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High Throughput Determination of Ricinine, Abrine, and Alpha-Amanitin in Drinking Water by Solid Phase Extraction and High Performance Liquid Chromatography Tandem Mass Spectrometry (HPLC/MS/MS)

Version 1.0

Office of Research and Development
National Homeland Security Research Center

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Centers for Disease Control and Prevention
Atlanta, GA 30333

U.S. Environmental Protection Agency
Cincinnati, OH 45268

Disclaimer

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Questions concerning this document or its application should be addressed to:

Erin Silvestri, MPH (EPA Project Officer)
U.S. Environmental Protection Agency
National Homeland Security Research Center
26 W. Martin Luther King Drive, MS NG16
Cincinnati, OH 45268
513-569-7619
Silvestri.Erin@epa.gov

Matthew Magnuson, PhD (EPA Technical Lead)
U.S. Environmental Protection Agency
National Homeland Security Research Center
26 W. Martin Luther King Drive, MS NG16
Cincinnati, OH 45268
513-569-7321
Magnuson.Matthew@epa.gov

Jennifer Knaack, PhD
Mercer University
3001 Mercer University Drive
Atlanta, GA 30341
678-547-6737
knaack_js@mercer.edu

Rudolph Johnson, PhD
Centers for Disease Control and Prevention
4770 Buford Highway, MS F-44
Atlanta, GA 30341
770-488-3543
Rmj6@cdc.gov

Acknowledgments

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Acronyms

ABR	abrine
AMAN	alpha-amanitin
CAS®	Chemical Abstracts Service
CCC	continuing calibration check
CDC	Centers for Disease Control and Prevention
CR	confirmation ratio
DL	detection limit
ESI	electrospray ionization
FD	field duplicate
HR_{pir}	half range for the predicted interval of results
HRL	highest reportable limit
HPLC	high performance liquid chromatography
i.d.	inside diameter
IDC	initial demonstration of capability
IS	internal standard
IQS	internal quantification standard
LC	liquid chromatography
LD_{50}	median lethal dose
LFB	laboratory fortified blank
LFSM	laboratory fortified sample matrix
LFSMD	laboratory fortified sample matrix duplicate
LRB	laboratory reagent blank
MRL	minimum reporting level
MRM	multiple reaction monitoring
MS/MS	tandem mass spectrometry
MSDS	Material Safety Data Sheet
m/z	mass to charge ratio
NHSRC	National Homeland Security Research Center
PIR	prediction interval of results
QC	quality control
QCS	quality control sample
RIC	ricinine
RPD	relative percent difference
SAP	Sampling and analytical procedure
SPE	solid phase extraction
SS	standard solution
TOC	Total Organic Carbon

Executive Summary

This document provides the standard operating procedure for determination of ricinine (RIC), abrine (ABR), and α-amanitin (AMAN) in drinking water by isotope dilution liquid chromatography tandem mass spectrometry (LC/MS/MS). This method is designed to support site characterization and inform site-specific cleanup goals of environmental remediation activities following a homeland security incident involving one or a combination of these analytes.

The method can be summarized as follows. A 50-mL drinking water sample is collected and preserved with sodium thiosulfate (80 mg/L) (a dechlorinating agent), and sodium Omadine™ (64 mg/L) (an antimicrobial preservative). An aliquot is combined with an internal standard mixture containing isotopically-labeled standards (for RIC and ABR) and/or internal quantification standard (for AMAN). The sample is then pipetted into a well of a preconditioned 96-well solid phase extraction plate and extracted. The extract is concentrated to dryness under nitrogen and heat and is then adjusted to a 100 μL volume in high performance liquid chromatography (HPLC)-grade water. RIC, ABR, and AMAN are separated from the sample matrix and identified by HPLC/MS/MS analysis, operated in multiple reaction monitoring mode or equivalent. 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 HPLC/MS/MS conditions. Quantitation is performed using the internal standard technique. Utilization of an isotopically labeled internal standard for RIC and ABR and an internal quantification standard for AMAN provides a high degree of accuracy and precision for sample quantitation by accounting for analyte recovery and analytical efficiency.

Accuracy and precision data have been generated in reagent water and in finished ground and surface waters that contain residual chlorine and/or chloramine that have been used as disinfectants. RIC, ABR, and AMAN can be analyzed up to 28 days after collection, although initial loss of analyte signal during the first few hours may be observed with ABR and AMAN in some water samples. The amount of loss is dependent on the water source and does not continue after this initial period. Thus, in an actual contamination incident, it is likely this initial signal loss would occur before the sample reaches the laboratory.

HIGH THROUGHPUT DETERMINATION OF RICININE, ABRINE, and ALPHA-AMANITIN IN DRINKING WATER

1. **SCOPE AND APPLICATION**

   1.1. This document is an isotope dilution liquid chromatography tandem mass spectrometry (LC/MS/MS) method for the determination of ricinine (1,2-dihydro-4-methoxy-1-methyl-2-oxo-3-pyridinecarbonitrile, Chemical Abstracts Services (CAS®) Registry Number® 524-40-3), abrine ((2S)-3-(1H-indol-3-yl)-2-(methylamino)propanoic acid, CAS Registry Number 526-31-8), and alpha-amanitin (cyclic(L-asparaginyl-4-hydroxy-L-prolyl-(R)-4,5-dihydroxy-L-isoleucyl-6-hydroxy-2-mercapto-L-tryptophylglycyl-L-isoleucylglycyl-L-cysteinyl), cyclic (4-8)-sulfide, (R)-S-oxide, CAS Registry Number 23109-05-9). 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 one or a combination of these analytes. (*Note:* Ricin, abrin, and α-amanitin are not toxins regulated under the Safe Drinking Water Act (as amended in 1986 and 1996) [1]. A description of the development of this method will be presented separately.

      1.1.1. Ricinine (RIC), abrine (ABR), and alpha-amanitin (AMAN) can be analyzed up to 28 days after collection. However, in some water samples, ABR and AMAN were observed to undergo an initial loss of analyte signal during the first few hours after ABR and AMAN were added to the sample (Section 8.4). The amount of loss is dependent on the water source, does not continue after this initial period, and will be discussed in more detail elsewhere [2]. Thus, in an actual contamination incident, it is likely this initial signal loss would occur before the sample reaches the laboratory. Section 13.5 provides suggestions for additional quality control steps that may be necessary should analysis of samples suspected of ABR or AMAN contamination results in unexpectedly low reported concentrations.

   1.2. Significance: Ricin and abrin are toxic lectins from *Ricinus communis* (castor bean) [3,4] and *Abrus precatorias* (jequirity pea) [5,6], respectively. Ricinine and abrine are small alkaloid molecules that are present in crude extracts of ricin and abrin, respectively, and can be used as biomarkers for these toxins [7-9]. Alpha-Amanitin is a highly toxic bicyclic octapeptide found in *Amanita phalloides* [10,11]. Methods for detecting these toxins in environmental matrices are necessary to characterize the contamination and to inform remediation and clearance of intentional or accidental contamination events. The method presented here can be applied for the quantification of ricinine, abrine, and alpha-amanitin in drinking water samples.

1.3.   Whether performed manually or with automation, the use of 96-well plates during solid phase extraction (SPE) produces two key benefits. The 96-well plates allow for extensive automation of the method, thereby enabling high throughput of samples, as might be required during environmental remediation. Additionally, the use of this format results in the ability to perform isotope dilution more economically since smaller amounts can be utilized.

1.4.   Isotopically-labeled RIC and ABR internal standards and an internal quantification standard (IQS) for AMAN are added equally to all unknowns, quality controls, and calibration standards. In addition to enabling accurate quantitation, internal standards also account for and resolve some of the QC issues surrounding analysis, including analysis efficiency and sample loss, in the intended use of these analytes. The overall QC approach utilizing quantitation and confirmation ions as well as either an isotopically-labeled analyte or internal quantification standard (see Section 3.9) greatly increases confidence that RIC, ABR, and AMAN, and not another molecule with similar fragmentation patterns, are being quantitated during analysis.

1.5.   For this method, accuracy and precision data have been generated in reagent water and in finished ground and surface waters that contain residual chlorine and/or chloramine that have been used as disinfectants.

1.6.   The QC approach included in this method has been single-laboratory verified by the Centers for Disease Control and Prevention (CDC), National Center for Environmental Health, Division of Laboratory Sciences, Emergency Response Branch, in the Chemical Terrorism Methods Development Laboratory and is designed to be sufficiently rigorous that network laboratories can successfully perform the method.

1.7.   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.

1.8.   Determining the detection limit (DL) is optional (Section 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.

1.9.   This method is intended for use by analysts skilled in the performance of solid phase extractions, the operation of high performance liquid chromatography/tandem mass spectrometry) HPLC/MS/MS instruments and the interpretation of the associated data.

1.10.   This method has been verified using only the conditions specified in the method. Alteration of this method is not recommended. However, equivalent equipment and consumables are acceptable when equivalent performance is demonstrated.

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, Arch Chemicals, Inc., Norwalk, CT). An aliquot is combined with an internal standard mixture containing isotopically-labeled standards (for RIC and ABR) and/or internal quantification standard (for AMAN). The sample is then pipetted into a well of a preconditioned 96-well solid phase extraction plate and extracted. The extract is concentrated to dryness under nitrogen and heat and is then adjusted to a 100 µL volume in HPLC-grade water. RIC, ABR, and AMAN are separated from the sample matrix and identified by HPLC/MS/MS analysis, operated in multiple reaction monitoring (MRM) mode or equivalent. 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 HPLC/MS/MS conditions. Quantitation is performed using the internal standard technique. Utilization of an isotopically labeled internal standard for RIC and ABR and an internal quantification standard for AMAN provides a high degree of accuracy and precision for sample quantitation by accounting for analyte recovery and analytical efficiency.

2.2. Due to site-specific circumstances during an environmental remediation activity, changes to the on-going calibration frequency, and number of continuing calibration checks (CCCs) may be necessary and appropriate. For example, this may be necessary when sample throughput requirements exceed available laboratory capacity or when a shorter initial demonstration of capability (IDC) is warrented. However, initial and ongoing QC requirements and acceptance criteria (see Section 9) should not be changed. Adopting steps such as a replacing ongoing recalibration with a calibration check only to save time may result in higher QC failure rates and perhaps less accurate quantitation. Laboratories 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 (Section 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 – for this method, the confirmation ion transition is the second most abundant ion transition for each analyte (See Confirmation Ratio, Section 3.4, below).

3.4.   CONFIRMATION RATIO (CR) – the peak area produced by the confirmation ion transition divided by the peak area produced by the quantitation ion transition. The confirmation ratio serves as an additional QC measure for analyte identification.

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 check the precision associated with sample collection, preservation, storage, and laboratory procedures.

3.8.   ISOTOPICALLY-LABELED INTERNAL STANDARD (IS) – 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.

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 (Section7.3.5.2). The LFB is analyzed exactly like a sample. Its purpose is to verify that the methodology is competently replicated and that 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.

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 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 (Section 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 –for this method, the quantitation ion transition is the most abundant ion transition for each analyte and internal standard (See Confirmation Ratio, Section 3.4, above). Only quantitation ion transitions are monitored for internal standards.

3.15. SECOND SOURCE QUALITY CONTROL SAMPLES – materials obtained from a source different from the original source 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 may 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 in the reagents and supplies used in this method might be encountered. Interfering compounds can be recognized by deviations in the sample quantitation/confirmation ratios from the calibration standard ratios and can also be monitored using appropriate LRBs. Any interference that results in quality control (QC) failure (Section 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 reagent-grade water followed by acetonitrile. Allow glass to dry completely before use. If the laboratory wishes to use a muffle oven for decontamination, then the appropriate measures should be taken to assure that

the muffle oven conditions are suitable to remove all traces of RIC, ABR, AMAN and other interferences.

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 11.2.5 for analyte confirmation.

5. SAFETY

5.1 The toxicity or carcinogenicity of each reagent used in this method has not been defined precisely. Each chemical should be treated as a potential health hazard, and exposure to these chemicals should be minimized. Each laboratory is responsible for maintaining an awareness of Occupational Safety and Health Administration regulations regarding safe handling of chemicals used in this method. A reference file of MSDSs should be made available to all personnel involved in the chemical analyses. Additional references to laboratory safety are available [12-14].

5.2 While this method determines ricinine and abrine, ricin and abrin may also be present in the samples. Ricin is toxic, and the rat oral LD_{50} has been reported between 1-20 mg toxin/kg body weight [15,16]. Abrin toxicity in beverages is reported to be within the same range in mice [17]. Alpha-Amanitin is also highly toxic and the LD_{50} due to ingestion is 0.1-0.3 mg toxin/kg body weight [10,11]. Ingestion is the primary reported route of exposure, but all other routes of exposure (e.g., inhalation, dermal contact, and eye exposure) should be avoided. Follow universal safety precautions when performing this procedure, including the use of a laboratory coat, safety glasses, latex or nitrile gloves, and a high quality ventilated chemical fume hood and/or biological safety cabinet.

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. Maintenance and repair of mechanical and electronic components should generally 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 validation 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 – Eppendorf 4780 (Eppendorf Co., Westbury, NY, or equivalent).

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

6.4   SOLID PHASE EXTRACTION (SPE) APPARATUS WITH 96 WELL PLATES

   6.4.1   96-WELL PLATE SPE– Strata™ X 60-mg 96-well plate (PN# 8E-S100-UGB), available from Phenomenex (Torrence, CA) or equivalent.

   6.4.2   PLATE SHAKER (ThermoFisher Scientific, Waltham, MA, or equivalent).

   6.4.3   96-WELL LIQUID HANDLER – Use a 96-well liquid handler equipped with a solid phase extraction manifold and vacuum system. These systems must be calibrated prior to use, according to vendor or laboratory specifications. In addition, these liquid handlers have to be used during laboratory method validation. The liquid handlers that have been shown to be compatible with this method include the Tomtec® Quadra 3 SPE (Tomtec, Inc. Hamden, CT), the Caliper Zephyr®, and the Caliper i1000 (Caliper Life Sciences, Hopkinton, MA). The selection of these liquid handlers has typically been based on cost and required sample throughput.

6.5   96-WELL NUNC® DEEP WELL PLATE – 2000 mL plate (Nunc PN# 278752 or equivalent). Must be compatible with 96-well liquid handler described in Section 6.4.3.

6.6   EXTRACT CONCENTRATION SYSTEM. The 96-well plate requires a compatible dry-down step for sample pre-concentration following extraction. The TurboVap™ 96 concentrator evaporator workstation (Zymark® Corp., Hopkinton, MA) has proven to be well suited for this application, but other evaporator systems which result in equivalent method performance could be used instead. Drying should be performed with nitrogen gas with a purity recommended by the manufacturer.

6.7   HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ELECTRON IONIZATION TANDEM MASS SPECTROMETRY (HPLC/MS/MS) SYSTEM

   6.8.1   HPLC COLUMN – Synergi™ 4µm Fusion-RP polar embedded C18 column (100 x 2.00 mm) (Phenomenex PN 00M-4371-D0 or equivalent).

6.8.2 HPLC SYSTEM – The HPLC system (e.g., Agilent® 1100 HPLC, Agilent Technologies, Santa Clara, CA; 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 described in Table 6-1.

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

Parameter	Setting			
HPLC Method	Gradient: Reservoir A = 10% Methanol in water, 5 mM formic acid Reservoir B = Acetonitrile, 5 mM formic acid			
	Time (min)	**%A**	**%B**	**Flow Rate (μL/min)**
	0	93	7	300
	0.5	93	7	300
	6.50	70	30	300
	7.00	70	30	500
	8.00	70	30	500
	8.02	40	60	500
	10.49	40	60	500
	10.50	93	7	500
	13.46	93	7	500
	13.50	93	7	300
Column type	Synergi 4μm Fusion-RP polar embedded C18 column (100 x 2.00 mm)			
Injection Volume	20 μL			
Autosampler Tray Temperature	4 °C			
Column Temperature	40 ± 5 °C			
Injection Settings	Draw Speed: 200 μL/min Eject Speed: 200 μL/min Injection Mode: Standard			
Needle Rinse Settings	Rinse Solvent: Mobile Phase A Needle Rinse Time: 10 seconds in flush port			
Typical retention time	ABR and ABR internal standard: 2.4 min RIC and RIC internal standard: 2.8 min AMAN (native only): 5.1 min AMAN internal standard: 8.6 min			

ABR, abrine; AMAN, alpha-amanitin; HPLC, high performance liquid chromatography; RIC, Ricinine

6.8.3 MASS SPECTROMETER (MS) – The MS/MS (Applied Biosystems API 4000 quadrupole ion trap mass spectrometer, Foster City, CA, or equivalent) must be capable of performing electron ionization with both

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positive and negative ion detection and has to be configured for multiple reaction monitoring (MRM) with a dwell time of 100 msec per ion. The MS/MS was configured as described in Table 6-2.

Table 6-2. Tandem Mass Spectrometer (MS/MS) Parameters

Parameter	Setting
MS Scan Mode	Multiple Reaction Monitoring (MRM)
Ionization Type	Electrospray ionization
Dwell Time	100 msec per ion
Curtain Gas	10
Source Temperature	550 (interface heater ON)
Ion Source Gas 1	75
Ion Source Gas 2	10
Collision Gas	High
Ion Spray Voltage	5500 in positive polarity mode -4500 in negative polarity mode
Entrance Potential	10 in positive polarity mode -10 in negative polarity mode

The multiple reaction monitoring (MRM) transition conditions for this method and their associated mass spectrometer settings should be set as stated in Table 6-3 (ion transitions used in this method are listed in Table 10-1):

Table 6-3. Mass Spectrometer conditions

Analyte	Time Post Injection (min)	Polarity	Declustering Potential	Collision Energy	Collision Cell Exit Potential
Ricinine Quantitation	0	Positive	51	41	8
Ricinine Confirmation	0	Positive	51	25	8
Ricinine Internal Standard	0	Positive	51	25	8
Abrine Quantitation	0	Positive	46	17	12
Abrine Confirmation	0	Positive	31	29	16
Abrine Internal Standard	0	Positive	46	17	12
Alpha-Amanitin Quantitation	3.5	Negative	-100	-38	-17
Alpha-Amanitin Confirmation	3.5	Negative	-100	-54	-13
Alpha-Amanitin Internal Quantification Standard	3.5	Negative	-110	-34	-10

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A typical chromatogram is shown in Figure 6-1:
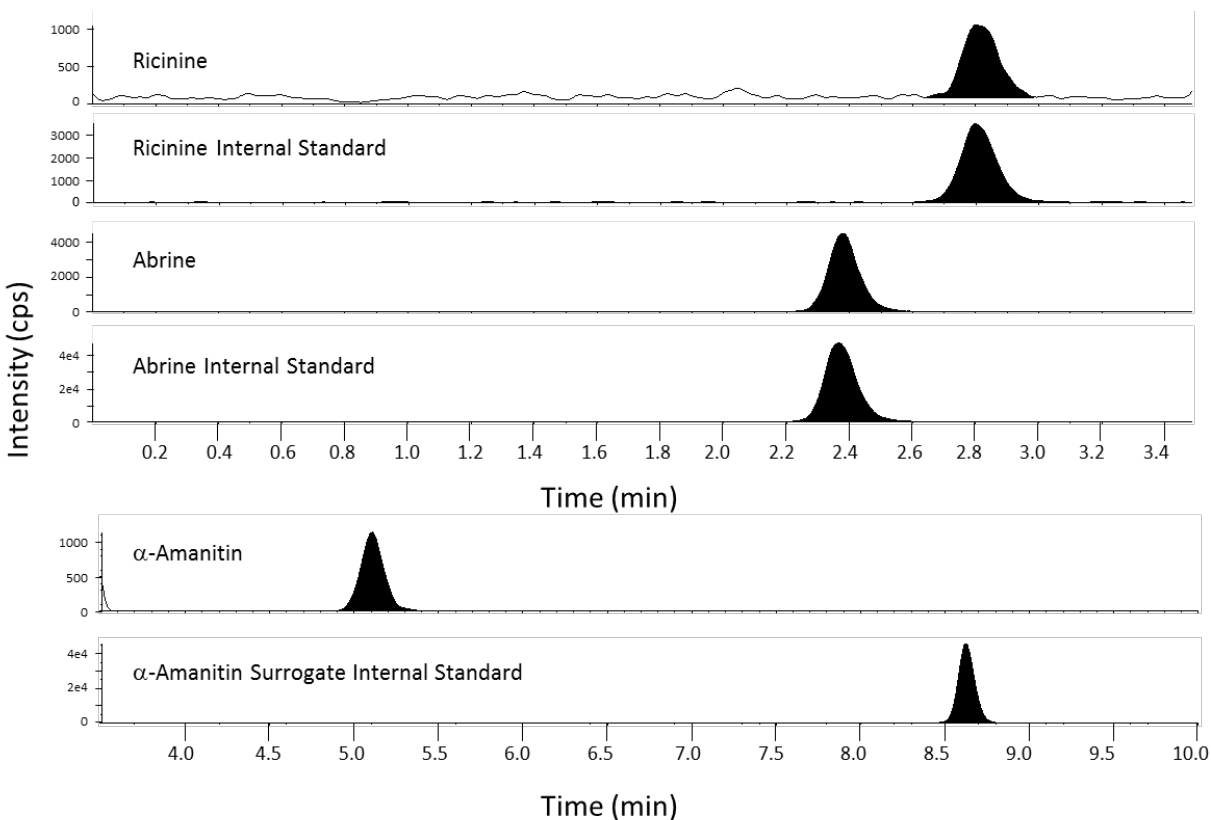
Figure 6-1. Chromatogram of typical analyte and internal standard peaks.

7.   REAGENTS AND STANDARDS (These reagents were used during the validation 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 ([Tedia®, Fairfield, OH] HPLC or equivalent grade water).

7.1.3 METHANOL - (CH_3OH, CAS# 67-56-1) – high purity, demonstrated to be free of analytes and interferences (Tedia HPLC or equivalent).

7.1.4 ACETONITRILE - (CH_3CN, CAS# 75-05-8) – high purity, demonstrated to be free of analytes and interferences (Tedia HPLC or equivalent).

7.1.5 FORMIC ACID - (HCOOH, CAS# 64-18-6) – reagent grade >95% purity, demonstrated to be free of analytes and interferences (Sigma®, Sigma-Aldrich Inc., St. Louis, MO; or equivalent).

7.1.6 SAMPLE PRESERVATION REAGENTS – the following sample preservation reagents are required for this method and should be demonstrated to be free of analytes and interferences:

  7.1.6.1 SODIUM THIOSULFATE ($Na_2S_2O_3$, CAS# 7772-98-7) – an additive used in sample collection (Sigma-Aldrich ≥ 98% pure, product number 72049 or equivalent).

  7.1.6.2 SODIUM OMADINE (C_5H_4NNaOS, CAS#: 3811-73-2) – an additive used for sample collection (Sigma-Aldrich > 96% pure, product number H3261 or equivalent)

7.2 REAGENT PREPARATION

7.2.1 5% METHANOL IN WATER – A 5%/95% (v/v) methanol/water solution is prepared through volumetric dilution with HPLC grade deionized water. Measure 10 mL of methanol using an appropriate pipette, volumetric flask, or graduated cylinder and pour into a clean, dry container with a capacity of 250 mL or more. Measure 190 mL of HPLC grade deionized water with a volumetric flask or graduated cylinder and pour into the same container with the methanol. Mix the solution well.

7.2.2 HPLC MOBILE PHASE A – A 10% methanol solution in HPLC-grade water with 5 mM formic acid. Measure 100 mL of methanol using an appropriate pipette, volumetric flask, or graduated cylinder with a 1 L capacity. Add 700 mL HPLC grade water to the cylinder. Add 192 uL 98% formic acid to the cylinder and fill the cylinder to 1 L with HPLC-grade water. Pour the solution into a clean dry container with a capacity of at least 1 L.

7.2.3 HPLC MOBILE PHASE B – A solution of acetonitrile with 5 mM formic acid. Measure 800 mL of methanol using an appropriate pipette,

volumetric flask, or graduated cylinder with a 1 L capacity. Add 192 uL 98% formic acid to the cylinder and fill the cylinder to 1 L with methanol. Pour the solution into a clean dry container with a capacity of at least 1 L.

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 measured accurately to minimize the addition of excess organic solvent to aqueous samples. Store all calibration and control materials at -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 AND INTERNAL QUANTIFICATION STANDARD SOLUTIONS – Each analyte requires a separate internal standard. RIC and ABR internal standards are isotopically-labeled. In this method, isotopically-labeled internal standards are chemicals that are structurally identical to the method analyte but substituted with ^{13}C or $^{13}C,D_3$. These isotopically-labeled internal standards have no potential to be present in water samples and are not method analytes. The internal standard for RIC is $^{13}C_6$-labeled ricinine and is custom-synthesized by Cerilliant (Round Rock, TX). The internal stand for ABR is $^{13}C,D_3$-labeled L-abrine and it is also custom-synthesized by Cerilliant. The internal standard for AMAN is structurally similar to AMAN but without isotopic labeling. The internal standard for AMAN is not known to be naturally-produced and is not an expected interference for this method. The internal standard for AMAN is available by custom synthesis through Invitrogen (Grand Island, NY). 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 labeled or internal quantification standards for RIC, ABR, and AMAN. Combine these together to make an internal standard stock solution.

7.3.2.1 INTERNAL STANDARD STOCK SOLUTION – Combine internal standards and internal quantification standards in HPLC-grade water for RIC, ABR, and AMAN so that the final concentration is approximately 85 ng/mL for RIC and 1265 ng/mL for ABR IS. The AMAN IQS should be 400 ng/mL. The stock solution was observed to be stable for at least one year when stored at -20 ± 5 °C.

7.3.3   ANALYTE STOCK STANDARD SOLUTIONS. Prepare or purchase individual stock solutions of RIC (Cerilliant, Round Rock, TX or

equivalent), ABR (Cerilliant, Round Rock, TX or equivalent. Please note that Cerilliant makes this product off-catalogue), and AMAN (Sigma-Aldrich, St. Louis, MO or equivalent) from a reliable source. These analytes will be used to generate the stock solutions below. Store these materials according to manufacturers' instructions.

7.3.3.1 ANALYTE STOCK STANDARD SOLUTION 1 for RIC and ABR (SS1-RIC/ABR) – Combine RIC and ABR into a single solution using a 2 mL volumetric flask. To make this solution, weigh out and reconstitute powdered RIC and ABR in HPLC-grade water or dilute purchased solutions of RIC and ABR into HPLC-grade water so that the final concentration of this solution is 10 mg/L RIC and 10 mg/L ABR.

7.3.3.2 ANALYTE STOCK STANDARD SOLUTION 2 for RIC and ABR (SS2-RIC/ABR) – Dilute SS1-RIC/ABR as follows: combine 100 µL SS1-RIC/ABR with 900 µL HPLC-grade water and mix well. The final concentration of this solution is 1 mg/L RIC and 1 mg/L ABR.

7.3.3.3 ANALYTE STOCK STANDARD SOLUTION 1 for AMAN (SS1-AMAN) – Weigh out and reconstitute powdered AMAN in HPLC-grade water or dilute a purchased solution of AMAN into HPLC-grade water using a 2 mL volumetric flask so that the final concentration of this solution is 10 mg/L AMAN.

7.3.3.4 ANALYTE STOCK STANDARD SOLUTION 2 for AMAN (SS2-AMAN) – Dilute SS1-AMAN as follows: combine 100 µL SS1-AMAN with 900 µL HPLC-grade water and mix well. The final concentration of this solution is 1 mg/L AMAN.

7.3.4 CALIBRATION STANDARD STOCK SOLUTIONS – Prepare the calibration standard stock solutions from dilutions of the analyte stock solutions in reagent water containing the preservatives specified in section 8.2.1. These standard calibration solutions were observed to be stable for at least one year when stored at -20 ± 5 °C.

7.3.4.1 PREPARATION OF CALIBRATION STANDARD STOCK SOLUTIONS – Calibration standard stock solutions may be prepared using the volumes listed in Table 7-1, below, using a 25 mL volumetric flask. Eight concentrations, along with the numbers of solutions, are for illustration purposes only. Other concentrations may be required in practice to meet site-specific performance and QC goals. (See Section 10.2.3 for considerations in selecting concentrations of calibration solutions.) All standards should be diluted with HPLC-grade water.

Table 7-1. Calibration Standard Stock Solution Volumes

Concentration (ng/mL)			Total Volume (mL)	Volume of Analyte Stock Standard Solution (µL)			
RIC	ABR	AMA		SS1-RIC/ABR	SS1-AMAN	SS2-RIC/ABR	SS2-AMAN
0	0	0	25				
0.5	0.5	2	25			12.5	50
0.7	0.7	3	25			17.5	75
1	1	5	25			25	125
5	5	10	25			125	250
30	30	50	25	75	125		
50	50	100	25	125	250		
100	100	200	25	250	500		
200	200	400	25	500	1000		

ABR, abrine; AMAN, alpha-amanitin; RIC, Ricinine; SS, standard solution

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 (section 7.3.4) and are prepared the same way as the calibration standards. They are prepared from an analyte source different from the calibration standard solutions as described more completely in Section 9.3.5.

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 are 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 1 ng/mL for RIC, 1 ng/mL for ABR, and 5 ng/mL for AMAN. The LFB-high for this demonstration is 50 ng/mL for RIC, 50 ng/mL for ABR, and 100 ng/mL for AMAN. LFB-low and LFB-high can be prepared as indicated in Table 7-1, in Section 7.3.4. In a particular laboratory, the LFBs should be selected from similar points in their calibration range (e.g., LFB-low should be approximately 10 times the MRL (Section 9.2.4) and LFB-high should be approximately 150 times the MRL).

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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 (Section 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 (Section 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 FOR COLLECTION

8.1.1 Samples should 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 Inc., Franklin Lakes, NJ; or equivalent).

8.1.2 Vessels should be prepared before sample collection with sodium thiosulfate and sodium Omadine according to Table 8-1 to fulfill the purpose(s) listed. The preservatives should be added to all samples, including initial and on-going QC samples.

8.2 SAMPLE COLLECTION - When sampling from a water tap, samplers should request guidance about how long to flush the tap, if needed. 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 may leach interfering analytes into the water sample. If analyte recovery is unacceptably low, additional experiments may need to be performed to determine optimal collection conditions.

When filling sample bottles, take care not to flush out the sample preservation reagents. Samples do not need to be collected headspace free. After collecting the sample, cap the bottle and agitate by hand to mix the sample with the preservation reagents. Keep the sample sealed from time of collection until analysis.

8.3 SAMPLE SHIPMENT AND STORAGE – Sample stability was tested at 4 °C. As a matter of practice, ensure that samples do not experience excessive heat above

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this temperature. It is recommended that all samples be iced, frozen (-20 °C), or refrigerated (4 °C) from the time of collection until extraction. During method development, no significant differences were observed between standards that were frozen or refrigerated.

Table 8-1. Preservative Concentrations and Purposes of Preservatives

Compound	Mass added to 50 mL water sample (mg)	Concentration in sample (g/L)	Purpose
Sodium thiosulfate	4	0.08	Dechlorinating agent
Sodium Omadine	3.2	0.064	Microbial inhibitor

8.4   SAMPLE HOLDING TIMES - Table 8-2 presents the RIC, ABR, and AMAN storage stability data. Samples were collected and stored as described in Section 8 at 4 °C in the presence or absence of preservatives. The data support a 28-day aqueous holding time for preserved RIC specified in Section 8.4. Preserved ABR and AMAN and samples can also be held by the laboratory for a maximum of 28 days at 4 °C, although the initial loss of ABR and AMAN (Table 8-2) in some water occurs within 5 hrs after ABR and AMAN are added to the water sample. In an actual contamination incident, this 5 hr period will very likely have elapsed prior to the sample arriving at the laboratory. See section 1.1.1 for discussion of appropriate application of this method for ABR and AMAN.

8.5   EXTRACT HOLDING TIMES – Water samples should be extracted as soon as possible but within the holding time (28 days) described in Section 8.4. Data generated during this study indicate that extracts are stable for at least 28 days when preserved and stored at 0 °C or lower. In practice, analysis should occur as soon as possible.

Table 8-2. Recoveries of Ricinine, Abrine, and Alpha-Amanitin in Preservative Over Time (n=3)

Analyte	Water Type	Percent Recovery				
		15 min	5 hr	7 days	14 days	28 days
Ricinine (50 µg/L)	Ground Water 1[b] (chlorine)	97.1 ± 0.8	105 ± 3	93 ± 5	99 ± 8	91 ± 10
	Surface Water 2[c] (chlorine)	103.1 ± 2.4	103 ± 3	95 ± 3	102 ± 3[a]	88 ± 1
	Surface Water 3[d] (monochloramine)	94.2 ± 2	102 ± 5	93 ± 11	107 ± 8	92 ± 7
	Surface Water 4[e] (monochloramine)	110.4 ± 9.5	113 ± 8	97 ± 6	99 ± 4	86 ± 10
	Surface Water 5[f] (chlorine)	104.3 ± 3.4	104 ± 3	95 ± 4	98 ± 2	90 ± 6
Abrine (50 µg/L)	Ground Water 1[b] (chlorine)	103.6 ± 4.4	14 ± 1	14 ± 0	14 ± 0	14 ± 0
	Surface Water 2[c] (chlorine)	103.2 ± 2.8	9 ± 0	8 ± 0	9 ± 0[a]	8 ± 0
	Surface Water 3[d] (monochloramine)	103.8 ± 1.5	107 ± 3	98 ± 6	91 ± 2	90 ± 4
	Surface Water 4[e] (monochloramine)	102.9 ± 0.8	103 ± 3	100 ± 5	97 ± 4	93 ± 5
	Surface Water 5[f] (chlorine)	102.7 ± 1.2	21 ± 1	21 ± 1	20 ± 1	19 ± 1
α-Amanitin (100 µg/L)	Ground Water 1[b] (chlorine)	103.6 ± 4.4	7 ± 1	6 ± 0	7 ± 1	8 ± 0
	Surface Water 2[c] (chlorine)	103.2 ± 2.8	0 ± 0	0 ± 0	0 ± 0[a]	0 ± 0
	Surface Water 3[d] (monochloramine)	103.8 ± 1.5	97 ± 8	80 ± 5	90 ± 20	110 ± 30
	Surface Water 4[e] (monochloramine)	102.9 ± 0.8	104 ± 8	90 ± 5	90 ± 20	90 ± 10
	Surface Water 5[f] (chlorine)	102.7 ± 1.2	30 ± 7	30 ± 10	26 ± 6	19 ± 1

[a] n (number of replicates) =2 for this data point.
[b] Total organic carbon (TOC) below detection limit in well-field; pH 7.6; hardness 500 mg/L; Chlorine 0.2-0.4 mg/L (monthly averages).
[c] TOC 0.3 mg/L; pH 8.9; hardness 17 mg/L; Chlorine 1.3 mg/L (monthly average).
[d] TOC 2.3 mg/L; pH 7.4; hardness 190 mg/L; Monochloramine 3.4 mg/L (monthly averages).
[e] TOC 7.6 mg/L; pH 9.2; hardness 65 mg/L; Monochloramine 2.4 mg/L (monthly averages).
[f] TOC 1.0 mg/L; pH 8.5; hardness 130 mg/L; Chlorine 0.8 mg/L (monthly averages).

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 consistent with 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. Laboratories are encouraged to institute additional QC practices to meet specific needs [18].

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 laboratory, the IDC helps ensure successful long-term implementation of the method in a variety of other laboratories. 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 (Section 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 (Section 9.2.1-9.2.4) and ongoing (Section 9.3), should not be changed. In addition, a shorter IDC may result in higher QC failure rates and less accurate quantitation in some concentration ranges. Laboratories 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, and autosampler vials/plates is 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 Section 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 (Section 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 requirement is a result of the rigor of the QC requirements in the lengthy IDC (Section 9.2.1-9.2.3), especially those associated with the LFBs (see Section 10.3.3). If a shorter IDC is required by site-specific conditions (see Section 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 (*Mean*) and standard deviation for the method analytes in these replicates. Determine the half range for the prediction interval of results (HR_{PIR}) for each analyte using the equation below:

$$HR_{PIR} = 3.963s$$

where *s* is the standard deviation and 3.963 is a constant value for seven replicates.

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

The Upper Predicted Interval of Results (PIR) Limit should be ≤150% recovery.

$$\frac{Mean + HR_{PIR}}{FortifiedConcentration} \times 100\% \leq 150\%$$

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The Lower PIR Limit should be ≥ 50% recovery.

$$\frac{Mean - HR_{PIR}}{FortifiedConcentration} \times 100\% \geq 50\%$$

9.2.4.3 The MRL is validated if both the upper and lower PIR limits meet the criteria described above (Section 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). The detection limit is a statistical determination of precision, and accurate quantitation is not expected at the DL. Replicate analyses for this procedure should be done over at least three days (i.e., both the sample preparation and the HPLC/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 may be estimated by selecting a concentration that is between 3 and 5 times the signal-to-noise ratio for the analyte peak. The appropriate fortification concentrations will be dependent upon the sensitivity of the HPLC/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 three, and two LFBs individually fortified on day five, 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 subtraction of values of analyte-free blanks
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 (Section 3.1) to confirm that potential background contaminants are not interfering with the identification or quantitation of method analytes. Running the LRB first may prevent unnecessary analysis if the LRB is invalid. Preparation of the LRB is described in Section 7.3.5.2. 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 analytes or other contaminants that interfere with the measurement of the 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 analytes are detected in the LRB at concentrations greater than 1/3 the MRL, then all data for the problem analyte(s) are considered invalid for all samples in the analysis batch.

9.3.2　ONGOING CALIBRATION. During development of this method, the analytical system was 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 may 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 Section 2.2, in some well-considered circumstances and in consultation with the sample submitter about increased QC and quantitation risk, it may be desirable not to perform recalibration at the beginning of each analysis batch and instead rely on CCC samples (as described in Section 9.3.3) to verify ongoing calibration. If so, the beginning CCC of each analysis batch should be at or below the MRL 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 and 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 Section 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, which are analyzed after the field samples. If a different IDC approach was used based on site-specific goals and tolerance of QC and quantitation risk, it may acceptable to run only 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 CCCs 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 (Section 9.2), each time a new analyte stock standard solution 1 for RIC/ABR or AMAN (SS1-RIC/ABR or SS1-AMAN, Section 7.3.3.1) is prepared, and, at least quarterly, analyze a QCS 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 $\pm$ 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) or INTERNAL QUANTIFICATION STANDARD (IQS) – The analyst should monitor the peak area of the IS or IQS in all injections during each

analysis day. The IS or IQS peak area must meet the criteria in both of the following two subsections.

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 or IQS may not be as predicted. Prepare new calibration solutions, QC samples, and field samples with an appropriately increased concentration of IS or IQS.

9.3.6.2 The IS or IQS response (peak area) in any sample 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 or IQS area in a chromatographic run does not meet these criteria, inject a second aliquot of that extract.

9.3.6.2.1 If the reinjected aliquot produces an acceptable IS or IQS response, report results for that aliquot.

9.3.6.2.2 If the reinjected aliquot fails the IS or IQS 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(IQS) recovery." Alternatively, prepare another aliquot of the sample as specified in Section 11.2 or collect a new sample and re-analyze.

9.3.7 LABORATORY FORTIFIED SAMPLE MATRIX (LFSM) and LFSM DUPLICATES (LFSMD) – The isotopically-labeled and internal quantification standards in this method also serve 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 native RIC, ABR, or AMAN background concentrations. 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 situation would apply 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 chromatographic and mass spectral properties identical to RIC, ABR, or AMAN. In this case, the laboratory should discuss with the submitter the number and frequency of LFSMs.

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 ≤30% for samples fortified at or above their native concentration. Greater variability may 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 ≤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 check the precision associated with sample collection, preservation, storage, and laboratory procedures. Some of these factors are out of 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 they 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 may 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 of 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 laboratory bias may also be present.)

10.   CALIBRATION AND STANDARDIZATION

10.1   All laboratory equipment should be calibrated according to manufacturers' 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. (Ongoing calibration is discussed in section 9.3.3.) 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 ELECTROSPRAY IONIZATION (ESI)-MS/MS TUNE
10.2.1.1   Calibrate the mass scale of the MS with the calibration compounds and procedures prescribed by the manufacturer.

10.2.1.2   Optimize the [M+H]+ for each method analyte and surrogate or labeled internal standard by infusing approximately 1.0-5.0 µg/mL of each analyte (prepared in water containing 15% methanol) directly into the MS at the chosen LC mobile phase flow rate (between 0.3 and 0.5 mL/min). Each analyte should be tuned separately using solutions containing only one analyte, internal quantification standard, or labeled internal standard. The MS parameters (voltages, temperatures, gas flows, etc.) are

varied until optimal analyte responses are determined. The method analytes may have different optima requiring some compromise between the optima. See Tables 6-2 and 6-3 for ESI-MS/MS conditions used in method development

10.2.1.3 Optimize product ions for each analyte, IS, or IQS by infusing approximately 1.0-5.0 µg/mL of each analyte (prepared in the initial mobile phase conditions) directly into the MS at the chosen LC mobile phase flow rate (between 0.3 and 0.5 mL/min). This tune can be done on a mix of the method analytes. The MS/MS parameters (collision gas pressure, collision energy, etc.) are varied until optimal analyte responses are determined. The MS/MS product ions used in this method are listed in Table 10-1. See Tables 6-2 and 6-3 for MS/MS conditions used in method development.

Table 10-1. Ion transitions monitored for Ricinine, Abrine, and Alpha-Amanitin

Analyte	Precursor Ion (*m/z*)	Quantitation Ion (*m/z*)	Confirmation Ion (*m/z*)
Ricinine Analyte	165	82	138
Internal Standard	171	144	-
Abrine Analyte	219	188	132
Internal Standard	223	188	-
Alpha-Amanitin Analyte	917.4	899.4	560.2
Internal Quantification Standard	887.5	843.5	-

10.2.1.4 Establish LC operating parameters that optimize resolution and peak shape. LC conditions used in method development can be found in Table 6-1. The LC conditions listed in Table 6-1 may not be optimum for all LC systems and may need to be optimized by the analyst. If possible, optimize chromatographic conditions so that a unique quantitation ion is available for each analyte that is free from interference due to an identical product ion in any co-eluting (or overlapping) peak(s).

10.2.2 INSTRUMENT CONDITIONS – Operational conditions are tabulated in Section 6.8.3. 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

syringe is functioning properly and (2) changing the septum as needed.

10.2.3 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 or internal quantification standard is added before extraction. In practice, the lowest concentration of the calibration standard should be at or below the MRL (Section 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 remaining points are distributed between these two extremes, with the majority of points in the concentration range where most unknowns are expected to fall.

10.2.4 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 laboratories, including the developers of this method, 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, except the lowest point, for the analyte should calculate to be 70 to 130 percent of its true value. The lowest point should calculate to be 50 to 150 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), this standard 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 must be adjusted accordingly. The resulting r^2 value should be greater than 0.980.

10.3 CONTINUING CALIBRATION CHECKS (CCCs). As described in Sections 9.3.2 and 9.3.3, up to four CCCs are used in conjunction with each analysis batch, depending on initial IDC approach and site specific data quality objectives. The LRBs, LFBs, and CCCs are not counted as the 20 samples that constitute an analysis batch.

10.3.1   Inject an aliquot of the appropriate concentration calibration solution 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 that 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 (Section 10.3.3.1) established via the IDC. Acceptability 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 (σ_{n-1}, sigma) of the replicate analysis in the IDC (Section 9.2.2). Section 13.3 presents sample values for these parameters obtained in the developer's laboratory, in which eight replicate analyses performed over no less than 10 days are used to establish the LFB-low and LFB-high limits (Section 9.2.2). If the CCC results do not meet the following criteria, the CCC 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 LFB solutions in section 7.3.5.2.

   10.3.3.1   If both the LFB-low and LFB-high results are within two times the standard deviation 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 ration of the low standard confirmation ion falls below 10, this signal level indicates that the instrumental sensitivity or SPE 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.
> 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 large amount of analyte is not seen in the remainder of the calibration samples, contamination of this particular sample is indicated. 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 is contaminated. If necessary, prepare new solutions.

11. PROCEDURE

11.1 SAMPLE PREPARATION

11.1.1 Samples are collected, preserved and stored as presented in Section 8. Allow samples to come to room temperature prior to analysis.

11.1.2 If using a TurboVap 96 evaporator system, set it to 50-60 °C. Follow manufacturers' directions for other equipment.

Note: Steps 11.1.3 through 11.1.8 can be performed using an automated liquid handler or a manual pipettor with a manual 96-well manifold. However, data presented in this document were collected using an automated liquid handler.

11.1.3 Fill 96 plate wells.

11.1.3.1 Into each well of the 96-well Nunc deep well plate (Nalge Nunc International, Rochester, NY), add 30 µL of the RIC/ABR/AMAN internal standard mixture (refer to section 7.3.2).

11.1.3.2 Into each sample well, add 200 µL of sample.

11.1.3.3 Into each blank well, add 200 µL of reagent water (for the LRB).

11.1.3.4 Into each calibration standard well, add 200 µL of RIC/ABR/AMAN calibration standard stock solutions (refer to sections 7.3.4).

11.1.3.5 Into each quality control well, add 200 µL of appropriate quality control material (refer to section 7.3.5).

11.1.4 Mix on the plate shaker for 2 min or mix by other appropriate means.

11.1.5 Plate SPE procedure: For each well on the Nunc plate filled in Section 11.1.3, perform the following steps and do not let wells go dry for more than 1 minute:

11.1.5.1 Condition the selected well on the Phenomenex® Strata-X 60-mg SPE well plate (Phenomenex, Torrance, CA) with 1125 µL of 100% methanol.

11.1.5.2 Condition the SPE plate with 1125 µL of deionized water.

11.1.5.3 Load 150 µL from the Nunc sample plate and draw through the SPE plate using positive or negative pressure.

11.1.5.4 Wash the SPE plate with 1125 µL of 5% methanol/95% water (Section 7.2.1).

11.1.5.5 Elute the sample with 800 µL of acetonitrile into a 96-well Nunc deep well plate.

11.1.6 Blow the sample to dryness using a gentle stream of nitrogen gas at 50-60 °C. If using a TurboVap evaporator system, set the flow rate to 40 flow units until approximately 50% has been evaporated. Then raise the flow rate to 50-75 flow units until dry. When using

systems other than the TurboVap, set the flow rate for the blow
down gas according to manufacturer's directions.

11.1.7 Add 100 μL of HPLC-grade water to reconstitute each sample and
vortex.

11.1.8 Transfer the water solution into appropriate autosampler vials or
a 96-well autosampler plate.

11.2 ANALYSIS OF SAMPLE EXTRACTS

11.2.1 Establish operating conditions as described in Section 10.2.2.

11.2.2 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.

11.2.3 Set up the available automation equipment and software as
specified by the manufacturer for batch analysis, paying particular
attention to the following potential sources of errors/complications:

11.2.3.1 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.

11.2.3.2 Check to be sure that the number and positions of
samples entered on the sequence setup page correspond
to the samples in the autosampler.

11.2.4 Run the automation sequence to analyze the batch of aliquots of
field and QC samples at appropriate frequencies (Section 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.

      11.2.5   COMPOUND IDENTIFICATION – The presumed peaks for RIC, ABR, and AMAN in the sample must appear in the same retention time window as the internal standard corresponding to each analyte (around 2.8 min for RIC, 2.4 min for ABR, and 5.1 min for AMAN in the developer's laboratory) and have similar chromatographic characteristics such as peak shape. This compound identification relies on expert judgment of the analyst because the retention times reported by the software are not always reliable. Identification of the peak as RIC, ABR, or AMAN 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 3.02 for RIC, 0.43 for ABR, and 0.13 for AMAN in the developer's laboratory.) This percent tolerance is based on running a new calibration curve with each batch. Depending on site-specific data quality objectives, this might not have been done. In this case, the CR should still be within 30% of the most recent calibration, which should have been analyzed no more than 30 days prior. Furthermore, no components of the analytical instrument should have been changed or recalibrated between the most recent CCC and analysis of the current batch of samples. Any changes to the instrument could result in this tolerance being exceeded.

12.   DATA ANALYSIS AND CALCULATIONS

12.1   Concentrations are calculated using the ions listed in Table 10-1. Use of other ions is not advised. If a particular instrument cannot produce the fragments listed in Table 10-1, that 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 LFB-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 and consistent with laboratory policy. This process 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 both the instrument and data storage databases onto external media according to individual laboratory guidelines.

13.   METHOD PERFORMANCE

13.1   ANALYTICAL IDENTIFICATION–Analyte identification using the approach described in Section 11.2.5 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 LABORATY MINIMUM REPORTING LEVELS and DETECTION LIMIT– The reportable range of results for RIC, ABR, and AMAN are summarized in Table 13-1, along with the DL determined from the IDC procedure described previously. The lowest calibration standard is used as the method reportable limit (MRL) and the DL calculated from the standard deviation of replicate measurements of that standard. The highest reportable limit (HRL) is based on the highest linear calibration standard.

Table 13-1. Method Performance (n=8 over 10 days)

Analyte	DL (µg/L)	MRL (µg/L)	HRL (µg/L)
Ricinine	0.09	0.50	200
Abrine	0.06	0.50	200
Alpha-Amanitin	0.54	2.0	400

DL, detection limit; HRL, highest reportable limit; MRL, minimum reportable level; n, number of replicates

13.3 SINGLE LABORATORY ACCURACY AND PRECISION for LFBs –
Single laboratory precision and accuracy data are 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 10 days for each analyte. The means, standard deviations, and relative standard deviations for the two LFBs are shown in Table 13-2.

Table 13-2. Single Lab Precision and Accuracy (n=8 over 10 days).

Analyte	QC Pool	Fortified Conc. (µg/L)	Mean of IDC Replicates (µg/L)	Standard Deviation (µg/L)	RSD (%)	Measured Conc. (%)[a]
Ricinine	LFB-Low	1.0	0.93	0.05	5.4	93 ± 5
	LFB-High	50	50	0.68	1.4	100 ± 1
Abrine	LFB-Low	1.0	0.97	0.04	4.1	97 ± 4
	LFB-High	50	51	0.45	0.88	102 ± 1
Alpha-Amanitin	LFB-Low	5.0	5.0	0.40	8.0	100 ± 8
	LFB-High	100	101	10.2	10.1	101 ± 10

[a]The measured concentration is expressed as a percent of that fortified $(x \pm \sigma_{n-1})$.
IDC, initial demonstration of capability; LFB, laboratory fortified blank; n, number of replicates; QC, quality control; RSD, relative standard deviation

13.4 SINGLE LABORATORY RECOVERY AND PRECISION FOR TAP
WATER MATRICES. Table 8-2 expresses percent mean recoveries for RIC, ABR, and AMAN in several different chlorinated and chloraminated tap waters derived from the types of sources (i.e., ground or surface water) indicated. Water quality parameters describing these sources are indicated in the footnotes. Percent recoveries were determined by dividing the measured concentration by the spiked concentration (number of replicates

(n)=3). No analytes or interferences were detected in the unspiked samples.

13.5   SPECIAL NOTE FOR ABR AND AMAN. The performance data for the method presented in Section 13 are from samples preserved as described in Section 8.1.2. As indicated in Section 8.4, ABR and AMAN exhibit an initial analytical signal loss that is dependent on the specific drinking water. Accordingly, if ABR and AMAN are unexpectedly low (or absent) for a particular drinking water, additional QC steps, such as additional LFSMs held at least 5 hours (see Section. 9.3.7), maybe required.

14.   POLLUTION PREVENTION

14.1   This method utilizes solid phase extraction to extract analytes from water. The method 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.

14.2   For information about pollution prevention that may be applicable to laboratory operations, consult "Less is Better: Laboratory Chemical Management for Waste Reduction" available from the American Chemical Society's Department of Government Relations and Science Policy on-line at http://portal.acs.org/portal/fileFetch/C/WPCP_012290/pdf/WPCP_012290.pdf (accessed May 2010).

15.   WASTE MANAGEMENT

15.1   Dispose of waste materials 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 store them temporarily in a chemical fume hood. Dispose of RIC, ABR, and AMAN in an appropriate waste stream as well.

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