

<sup>e</sup>TOC 0.3 mg/L; pH 8.9; hardness 17 mg/L; Chlorine 1.3 mg/L (monthly averages)

14. POLLUTION PREVENTION

14.1 This method utilizes solid phase extraction to extract analytes from water. It requires the use of reduced volumes of organic solvent and very small quantities of pure analytes, thereby minimizing the potential hazards to both the analyst and
the environment as compared to the use of large volumes of organic solvents in conventional liquid-liquid extractions.


15. **WASTE MANAGEMENT**

15.1 Dispose of waste materials must be in compliance with the individual laboratory’s chemical hygiene plan, as well as federal, state, and local regulations. Always dispose of solvents and reagents in an appropriate container clearly marked for waste products and, if temporary storage is needed, store them in a chemical fume hood.

15.2 VX is a highly lethal cholinesterase inhibitor. Dispose of VX in an appropriate waste stream as well. Unused VX-containing solutions shall be diluted no less than 3x initial volume with freshly prepared 5% solution of hypochlorite prior to disposal. Hypochlorite bleach solution should not be more than one week old. Unused solutions containing VX should then be placed in waste collection container labeled as corrosive or flammable waste as appropriate. Pipettor tips used to aspirate VX-containing solution will be fully immersed in freshly prepared 5% solution of hypochlorite, with bleach solution drawn into pipettor tip, prior to disposal in an appropriate container.
16. REFERENCES


