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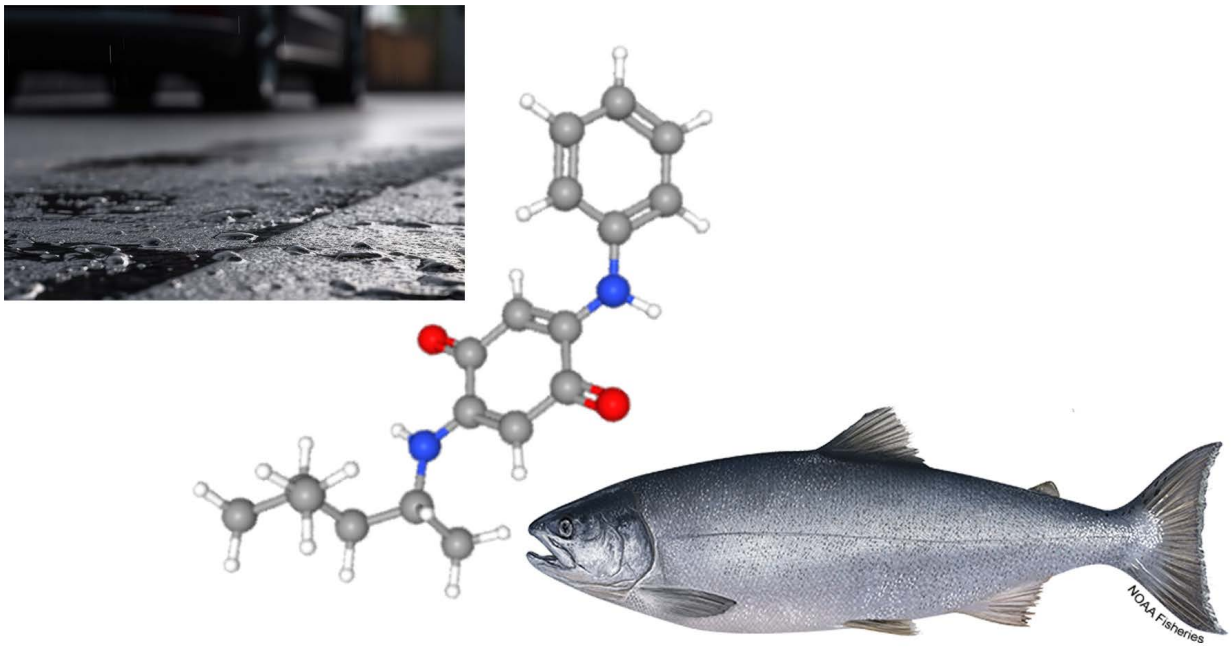
DRAFT Method 1634

Determination of 6PPD-Quinone in Aqueous Matrices Using Liquid Chromatography with Tandem Mass Spectrometry (LC/MS/MS)



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Notice

This document represents the first draft of Method 1634 for 6PPD-Q currently under development by the EPA Office of Water, Engineering and Analysis Division (EAD), in collaboration with EPA Region 10 and Eurofins Environment Testing Northern California. **This method is not approved for Clean Water Act compliance monitoring unless and until it has been proposed and promulgated through rulemaking.**

A single-laboratory validation study tested the method on surface water and stormwater runoff matrices and the report on the results of that study has been prepared. A multi-laboratory validation study is anticipated at a later date. The Office of Water may use the results from the multi-laboratory validation study to finalize the method and add formal performance criteria.

In the meantime, the Office of Water is releasing this draft on its web site. Laboratories, regulatory authorities, and other interested parties are encouraged to review the method, and where appropriate, utilize it for their own purposes, with the explicit understanding that this is a draft method, subject to revision.

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Determination of 6PPD-Quinone in Aqueous Matrices Using Liquid Chromatography with Tandem Mass Spectrometry (LC/MS/MS)

1.0 Scope and Application

1.1 Method 1634 is for the determination of 6PPD-quinone (6PPD-Q), also called 2-anilo-5-[(4-methylpentan-2-yl)amino]cyclohexa-2,5-diene-1,4-dione, or N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylene-diamine-quinone (see Table 1) in aqueous matrices, predominantly stormwater and surface water, by either liquid chromatography-tandem mass spectrometry (LC/MS/MS). 6PPD-Q is formed in the environment from the conversion of the tire additive N-(1,3-dimethylbutyl)-N'-phenyl-*p*-phenylenediamine (PPD) in the presence of ozone.

1.2 This method was developed for use in Clean Water Act (CWA) programs for aqueous samples from stormwater and surface water sources. Other applications and matrices may be possible, which may or may not require modifications of sample preparation, chromatographic conditions, etc.

1.3 The instrumental portions of this method are for use only by analysts experienced with LC/MS/MS instruments or under the close supervision of such qualified persons. Each laboratory that uses this method must demonstrate the ability to generate acceptable results using the procedure in Section 9.2.

1.4 This method is "performance-based," which means that the analyst may make modifications to improve performance (e.g., overcome interferences, or improve the sensitivity, accuracy or precision of the results) without additional EPA review, *provided that* all performance criteria in this method are met. Requirements for establishing equivalency are in Section 9.1.2, and include 9.1.2.2 c. For CWA uses, additional flexibility is described at 40 CFR 136.6. The laboratory must document changes in performance, sensitivity, selectivity, precision, recovery, etc., that result from modifications within the scope of 40 CFR Part 136.6, and Section 9 of this method, and document how these modifications compare to the specifications in this method. Changes outside the scope of 40 CFR Part 136.6 and Section 9 of this method may require prior review or approval.

2.0 Summary of Method

Aqueous samples are prepared and extracted using solid-phase extraction (SPE) procedures. Analyses are conducted by LC/MS/MS to identify 6PPD-Q based on retention time and multiple reaction monitoring (MRM) transitions and quantify it by isotope dilution techniques in environmental samples.

2.1 An aliquot of the extracted internal standard (EIS) $^{13}C_6$-6PPD-Q solution is spiked into a 250-mL aqueous sample. Aqueous samples are extracted using an SPE cartridge. 6PPD-Q is eluted from the cartridge with acetonitrile and brought to a final volume of 10 mL.

2.2 The extract (SPE eluent) is spiked with the non-extracted internal standard (NIS) D_5-6PPD-Q solution and injected on the high performance liquid chromatograph (HPLC) equipped with a C18 column interfaced to a tandem mass spectrometer (LC/MS/MS). The 6PPD-Q is identified by comparing the acquisition of the mass transitions and retention time to reference spectra and retention time for the calibration standards acquired under identical LC/MS/MS conditions.

2.2.1 The target analyte and the isotopically labeled standards are identified through peak analysis of the Multiple Reaction Monitoring (MRM) transitions and retention time comparison (Section 10.3).

2.2.2 Quantitative determination of 6PPD-Q concentration is made using the primary quantitation ion (Q1) with respect to the EIS, and the results for 6PPD-Q are recovery corrected by the isotope dilution method.

2.2.3 The EIS recoveries are determined similarly against the NIS and are used as general indicators of overall analytical quality.

2.2.4 The quality of the analysis is assured through reproducible calibration and testing of the extraction and LC/MS/MS systems.

3.0 Definitions

Definitions are provided in the glossary at the end of this method.

4.0 Contamination and Interferences

4.1 Method interferences may be caused by contaminants in solvents, reagents (including reagent water), sample bottle and caps, glassware, solvent bottles, squirt bottles, and other processing apparatus may yield artifacts and elevated baselines causing misinterpretation of chromatograms. These items must be routinely demonstrated to be free from interferences by analyzing laboratory reagent blanks (Section 9.5) using the same conditions as for samples. Subtracting blank values from the sample results is not permitted.

4.2 Matrix interferences may result from contaminants co-extracted from samples. The extent of matrix interferences will depend on the nature of the sample. Some stormwater matrices collected during the winter months contained compounds that interfered with the quantitation ions of $^{13}C_{12}$-6PPD-Q, which was being considered as an EIS. As a result, this compound is not recommended for use with this method.

4.3 Interferences may also come from the SPE cartridges used to extract water samples. The use of field and laboratory reagent blanks can provide information regarding to the presence or absence of such interferences. The testing of different brands and/or lot numbers of SPE devices may be needed to ensure that the contamination does not preclude analyte identification and quantitation.

5.0 Safety

5.1 It is not the intent for this method to cover all the potential safety problems associated with its use. It is the responsibility of the user of the method to follow appropriate safety, waste disposal and health practices under the assumption that all samples and reagents are potentially hazardous. Safety glasses, gloves, lab coats and closed-toed, nonabsorbent shoes are a minimum.

5.2 The laboratory is responsible for maintaining a current awareness file of Occupational Safety and Health Administration (OSHA) regulations regarding the safe handling of the chemicals specified in this method. A reference file of safety data sheets (SDSs) should also be made available to all personnel involved in these analyses. It is also suggested that the laboratory perform personal hygiene monitoring of each analyst who uses this method and that the results of this monitoring be made available to the analyst. Additional information on laboratory safety can be found in References 1 – 4.

5.3 Specific Safety Concerns or Requirements

5.3.1 Make certain the vacuum exhaust hose used during the filtering is securely anchored inside of a fume hood to prevent vapors from being released into the working environment.

5.3.2 Eye protection that satisfies ANSI Z87.1 (Reference 5), laboratory coat, and chemically resistant gloves must be worn while samples, standards, solvents, and reagents are being handled. Nitrile gloves provide adequate levels of protection against the chemicals used in this method.

5.3.3 Exposure to chemicals must be maintained as low as reasonably achievable; therefore, all samples should be opened, transferred and prepared in a fume hood. Solvent and waste containers will be kept closed unless transfers are being made.

5.4 Safety Information for Primary Reagents Used

The following is a list of the materials used in this method, which have a serious or significant hazard rating as per the information in the SDSs. **This list does not include all materials used in the method. The table contains a summary of the primary hazards listed in the SDS for each of the materials listed in the table.** A complete list of materials used in the method can be found in the reagents and materials section.

Material[1]	**Hazards**	**Exposure Limit[2]**	**Signs and symptoms of exposure**
Acetic Acid	Corrosive Poison Flammable	10 ppm-TWA 15 ppm-STEL	Contact with concentrated solution may cause serious damage to the skin and eyes. Inhalation of concentrated vapors may cause serious damage to the lining of the nose, throat, and lungs. Breathing difficulties may occur.
Acetonitrile	Flammable Poison Irritant	20 ppm- TWA	Liquid and vapor can irritate the eyes, nose, throat and lungs. Symptoms of exposure look like cyanide exposure and can include pink coloring of the skin, dilated pupils, headache, nausea and vomiting, dizziness, weakness, stiffness of the lower jaw, anxiety, pain and tightness in the chest, rapid breathing and pulse, irregular heartbeat, shortness of breath, convulsions, loss of consciousness and death.
Ammonium Hydroxide	Corrosive Poison	50 ppm-TWA	Severe irritant. Effects from inhalation of dust or mist vary from mild irritation to serious damage to the upper respiratory tract. Symptoms may include sneezing, sore throat or runny nose. Contact with skin can cause irritation or severe burns and scarring with greater exposures. Causes irritation of eyes, and with greater exposures it can cause burns that may result in permanent damage, including blindness. Brief exposure to 5000 PPM can be fatal.
Formic Acid	Flammable Irritant Corrosive Toxic	5 ppm – TWA 10 ppm – STEL	Extremely destructive on contact with skin, mucous membranes, eyes, upper respiratory tract. Inhalation may result in spasms, inflammation and edema. Symptoms include burning sensation, coughing, wheezing, shortness of breath, headache, nausea, vomiting and depression.
Methanol	Flammable Poison Irritant	200 ppm-TWA 250 ppm – STEL	A slight irritant to the mucous membranes. Toxic effects exerted upon nervous system, particularly the optic nerve. Symptoms of overexposure may include headache, drowsiness, and dizziness. Methyl alcohol is a defatting agent and may cause skin to become dry and cracked. Skin absorption can occur; symptoms may parallel inhalation exposure. Irritant to the eyes.
Always add acid to water to prevent violent reactions.			
Exposure limit refers to the OSHA regulatory exposure limit.			

5.5 Training – Workers must be trained in the proper method of removing contaminated gloves and clothing without contacting the exterior surfaces.

5.6 Personal hygiene – Hands and forearms should be washed thoroughly after each manipulation and before breaks (coffee, lunch, and shift).

5.7 Waste Handling – Good technique includes minimizing contaminated waste. Plastic bag liners should be used in waste cans. Janitors and other personnel should be trained in the safe handling of waste.

5.8 Decontamination

5.8.1 Decontamination of personnel – Use any mild soap with plenty of scrubbing action.

5.8.2 Glassware, tools, and surfaces – Satisfactory cleaning may be accomplished by rinsing with acetonitrile, then washing with any detergent and water. If glassware is first rinsed with solvent, the wash water may be disposed of in the sewer. Given the cost of disposal, it is prudent to minimize solvent wastes.

5.8.3 Laundry – Clothing known to be contaminated should be collected in plastic bags. Persons that convey the bags and launder the clothing should be advised of the hazard and trained in proper handling. The clothing may be put into a washer without contact if the launderer knows of the potential problem. The washer should be run through a cycle before being used again for other clothing.

6.0 Equipment and Supplies

Note: *Brand names, suppliers, and part numbers are for illustration purposes only and no endorsement is implied. Equivalent performance may be achieved using apparatus and materials other than those specified here. Meeting the performance requirements of this method is the responsibility of the laboratory.*

The presence of 6PPD-Q in the environment is still being delineated. Because 6PPD-Q may be ubiquitous in the environment, all disposable equipment (including, but not limited to vials, pipet tips, and SPE manifold parts) that comes in contact with a sample or extract is subject to periodic QC checks. QC checks should include either a rinse with DI water or an extraction of the equipment with NH_4OH/acetonitrile to mimic the usage encountered during sample preparation.

6.1 2-mL autosampler vials, polypropylene, with polypropylene screw caps, or Agilent PTFE-lined LC screw caps, or equivalent.

Note: *Evaporation from the vials may occur after injection because the polypropylene caps do not reseal after injection. Caps should be replaced after multiple injections.*

6.2 Balances

6.2.1 Analytical balance capable of accurately weighing to the nearest 0.1 mg.

6.2.2 Top loading – Capable of weighing 10 mg.

6.3 250-mL amber glass bottles with polytetrafluoroethylene-lined (PTFE) caps.

6.4 15-mL polypropylene centrifuge tubes with polypropylene screw caps.

6.5 Miscellaneous laboratory apparatus (beakers, test tubes, volumetric flasks, pipettes, etc.). All volumetric glassware must be Class A.

6.6 Pipettes, auto-pipets, and other equipment used to prepare standards and reagents.

6.7 Solid-phase extraction (SPE) system

6.7.1 SPE Cartridges – 200-milligram/6-cc SPE cartridges containing Strata-XL, 100-µm polymeric reversed-phase (Phenomenex, PN 8B-S043-FCH), or equivalent.

6.7.2 Vacuum extraction manifold – Supelco Visiprep, or equivalent. A manual vacuum manifold with column adapters, disposable liners, and column reservoirs for cartridge extraction.

6.7.3 Automated SPE manifolds may be used as long as volumes and conditions remain consistent with the manual SPE manifold.

6.8 Volumetric flask, Class A, various sizes, 2.0-mL to 100-mL, as appropriate.

6.9 Vortex Mixer – Fisherbrand™ analog vortex mixer, Cat. No. 02-215-414, or equivalent variable speed mixer.

6.10 Liquid Chromatograph/Tandem Mass Spectrometer (LC/MS/MS) – The instrumentation consists of an HPLC equipped with a refrigerated autosampler, an injection valve, a column heater, and a pump capable of variable flow rate connected to a tandem MS/MS operated in positive ion electrospray (ESI) mode.

Note: *This method has been validated using LC/MS/MS. Higher resolution mass spectrometers may be used as long as they can be operated in the MRM mode and meet the sensitivity and QC requirements of this method.*

6.10.1 SCIEX 5500 Triple Quad MS, or equivalent, capable of collecting at least 10 scans across a chromatographic peak and a data system. The source is operated in positive ion electrospray (ESI) mode.

6.10.2 Shimadzu CTO-20AC HPLC equipped with at least 2 LC-20AD pumps and one DGU-20 degassing unit, or equivalent.

6.10.3 Phenomenex Kinetex XB C18, 3.6 µm, 4.6 mm x 100 mm, Part No. 00D-4744-E0, or equivalent. This solid-core column (versus a porous particle column) showed reduced tailing for the analyte.

6.11 Sampling equipment for discrete or composite sampling

6.11.1 Sample bottles and caps

6.11.1.1 Liquid samples (waters, sludges and similar materials containing 5 percent solids or less) – Sample bottle, amber glass, 250 mL, with screw cap. Use of pre-cleaned bottles purchased from suppliers who provide a certificate of analysis is encouraged. Each lot of bottles should be checked for background contaminants prior to use.

6.11.1.2 If amber bottles are not available, samples must be protected from light.

6.11.1.3 Bottle caps – Threaded to fit sample bottles. Caps must be lined with fluoropolymer.

6.11.1.4 Cleaning – If pre-cleaned bottles are not used, employ the steps below to clean bottles.

6.11.1.4.1 Bottles are detergent water washed, then solvent rinsed before use.

6.11.1.4.2 Liners are detergent water washed and rinsed with reagent water (Section 7.2.1).

6.11.2 Compositing equipment – Automatic or manual compositing system incorporating glass containers cleaned per bottle cleaning procedure above. Only glass or fluoropolymer tubing must be used. If the sampler uses a peristaltic pump, a minimum length of compressible silicone rubber tubing may be used in the pump only. Before use, the tubing must be thoroughly rinsed with methanol, followed by repeated rinsing with reagent water to minimize sample contamination. An integrating flow meter is used to collect proportional composite samples.

7.0 Reagents and Standards

7.1 Reagent grade chemicals shall be used, whenever available, to prepare all samples, blanks and other QC samples. Unless otherwise indicated, the reagents shall conform to the specifications of the Committee on the Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided the reagent is of sufficiently high purity to permit use without lessening the accuracy of the results.

7.1.1 Acetonitrile, HPLC Grade, or equivalent.

7.1.2 Methanol, HPLC Grade, or equivalent.

7.1.3 Reagent Water – HPLC grade water, distilled or de-ionized water, that has been demonstrated to be free of 6PPD-Q (e.g., below the MDL).

7.1.4 50:50 Methanol:Water (v/v): dilute 500 mL methanol (Section 7.1.2) with 500 mL HPLC grade reagent water (Section 7.1.3). Store in a polypropylene bottle and seal with a screw cap.

7.1.5 Formic Acid, CH_2O_2, Certified ACS Grade, Fisher PN A118P-500, or equivalent.

7.1.6 Formic Acid, 0.2% in methanol: Add 2 mL of formic acid (Section 7.1.5) into 998 mL of HPLC grade methanol (Section 7.1.2).

7.1.7 Formic Acid, 0.2% in water: Add 2 mL of formic acid (Section 7.1.5) into 998 mL of reagent water (Section 7.1.3).

7.1.8 Acetic acid, glacial, Fisher PN BP2401-500 or equivalent.

7.1.9 Acetic acid, 11% in water: Add 22 mL of acetic acid (Section 7.1.8) to 178 mL of reagent water (Section 7.1.3).

7.1.10 Ammonium hydroxide (NH_4OH), concentrated (28-30%), Reagent Grade, Spectrum Chemical, PN 18-600-616, or equivalent.

7.1.11 Ammonium hydroxide (NH_4OH), 0.2% in acetonitrile: Add 2mL of ammonium hydroxide (Section 7.1.10) into 998 mL of acetonitrile (Section 7.1.1).

7.2 Standard Solutions

Prepare from materials of known purity and composition or purchase as solutions or mixtures with certification to their purity, concentration, and authenticity. Observe the safety precautions in Section 5.

When not being used, store standard solutions in the dark at less than 6 °C, but not frozen, unless the vendor recommends otherwise, in screw-capped vials with PTFE-lined caps. The laboratory must maintain records of the certificates for all standards, as well as records for the preparation of intermediate and working standards, for traceability purposes.

Allow all solutions to warm to room temperature prior to use. Mix using a vortex mixer prior to taking aliquots for use. Standards may be scaled up or down as needed. Standards should not be used past the expiration date listed on the standard. If no expiration date is provided, or if standard solutions used for quantitative purposes are kept beyond 6 months (Sections 7.2.1 through 7.2.9), they should be assayed periodically (e.g., every 6 months) against certified standard reference materials (SRMs) from the National Institute of Science and Technology (NIST), if available, or certified reference materials from a source accredited under ISO Guide 17034 that attests to the concentration, to assure that the composition and concentrations have not changed.

7.2.1 6PPD-Q stock solution, 100 µg/mL, Cambridge Isotope Laboratories CIL ULM-12288-1.2, or equivalent.

7.2.2 Stock solutions for D_5-6PPD-Q (HPC Standards Catalogue Number 688151 or equivalent) and $^{13}C_6$-6PPD-QCIL (Cambridge Isotope Laboratories CLM-12293-1.2 or equivalent), 100 µg/mL in acetonitrile. These isotopically labeled analogs are used as the non-extracted internal standard (NIS) and extracted internal standard (EIS), respectively.

7.2.3 6PPD-Q Intermediate (IM) solution (20 ng/mL) – Dilute 50 µL of the 6PPD-Q stock (Section 7.2.1) to a final volume of 250 mL in acetonitrile (Section 7.1.1). This solution is used to prepare the calibration solutions and for sample fortification. Store the solution in a polypropylene bottle at 0 – 6 °C. The solution can be used for up to 6 months.

7.2.4 $^{13}C_6$-6PPD-Q EIS Solution (20 ng/mL) – Dilute 50 µL of a 100 µg/mL stock solution containing $^{13}C_6$-6PPD-Q (Section 7.2.2) to a final volume of 250 mL in acetonitrile. Store the solution in a polypropylene bottle at 0 – 6 °C. The solution can be used for up to 6 months.

7.2.5 D_5-6PPD-Q NIS Solution (20 ng/mL) – Dilute 50 µL of a 100 µg/mL stock solution containing D_5-6PPD-Q (Section 7.2.2) to a final volume of 250 mL in acetonitrile. Store the solution in a polypropylene bottle at 0 – 6 °C. The solution can be used for up to 6 months.

7.2.6 6PPD-Q Calibration Solutions – Calibration Solutions are prepared from the IM, EIS, and NIS Solutions above (Sections 7.2.3 – 7.2.5). Refer to Table 2 for the preparation of the calibration standards (V1 – V7). Store the calibration standard solutions in polypropylene bottles at 0 – 6 °C. The standards can be used for up to 6 months.

7.2.7 Second-source Standards – If available, the second-source solutions for the native analyte should be made using material from a vendor and/or lot other than that used for the calibration and spiking solutions. The second-source stock and intermediate solutions are prepared in the same manner as the primary source, as described in Sections 7.2.3.

7.2.8 6PPD-Q Calibration Verification (VER) Standard – Prepare the VER standard as described in Table 2 for the L4 (0.5 ng/mL) calibration standard. Store the calibration standard solution in a polypropylene bottle at 0 – 6 °C. The standard can be used for up to 6 months.

7.2.9 6PPD-Q Instrument Sensitivity Check (ISC) Standard – Prepare the ISC standard at the concentration of the lowest calibration standard that is within the quantitation range. The concentration of ISC will be laboratory-specific, based on the demonstrated quantitation range in each laboratory (i.e., it is not necessarily the lowest calibration standard analyzed). Store the calibration standard solutions in polypropylene bottles at 0 – 6 °C. The standard can be used for up to 6 months.

7.2.10 Mass calibration and mass calibration verification solutions – Use the mass calibration solution specified by the instrument manufacturer.

8.0 Sample Collection, Preservation, Storage, and Holding Times

8.1 Aqueous samples that flow freely are collected as grab samples that are free of headspace or in refrigerated bottles using automatic sampling equipment. Collect aqueous samples in 250-mL amber glass jars with PTFE-lined caps.

8.2 Protect aqueous samples from light (in amber glass containers) at less than or equal to 6 ºC (but keep from freezing) from the time of collection until sample preparation at the laboratory.

8.3 Aqueous samples must be extracted within 14 days from sample collection.

8.4 Extracts must be stored at 0 – 6 °C and analyzed within 28 days from extraction.

9.0 Quality Control

9.1 Each laboratory that uses this method is required to operate a formal quality assurance program (Reference 6). The minimum requirements of this program consist of an initial demonstration of laboratory capability, analysis of samples spiked with labeled compounds to evaluate and document data quality, and analysis of standards and blanks as tests of continued performance. Laboratory performance is compared to established performance criteria to determine if the results of analyses meet the performance characteristics of the method.

9.1.1 The laboratory must make an initial demonstration of the ability to generate acceptable precision and recovery with this method. This demonstration is given in Section 9.2.

9.1.2 In recognition of advances that are occurring in analytical technology, and to overcome matrix interferences, the laboratory is permitted certain options to improve separations or lower the costs of measurements. These options include alternative extraction, concentration, and cleanup procedures, and changes in sample volumes, columns, and detectors. Alternative determinative techniques and changes that degrade method performance are *not* allowed without prior review and approval.

Note: *For additional details about the flexibility to make modifications without prior EPA review, see 40 CFR Part 136.6.*

9.1.2.1 Each time a modification is made to this method, the laboratory is required to repeat the procedure in Section 9.2. If calibration will be affected by the change, the instrument must be recalibrated per Section 10.0. Once the modification is demonstrated to produce results equivalent or superior to results produced by this method as written, that modification may be used routinely thereafter, so long as the other requirements in this method are met (e.g., labeled compound recovery).

9.1.2.2 The laboratory is required to maintain records of modifications made to this method. These records include the following, at a minimum:

a) The names, titles, business addresses, and telephone numbers of the analyst(s) that performed the analyses and modification, and of the quality control officer that witnessed and will verify the analyses and modifications.

b) A listing of pollutant(s) measured, by name and CAS Registry number.

c) A narrative stating reason(s) for the modifications (see Section 1.6).

d) Results from all quality control (QC) tests comparing the modified method to this method, including:

i. Calibration (Section 10)
ii. Calibration Verification (VER) (Section 10.5 and 13.4)
iii. Initial precision and recovery (Section 9.2.1)
iv. Labeled compound recovery (Section 9.3)
v. Analysis of blanks (Section 9.5)
vi. Accuracy assessment (Section 9.4)

e) Data that will allow an independent reviewer to validate each determination by tracing the instrument output (peak height, area, or other signal) to the final result. These data are to include:

i. Sample numbers and other identifiers
ii. Extraction dates
iii. Analysis dates and times
iv. Analysis sequence/run chronology
v. Sample weight or volume (Section 11)
vi. Extract volume prior to SPE (Section 11)
vii. Extract volume after SPE (Section 11)
viii. Final extract volume prior to injection (Section 11.3)
ix. Injection volume (Section 12.3)
x. Dilution data, differentiating between dilution of a sample or extract (Section 14.3)
xi. Instrument identification
xii. Column (dimensions, liquid phase, solid support, film thickness, etc.)
xiii. Operating conditions (temperatures, temperature program, flow rates, eluent)
xiv. Detector (type, operating conditions, etc.)
xv. Chromatograms, printer tapes, and other recordings of raw data
xvi. Quantitation reports, data system outputs, and other data to link the raw data to the results reported

9.1.2.3 Alternative LC columns and column systems – If a column or column system other than those specified in this method is used, that column or column system must meet all the requirements of this method, including peak shape and separation for coeluting compounds.

9.1.3 Analyses of method blanks are required on an on-going basis to demonstrate the extent of background contamination in any reagents or equipment used to prepare and analyze field samples (Section 4.1). The procedures and criteria for analysis of a method blank are described in Section 9.5.

9.1.4 The laboratory must spike all samples with the EIS compound to monitor method performance. This test is described in Section 9.3. When results of these spikes indicate atypical method performance for samples, the samples are diluted to evaluate whether the performance issue is caused by the sample matrix. Procedures for dilution are given in Section 14.3.

9.1.5 The laboratory must, on an ongoing basis, demonstrate through calibration verification and the analysis of the ongoing precision and recovery standard (OPR) and blanks that the analytical system is in control. These procedures are given in Sections 13.1 through 13.7.

9.1.6 The laboratory should maintain records to define the quality of data generated. Development of accuracy statements is described in Section 9.4.

9.2 Initial Demonstration of Capability

9.2.1 Initial precision and recovery (IPR) – To establish the ability to generate acceptable precision and recovery, the laboratory must perform the following operations for each sample matrix type to which the method will be applied by that laboratory.

9.2.1.1 Extract, concentrate, and analyze four aliquots of reagent water, spiked with 0.5 mL of the 6PPD-Q intermediate solution (Section 7.2.3) and 0.5 mL of the $^{13}C_6$-6PPD-Q EIS Solution (Section 7.2.4). A 0.5-mL aliquot of the D_5-6PPD-Q NIS solution (Section 7.2.5) is added after extraction, but before analysis. At least one method blank must be included. All sample processing steps that are to be used for processing samples, including preparation, extraction and concentration (Sections 11.3 – 11.8) must be included in this test. Calculate the concentration of 6PPD-Q by isotope dilution, relative to $^{13}C_6$-6PPD-Q.

9.2.1.2 Using results of the set of the four analyses, compute the average percent recovery (R) of 6PPD-Q in the samples and the relative standard deviation (RSD) of the recovery.

9.2.1.3 Compare the average percent recovery and the RSD with the corresponding interim limits for initial precision and recovery (70-130% recovery and 20% RSD). If the average recovery and the RSD meet the acceptance criteria, system performance is acceptable, and analysis of blanks and samples may begin. If, however, the RSD exceeds the precision limit or the average percent recovery falls outside the range for recovery, system performance is unacceptable for that compound. Correct the problem and repeat the test (Section 9.2).

Note: *The interim criteria for initial precision and recovery above were easily achieved during the single-laboratory validation. Final criteria will be developed after the method has undergone a multi-laboratory validation study.*

9.2.2 Method detection limit (MDL) – The laboratory must establish an MDL for 6PPD-Q using the MDL procedure at 40 CFR Part 136, Appendix B (Reference 10). The minimum level of quantification (ML) is then calculated using the approach described in Section 20 (see ML in Glossary) or from another recognized source.

9.3 To assess method performance on the sample matrix, the laboratory must spike all samples with the EIS solution (Section 7.2.4) and all sample extracts with the NIS solution (Section 7.2.5) as described above in Section 9.2.

9.3.1 Analyze each sample according to the procedures in Sections 11.0 through 14.0.

9.3.2 Compute the percent recovery of the EIS using the non-extracted internal standard method (Section 13.8 and 14.2) and the equation in Section 13.5.2.

9.3.3 The recovery of the EIS ($^{13}C_6$-PPD-Q) must be within the limits of 25 – 200%.

9.4 Records of the recoveries of EIS and NIS compounds from samples must be maintained and should be assessed periodically.

9.4.1 After the analysis of 30 samples of a given matrix type for which the labeled compounds pass the tests in Section 9.3, compute the average percent recovery (R) and the standard deviation of the percent recovery (S_R) for the labeled compounds only. Express the assessment as a percent recovery interval from $R - 2S_R$ to $R + 2S_R$ for each matrix. For example, if R = 90% and S_R = 10% for analyses of aqueous matrices, the recovery interval would be expressed as 70 to 110%.

9.4.2 Update the accuracy assessment for each labeled compound in each matrix on a regular basis.

9.5 Method blanks – A method blank is analyzed with each sample batch (Section 4.1) to demonstrate freedom from contamination. The matrix for the method blank must be similar to the sample matrix for the batch, e.g., a 250-mL reagent water blank (Section 7.1.3).

9.5.1 Analyze the extract of the method blank.

9.5.2 If 6PPD-Q is found in the blank at a concentration greater than ½ the ML for the 6PPD-Q, or at a concentration greater than one-tenth the concentration in a sample in the extraction batch, whichever is greater, analysis of samples must be halted, and the problem corrected. (The laboratory may adopt more stringent acceptance limits for the method blank, at their discretion.) If the contamination is traceable to the extraction batch, samples affected by the blank must be re-extracted and the extracts re-analyzed. If, however, continued re-testing results in repeated blank contamination, the laboratory must document and report the failures (e.g., as qualifiers on results), unless the failures are not required to be reported as determined by the regulatory/control authority. Results associated with blank contamination for an analyte regulated in a discharge cannot be used to demonstrate regulatory compliance. QC failures do not relieve a discharger or permittee of reporting timely results.

9.6 The specifications contained in this method can be met if the apparatus used is calibrated properly and then maintained in a calibrated state. The standards used for initial calibration (Section 10.4), calibration verification (Sections 10.5 and 13.2), and for initial (Section 9.2.1) and ongoing (Section 13.4) precision and recovery should be prepared from the same source, so that the most precise results will be obtained. A second-source standard is used periodically to verify instrument performance by confirming the calibration standards.

9.7 Laboratory duplicates – A laboratory duplicate analysis is not required for NPDES compliance monitoring. If a particular project specifically requests a laboratory duplicate be performed, then the requestor is responsible for providing an additional container of that sample and must provide their acceptance criterion for the relative percent difference (% RPD) of such laboratory duplicates.

9.8 Depending on specific program requirements, field replicates may be collected to determine the precision of the sampling technique, and spiked samples may be required to determine the accuracy of the analysis for certain projects.

9.9 Matrix spikes/Matrix spike duplicates (MS/MSDs) generally are **not** required for isotope dilution methods because any deleterious effects of the matrix should be evident in the recoveries of the labeled compounds spiked into every sample. If a particular project specifically requests MS/MSD analyses be performed, then the requestor is responsible for providing additional containers of that sample and must provide their acceptance criterion for the relative percent difference (% RPD) of the results of the MS/MSD pair. If MS/MSDs are requested, follow the procedure described below.

9.9.1 An MS/MSD pair consists of two aliquots of a selected field sample spiked with the target analyte at a known concentration, as described in Section 11.1.2. The MS/MSD pair must be processed in the same manner and at the same time as the associated samples (Section 11 – 14).

9.9.2 Calculate the recovery of 6PPD-Q is each aliquot and the relative percent difference (RPD) between the recoveries in the two aliquots. Any MS/MSD recoveries outside of the project-specified control limits must be associated with an OPR analysis that meets the acceptance criteria in Section 13.4, otherwise the poor MS/MSD recoveries shall be attributed to poor laboratory performance.

10.0 Calibration and Standardization

10.1 Mass Calibration

LC/MS/MS – The mass spectrometer must undergo mass calibration to ensure accurate assignments of m/z's by the instrument. This mass calibration must be performed at least annually or as recommended by the instrument manufacturer, whichever is more frequent, to maintain instrument sensitivity and stability. Mass calibration must be repeated on an as-needed basis (e.g., QC failures, ion masses fall outside of the required mass window, major instrument maintenance, or if the instrument is moved). Mass calibration must be performed using the calibration compounds and procedures prescribed by the manufacturer. If the instrument manufacturer suggests more frequent mass calibration (e.g., for higher resolution mass spectrometers), follow the vendor's recommendations.

10.2 Optimizing Multiple Reaction Monitoring (MRM) Conditions – Multiple Reaction Monitoring (MRM) analysis is required to achieve better sensitivity than full-scan analysis. The default parent ions, quantitation ions (Q1), and confirmation (Q2) ions that were monitored during the validation of this method are listed in Table 7 for the target analyte, the EIS, and the NIS.

10.2.1 Prior to the analysis of any samples, including IPR/OPR, blanks, or calibration standards, the MS system must be optimized for each analyte/standard. Mass calibration should be verified when conditions have been optimized, by acquiring a full-scan mass spectrum of the target analyte ions. The target analyte ions should be within approximately 0.2 m/z of the expected mass.

10.2.2 The $[M+H]^+$ ion for each target analyte is optimized by infusing approximately 1.0 µg/mL of each analyte into a tee fitting with eluent flowing at about 0.1 mL/min. With the collision gas turned off, the entrance and exit slits are set per vendor's recommendation to allow the parent ion to pass uninhibited through the second and third quadrupoles to the detector.. Vary the MS parameters (capillary voltage, temperatures, gas flows, etc.) for the parent ions for each analyte until optimal analyte responses are determined.

10.2.3 With the infusion still running, next turn on the collision gas, set the entrance slit to vendor recommended settings to achieve Collision Induced Dissociation (CID) and vary the collision voltage and collision gas to get optimal conditions for each analyte. A compromise setting for the collision gas will have to be made for product ions.

10.2.4 After MS calibration and optimization and LC/MS/MS calibration, the same LC/MS/MS conditions must be used for analysis of all standards, blanks, IPR and OPR standards, and samples.

10.2.5 Mass Calibration Verification

The mass calibration must be verified prior to the analysis of any standards and samples and after each subsequent mass calibration. Each laboratory must follow the instructions for their instrument software to confirm the mass calibration, mass resolution, and peak relative response. In addition to the mass calibration verification performed using a standard specified by the manufacturer, the mass calibration must also be verified with respect to the ion masses monitored by this method.

10.2.5.1 Check the instrument mass resolution to ensure that it is at least unit resolution. Inject a mid-level calibration standard under LC/MS/MS conditions to obtain the retention times of each method analyte. During MS/MS analysis, fragment a small number of selected precursor ion $[M+H]^+$ and choose the most abundant product ion. The product ions (also the quantification and confirmation ions) chosen during method development are listed in Table 4, although these will be instrument dependent. Unit resolution must meet the manufacturer's criteria.

10.2.5.2 Check the mass calibration by measuring the amount of peak drift from the expected masses. If the peak apex has shifted more than approximately 0.2 m/z, recalibrate the mass axis following the manufacturer's instructions.

10.3 LC/MS/MS Instrument and Chromatographic Conditions

10.3.1 The HPLC and MS operating conditions may be optimized for compound separation and sensitivity. The same optimized operating conditions must be used for the analysis of all standards, blanks, IPR and OPR standards, and field samples. The conditions below are a suggested starting point and may need to be adjusted for the specific HPLC column and LC/MS/MS system employed.

Example HPLC Conditions (Shimadzu HPLC)

Column	Phenomenex Kinetex C18 3.5 µm, 3.6 mm x 100mm				
Column Temperature	45 °C				
Mobile Phase Composition	A = 0.2% Formic acid in Water B = Acetonitrile				
	Time (min.)	%A	%B	Curve	Flow Rate mL/min.
	0.0	90	10	0	0.60
	1.0	90	10	0	0.60
	3.0	45	55	0	0.60
Gradient Program	6.0	1	99	0	0.60
	8.0	1	99	0	0.60
	8.50	90	10	0	0.60
	9.0	90	10	0	0.60
	Maximum Pressure limit = 7,500 psi				
Injection Size	20 µL (fixed amount throughout the sequence)				
Run Time	10.0 minutes				

Recommended Mass Spectrometer Interface Settings

MS Interface Mode	ESI Positive Ion
Ion Spray Voltage	5.5 kV
Desolvation Temp	600 °C
Curtain Gas (nitrogen) Flow	305 psi
Collision Gas (nitrogen) Flow	58 psi

Example Mass Spectrometer Scan Settings

Compound	Reaction (MRM)	Accumulation Time (sec)	Declustering Potential (V)	EP (V)	Collision Energy (V)	CXP (V)	Typical RT (Min)
6PPD-Q	299.2 > 215.1	0.1	22	10	25	9	7.2
6PPD-Q	299.2 > 241.1	0.1	100	10	38	9	7.2
D_5-6PPD-Q	304.2>220.1	0.1	106	10	23	9	7.2
$^{13}C_6$-6PPD-Q	305.2 > 221.1	0.1	106	10	23	9	7.2

CXP = Collision cell exit potential
EP = Entrance Potential

10.3.2 Retention Time Calibration

10.3.2.1 Inject solution(s) containing the target analyte and the EIS and NIS compounds to determine their retention times. The laboratory may want to inject analytes/ compounds separately the first time they perform the calibration. The target compound will elute slightly before or with the labeled analog. Store the retention time (RT) for each compound in the data system.

10.3.2.2 Once RT windows have been confirmed for the target analyte, EIS, and NIS compound, then once per ICAL and at the beginning of the analytical sequence, the position of the target analyte, EIS, and NIS peaks shall be set using the midpoint standard of the ICAL curve when ICAL is performed. When an ICAL is not performed, the initial VER retention times or the midpoint standard of the ICAL curve can be used to establish the RT window position.

10.3.2.3 The RTs for the target analyte, EIS, and NIS compounds must fall within 0.4 minutes of the predicted retention times from the midpoint standard of the

ICAL or initial daily VER, whichever was used to establish the RT window position for the analytical batch.

10.3.2.4 For all target analytes with exact corresponding isotopically labeled analogs, target analytes must elute within 0.1 minutes of the associated EIS compound. (The laboratory may use relative retention times (RRTs) of the target analytes and their labeled analogs as an alternative, provided that they also develop corresponding RRT acceptance criteria that are at least as stringent as those described here.)

10.4 Initial calibration

Initial calibration is performed using a series of at least six solutions, with at least five of the six calibration standards being within the quantitation range, and with the lowest standard at or below the LOQ. (If a second-order calibration model is used, then one additional concentration is required, with at least six of the seven calibration standards within the quantitation range.) The initial calibration solutions contain the entire suite of EIS and NIS compounds, and target analytes. Calibration is verified with a calibration verification (VER) standard at least once every ten injections of a field sample extract, by analysis of a mid-level calibration solution. Calibration verification uses the mean RRs or RFs determined from the initial calibration to calculate the analyte concentrations in the verification standard.

Note: *Six calibration standards is the minimum number that must be used in the initial calibration; however, the laboratory may use more standards, as long as the criteria in Section 10.3.3.3 can be met.*

Prior to the analysis of samples, and after the mass calibration verification has met all criteria in Sections 10.1.4 and 10.1.7, each LC/MS/MS system must be calibrated at a minimum of six standard concentrations (Section 7.3.4 and Table 4). This method procedure calibrates and quantifies the target analyte using the isotopically labeled compound added to the sample prior to extraction, by isotope dilution.

10.4.1 Initial calibration frequency

Each LC/MS/MS system must be calibrated whenever the laboratory takes an action that changes the chromatographic conditions or might change or affect the initial calibration criteria, or if either the VER or Instrument Sensitivity Check (ISC) acceptance criteria have not been met.

10.4.2 Initial calibration procedure

Prepare calibration standards containing the target compound, EIS, and NIS compounds, at the concentrations described in Table 4. Analyze each calibration standard by injecting 2.0 µL (this volume may be changed to improve performance).

Note: *The same injection volume must be used for all standards, samples, blanks, and QC samples. This chromatographic method should accommodate injections up to 50 uL.*

10.4.3 Initial calibration calculations

10.4.3.1 Instrument sensitivity

Sufficient instrument sensitivity is established if a signal-to-noise ratio ≥ 3:1 for the quantification ions and the confirmation ions for the target analyte, or ≥ 10:1 for the EIS and NIS, which only have a quantification ion, can be achieved when analyzing the lowest concentration standard within the quantitation range that the laboratory includes in its assessment of calibration linearity.

10.4.3.2 Response Ratios (RR) and Response Factors (RF)

The response ratio (RR) for the native compound calibrated by isotope dilution is calculated according to the equation below, separately for each of the calibration standards, using the areas of the quantification ions (Q1) with the m/z shown in Table 4. RR is used for the target analyte.

$$RR = \frac{Area_t \, M_{EIS}}{Area_{EIS} \, M_t}$$

where:

$Area_t$ = The measured area of the Q1 m/z for the target analyte
$Area_{EIS}$ = The measured area at the Q1 m/z for the EIS in the calibration standard
M_{EIS} = The mass of the isotopically the EIS in the calibration standard
M_t = The mass of the target analyte in the calibration standard

A response factor (RF_s) is calculated for the EIS compound in the calibration standard using the equation below.

$$RF_s = \frac{Area_{EIS} \, M_{NIS}}{Area_{NIS} \, M_{EIS}}$$

where:

$Area_{EIS}$ = The measured area of the Q1 m/z for the EIS compound added to the sample before extraction
$Area_{NIS}$ = The measured area at the Q1 m/z for the NIS compound in the calibration standard
M_{NIS} = The mass of the NIS compound in the calibration standard
M_{EIS} = The mass of the EIS compound added to the sample before extraction

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Note: *Other calculation approaches may be used, such as a weighted linear regression or non-linear regression, based on the capability of the data system used by the laboratory. If used, the regression must be weighted inversely proportional to concentration and must not be forced through zero. Analysts should consult their instrument vendor for details on regression calibration models. When using a weighted regression calibration, linearity must be assessed using Option 2 below.*

10.4.3.3 Instrument Linearity

One of the following two approaches must be used to evaluate the linearity of the instrument calibration:

Option 1: Calculate the relative standard deviation (RSD) of the RR or RF values for each target analyte and isotopically labeled compound for

all the initial calibration standards that were analyzed. The RSD must be ≤ 20% to establish instrument linearity.

$$mean\ RR\ or\ RF = \frac{\sum_{i=1}^{n}(RR\ or\ RF)_i}{n}$$

$$SD = \sqrt{\frac{\sum_{i=1}^{n}(RR\ or\ RF_i - \text{mean RR or RF})^2}{n}}$$

$$RSD = \frac{SD}{mean}\ x\ 100$$

where:

$RR\ or\ RF_i$ = RR or RF for calibration standard *i*

n = Number of calibration standards

Option 2: Calculate the relative standard error (RSE) for each target analyte and EIS compound for all the initial calibration standards that were analyzed. The RSE for all target analytes and EIS compounds must be ≤ 20% to establish instrument linearity.

$$RSE = 100 \times \sqrt{\sum_{i=1}^{n} \frac{\left[\frac{x_i' - x_i}{x_i}\right]^2}{n-p}}$$

where,

x_i = Nominal concentration (true value) of each calibration standard

x'_I = Measured concentration of each calibration standard

n = Number of standard levels in the curve

p = Type of curve (2 = linear, 3 = quadratic)

In addition, although not required, it may be useful to compare the actual responses for each standard to the calibration model. Differences outside of a window of 70 – 130% of the modeled concentration may be cause for concern.

Note: *The correlation coefficient, r, and the coefficient of determination, r^2, are no longer considered appropriate metrics for linearity and shall not be used in conjunction with this method.*

10.4.3.4 Non-extracted Internal Standard Area

Each time an initial calibration is performed, use the data from all the initial calibration standards used to meet the linearity test in Section 10.3.3.3 to calculate the mean area response for the NIS compound, using the equation below.

$$Mean\ Area_{NIS} = \frac{\sum Area_{NIS}}{n}$$

where:

$Area_{NIS}$ = Area counts for the NIS

n = The number of ICAL standards (the default value is n = 6). If a different number of standards is used for the ICAL, for example, to increase the calibration range or by dropping a point at either end of the range to meet the linearity criterion, change 6 to match the actual number of standards used.

Record the mean areas for the NIS compound for use in evaluating results for sample analyses (see Section 13.8).

10.4.4 Initial calibration corrective actions

If the instrument sensitivity or the instrument linearity criteria for initial calibration are not met, inspect the system for problems and take corrective actions to achieve the criteria. This may require the preparation and analysis of fresh calibration standards or performing a new initial calibration. All initial calibration criteria must be met before any samples or required blanks are analyzed.

10.5 Calibration Verification (VER)

A VER standard is analyzed prior to the analysis of samples and blanks, at the end of an analytical run sequence containing samples, and after every 10 field samples to determine if the calibration is valid. The exception is after an acceptable initial calibration is run, when 10 samples may be analyzed before a VER is required.

11.0 Sample preparation

This method currently does not require or prescribe to any cleanup other than what might occur during the solid-phase extraction step. Aqueous samples are prepared as described in Section 11.1 and extracted through an SPE cartridge (Section 11.2).

11.1 Aqueous sample preparation

Aqueous samples and QC aliquots are prepared and extracted by either a manual or an automated cartridge-based SPE.

11.1.1 The sample container is weighed before and after extraction to determine the initial volume (assuming a density of 1.0 g/mL).

11.1.2 The $^{13}C_6$-6PPD-Q EIS and 6PPD-Q spike solutions are vortexed prior to use. Add 0.5 mL of the EIS solution (20 ng/mL) to each sample and QC sample. Add 0.5 mL of the 6PPD-Q spike solution to all OPRs, and if MS/MSD analyses are requested, add an appropriate amount to those aliquots to achieve a matrix spike concentration at least 3 to 5 times the background concentration in the original sample.

11.1.3 Seal the sample bottles with screw caps and mix the contents well by inverting and shaking before loading onto the SPE cartridge. Proceed to Section 11.2.

11.2 Solid-Phase Extraction by Manual or Automated Cartridge

Note: *Consult manufacturer's manual for instructions that are specific to the brand and model of the automated cartridge SPE system being used.*

11.2.1 The SPE cartridges (Section 6.10.1, are conditioned as follows in Section 11.2.2 – 11.2.4.

Note: *At no point should the cartridge be allowed to go dry until the final elution step with acetonitrile. The solvent/sample level should be stopped at the top of the column before the next liquid is added for all steps.*

11.2.2 Wash the cartridge with 5.0 mL of acetonitrile.

11.2.3 Wash with 5.0 mL of reagent water (Section 7.1.3), making sure to close the valve when the water reaches the top of the packing material.

11.2.4 Add ~5 mL of reagent water (Section 7.1.3) to each cartridge. If it goes dry, repeat Sections 11.2.2 – 11.2.4.

11.2.5 Samples (including all blanks, OPR, and if used, MS, MSD) are added to the columns. The entire aliquot of the sample is pulled through the cartridge at a flow rate of approximately 10-15 mL/min. Care should be taken not to allow sample to go dry before the completion of the sample loading and rinsing steps.

11.2.6 If the SPE column should plug (flow rate <1 drop per minute) prior to the entire contents of the sample container passing through the column, do the following:

1. Stop adding sample to the reservoir.
2. Return any remaining sample volume back to the original container.
3. Weigh the container and record this weight.
4. Determine the volume of the sample extracted by subtracting the remaining sample weight from the initial weight.
5. Proceed to Section 11.2.9, noting that additional vacuum or pressure might be needed to elute the SPE column.

11.2.7 After the entire sample has been loaded onto the column, rinse the sample bottle with 5 mL of 50:50 methanol:reagent water and pour onto the column reservoir.

11.2.8 After the rinse has completely passed through the cartridge, allow the cartridge to dry under high vacuum (10-15-inch Hg) for at least 5 minutes.

11.2.9 The SPE eluent is collected using 15-mL polypropylene centrifuge tubes as receiving tubes in the SPE manifold.

11.2.10 Sample bottles are rinsed with 5 mL of acetonitrile and the rinsate is transferred to the column reservoir onto the cartridges. Analytes are eluted from the cartridge by pulling the sample through using low vacuum such that the solvent exits the cartridge in a drop-wise fashion. If using a siphon or automated SPE system, the rinse should include the original sample bottle and the flow path.

Note: *If the SPE column became plugged and the steps in Section 11.2.6 were performed, then* ***do not*** *rinse the sample bottle. Any potential loss of analyte that might adhere to the walls of the bottle is a minor concern at that point.*

11.2.11 The step in 11.2.10 is repeated with a second 4-mL aliquot of acetonitrile. The total volume collected should be approximately 9-10 mL.

11.2.12 Proceed to Section 11.3 for final volume.

11.3 Final Extract Volume

11.3.1 Add 0.5 mL of the NIS solution (Section 7.2.5) to the sample extract immediately prior to injection to minimize the possibility of loss by evaporation, adsorption, or reaction. If an

extract is to be reanalyzed and evaporation has occurred, **do not add** additional NIS. Rather, bring the extract back to its previous volume with acetonitrile.

11.3.2 The polypropylene centrifuge tubes are tightly sealed, inverted several times and then vortexed.

11.3.3 Approximately 1.5 mL of the vortexed sample is transferred to a 2.0-mL polypropylene autosampler vial with a clean plastic pipet, or with an automatic pipet/plastic tip.

11.3.4 The autosampler vial is sealed with a polypropylene screw cap. Proceed to Section 12.0.

12.0 Instrumental Analysis

12.1 Analysis is performed on a LC/MS/MS to identify 6PPD-Q and the two labeled compounds based on retention times and MRM transitions and to quantify 6PPD-Q by the isotope dilution technique. The retention times, ions to be acquired for isotope dilution quantification, and MRM transitions to be monitored are listed in Table 4.

12.2 Perform mass calibration (Section 10.1), establish the operating conditions (Sections 10.2 and 10.3), and perform an initial calibration (Section 10.4) at the frequencies described in the sections prior to analyzing samples. Only after the criteria in these sections have been met, may blanks, MDLs, IPRs/OPRs, and samples be analyzed.

12.3 Inject 2-50 µL of the concentrated extract. The volume injected must be identical to the volume used for calibration (Section 10.5). If necessary, extracts are diluted with solvent (Section 14.3) to bring all target responses within the calibration range.

12.4 If samples are to be analyzed immediately after the initial calibration, a recommended analytical run sequence is as follows:

1. Instrument Blank
2. Instrument Sensitivity Check (ISC)
3. Initial calibration (a minimum of 6 calibration standards)
4. Method Blank
5. OPR
6. Up to 10 injections of sample extracts, diluted extracts, laboratory duplicate extracts and MS/MSD extracts (if requested)
7. VER
8. Instrument Blank
9. Up to 10 more injections of sample extracts, diluted extracts, laboratory duplicate extracts and MS/MSD extracts (if requested)
10. VER

12.5 If samples are not analyzed immediately after the initial calibration, then the following routine run sequence is recommended:

1. Instrument Blank
2. Instrument Sensitivity Check (ISC)
3. VER
4. Method Blank
5. OPR

6. Up to 10 injections of sample extracts, diluted extracts, laboratory duplicate extracts and MS/MSD extracts (if requested)
7. VER
8. Instrument Blank
9. Up to 10 more injections of sample extracts, diluted extracts, laboratory duplicate extracts, and MS/MSD extracts (if requested)
10. VER

13.0 Performance Tests

The following performance tests must be successfully completed as part of each routine instrumental analysis shift described in Sections 12.4 and 12.5 above (also see Table 9).

13.1 Instrument Sensitivity Check

The ISC solution in Section 7.2.9 is used to check the instrument sensitivity. The signal-to-noise of the ISC must be greater than or equal to 3:1 for the quantitation and confirmation ions that exist and must meet the ion ratio requirements in Section 14.1.3. If this requirement cannot be met, the problem must be corrected before analyses can proceed. In addition, the measured concentration of 6PPD-Q in the ISC must fall within ± 50% of its nominal concentration. If that requirement cannot be met, analysis must be halted, and the sensitivity of the LC/MS/MS system adjusted before analysis of field or QC samples.

13.2 Calibration Verification (VER)

After a passing instrument sensitivity check (Section 13.1), prior to the analysis of any samples, analyze a mid-level calibration standard (Section 7.3.4). This VER analysis is not required if samples are run immediately following the initial calibration sequence described in Section 12.4).

13.2.1 The calibration is verified by analyzing a VER standard at the beginning of each analytical sequence, every ten samples or less, and at the end of the analytical sequence.

13.2.2 Calculate concentration for each target analyte and EIS compound in the VER using the equation in Section 14.2.

13.2.3 The recovery of target analyte and EIS compound for the VER must be within 70 - 130%.

13.2.4 If the VER criterion in Section 13.2.3 is not met, recalibrate the LC/MS/MS instrument according to Section 10.4 and reanalyze any extracts that were analyzed between the last passing VER and the one that failed with the following exception. If an analyte in the VER failed because of high recovery, but that analyte was not detected in a sample extract, then that sample extract need not be reanalyzed.

13.2.5 Ion abundance ratios

Using the data from the VER standard, compute the ion abundance ratio (IAR) for each target analyte listed with a confirmation ion mass in Table 4, using the equation below. These ion abundance ratios will be used a part of the qualitative identification criteria in Section 14.1.

$$IAR = \frac{Area_{Q1}}{Area_{Q2}}$$

where:

IAR = Ion abundance ratio

$Area_{Q1}$ = The measured area of the Q1 m/z for the analyte in the mid-point calibration standard or daily VER standard, depending on the analyte concentration, as described in Section 14.1.3

$Area_{Q2}$ = The measured area of the Q2 m/z for the analyte in the mid-point calibration standard or daily VER standard, depending on the analyte concentration, as described in Section 14.1.3

Construct an acceptance window for the IAR of each target analyte as 50% to 150% of the IAR in the mid-point calibration standard or VER standard as applicable per Section 14.1.3.

13.3 Retention Times and Resolution

13.3.1 6PPD-Q must elute within ± 0.1 minutes of the EIS $^{13}C_6$-6PPD-Q EIS

13.3.2 The retention times of 6PPD-Q, $^{13}C_6$-6PPD-Q, and D_5-6PPD-Q must be within ± 0.4 minutes of their retention times in the ICAL or VER used to establish the RT windows.

13.4 Ongoing Precision and Recovery (OPR)

13.4.1 Analyze the extract of the OPR prior to analysis of samples from the same batch to ensure the analytical process is under control.

13.4.2 Compute the percent recoveries of 6PPD-Q and $^{13}C_6$-6PPD-Q using the equation below:

$$Recovery\ (\%) = \frac{Concentration\ found\ (ng/L)}{Concentration\ spiked\ (ng/L)}\ x\ 100$$

13.4.3 The acceptance criterion for OPRs is a recovery between 70 – 130%. If 6PPD-Q meets the acceptance criteria, system performance is acceptable, and analysis of blanks and samples may proceed. If, however, the recovery falls outside of the range given, the extraction processes are not being performed properly for the target compound. In this event, correct the problem, reprepare, extract, and clean up the sample batch and repeat the ongoing precision and recovery test.

13.4.4 Update QC charts to form a graphic representation of continued laboratory performance. Develop a statement of laboratory accuracy for the target analyte by calculating the average percent recovery (R) and the standard deviation of percent recovery (SR). Express the accuracy as a recovery interval from R – 2SR to R + 2SR. For example, if R = 95% and SR = 5%, the accuracy is 85 to 105%.

13.5 EIS Recoveries

EIS recoveries for field samples should fall within the recovery acceptance criteria of 25–200%.

13.6 Instrument blank

Prior to the start of the analytical sequence, analyze an instrument blank (acetonitrile only) to ensure no instrument contamination has occurred. In addition, an instrument blank should be

analyzed any time carryover contamination is suspected (i.e., after a sample with high analyte concentrations is analyzed), and when new lots of solvent are purchased.

13.7 Method blank

After the analysis of the instrument blank and prior to the analysis of samples, analyze a method blank. The results must meet the criterion in Section 9.5.

13.8 Non-extracted internal standard (NIS)

Calculate the ratio of the NIS areas from the QC and field samples relative to the mean area of the corresponding NIS in the most recent initial calibration to check for possible bad injections of the NIS solution or loss of instrument sensitivity.

The NIS areas in the field and QC samples must be within 50 to 200% of the mean area in the most recent initial calibration. If the areas are low for all the field samples and QC samples in the batch, it suggests a loss of instrument sensitivity, while low area in only some field or QC samples suggests a possible bad injection or matrix effect.

14.0 Data Analysis and Calculations

14.1 Qualitative determination LC/MS/MS Peak Identification

6PPD-Q and the EIS are identified in a standard, blank, or sample when all the criteria in Sections 14.1.1 through 14.1.4 are met.

14.1.1 Peak responses for 6PPD-Q must be at least three times the background noise level (signal-to-noise ratio [S/N] ≥3:1) and the EIS response must have S/N of at least 10:1. If the S/N ratio is not met due to high background noise, the laboratory must correct the issue (e.g., perform instrument troubleshooting and any necessary maintenance , such as cleaning the ion source, or replacing the LC column) and the instrument must be recalibrated (Section 10). If the S/N ratio is not met, but the background is low, then the analyte is to be considered a non-detect.

14.1.2 Target analyte, EIS compound, and NIS compound RTs must fall within ± 0.4 minutes of the predicted retention times from the midpoint standard of the ICAL or initial daily VER, whichever was used to establish the RT window position for the analytical batch. For all target analytes with exact corresponding isotopically labeled analogs, target analytes must elute within ± 0.1 minutes of the associated EIS.

14.1.3 For concentrations at or above the Minimum Level (ML) or LOQ, the IAR must fall within ± 50% of the IAR observed in the mid-point initial calibration standard. If project-specific requirements involve reporting sample concentrations below the ML or LOQ, the IAR must fall within ± 50% of the IAR observed in the daily VER (see Section 13.2.5).

14.1.4 If the field sample result does not all meet the criteria stated in Sections 14.1.1 through 14.1.3, and all sample preparation avenues (e.g., sample dilution, re-extraction, etc.) have been exhausted, the result must be reported with a data qualifier alerting the data user that the result could not be confirmed because it did not meet the method required criteria and therefore should be considered an estimated value. If the criteria listed above are not met for the standards, the laboratory must stop analysis of samples immediately and correct the issue.

14.2 Quantitative determination

Concentration of 6PPD-Q is determined with respect to the EIS, which is added to the sample prior to extraction. The recovery of the EIS is determined with respect to the NIS using the mean response ratio (RR) or mean response factor (RF) from the most recent multi-level initial calibration (Section 10.4) as follows. For 6PPD-Q:

$$Concentration\ (ng/L\ ) = \frac{Area_{6PPDQ}\ x\ M_{EIS}}{Area_{EIS}\ x\ RR} \times \frac{1}{sample\ volume\ (L)}$$

where:

$Area_{6PPD\text{-}Q}$ = The measured area of the Q1 *m/z* for 6PPD-Q
$Area_{EIS}$ = The measured area at the Q1 *m/z* for the EIS
M_{EIS} = The mass of the EIS added to the sample prior to extraction (nominally 100 ng)
RR = Mean response ratio used to quantify 6PPD-Q by isotope dilution (Section 10.4.3.1)

And for the EIS:

$$Concentration\ (ng/L) = \frac{Area_{EIS}\ M_{NIS}}{Area_{NIS}\ x\ RF} \times \frac{1}{sample\ volume\ (L)}$$

where:

$Area_{EIS}$ = The measured area at the Q1 *m/z* for the EIS
$Area_{NIS}$ = The measured area of the Q1 *m/z* for the NIS
M_{NIS} = The mass of the NIS added to the sample extract
RF = Mean response factor used to quantify the EIS by the NIS (Section 10.4.3.1)

Results for the target compound are recovery corrected by virtue of the use of isotope dilution quantification. EIS concentrations are determined similarly against the NIS and are used as general indicators of overall analytical quality.

14.3 Sample dilutions

14.3.1 If the Q1 area for 6PPD-Q exceeds the calibration range of the system, dilute the sample extract with acetonitrile by the factor necessary to bring the concentration within the calibration range and analyze an aliquot of this diluted extract. If the response for the EIS in the diluted extract meets the S/N and retention time requirements in Sections 14.1.1 and 14.1.2, and the EIS meets the method recovery criteria, then the target compound may be quantified using the EIS response. Adjust the sample-specific MDL and Minimum Level to account for the dilution.

Note: *Given that the NIS is diluted proportionally with the EIS, the method EIS recovery criteria should still be met even when the extract is diluted. If the diluted sample EIS recovery is below the method criteria, but the S:N for the EIS is at least 10:1, the result represents an estimated value that may have some use. For example, some clients may accept this estimated quantity if the result is well above any project criteria, especially if additional sampling is planned for that site.*

If the EIS response in the diluted extract does not meet the S/N and retention time requirements, then the compound cannot be measured reliably by isotope dilution in the diluted extract. In such cases, the laboratory must take a smaller aliquot of any affected aqueous sample and dilute it to 250 mL with reagent water and analyze the diluted aqueous sample. Adjust the calibration range, MDL, and Minimum Level to account for the dilution.

14.3.2 Because of the dilution, the NIS response in the diluted extract is no longer required to be within ±50% of the response (peak area) in the most recent VER.

14.3.3 If the EIS recovery is greater than 200%, re-analyze the extract, at dilution if needed.

14.4 Reporting of results

The data reporting practices described here are focused on Clean Water Act National Pollutant Discharge Elimination System (NPDES) monitoring needs and other Clean Water Act programs and may not be relevant to other uses of the method.

14.4.1 Report results for wastewater samples in ng/L. (Other units may be used if required in a permit.) Report all QC data with the sample results.

14.4.2 Reporting level

Unless specified otherwise by a regulatory authority or in a discharge permit, results for analytes that meet the identification criteria are reported down to the concentration of the ML established by the laboratory through calibration of the instrument (see the glossary for the derivation of the ML). EPA considers the terms "reporting limit," "quantitation limit," "limit of quantitation," and "minimum level of quantitation" to be synonymous.

14.4.2.1 Report a result for each analyte in each field sample or QC standard at or above the ML to 3 significant figures. Report a result for each analyte found in each field sample or QC standard below the ML as "<ML," where ML is the concentration of the analyte at the ML, or as required by the regulatory/control authority or permit. Each laboratory must establish their own MDL and ML for 6PPD-Q as described in Section 9.2.2.

14.4.2.2 Report a result for each analyte in a blank at or above the MDL to 2 significant figures. Report a result for each analyte not found in a blank above the MDL as "<MDL," where MDL is the concentration of the analyte at the MDL, or as required by the regulatory/control authority or permit. Blank subtraction is not allowed, unless explicitly requested or required by a regulatory authority or in a permit. In that case, both the sample result and the blank result must be reported together.

14.4.2.3 Report a result for an analyte found in a sample or extract that has been diluted at the least dilute level at which the area at the quantitation *m/z* is above the ML and within the calibration range) and with EIS recovery within the respective QC acceptance criteria.

14.4.2.4 Report the recovery of the EIS compound for all field samples and QC standards. If a sample extract was diluted and analyzed, report the EIS recoveries from both the original analysis and the analysis of the dilution.

14.4.3 Results from tests performed with an analytical system that is not in control (e.g., that does not meet acceptance criteria for any QC tests in this method) must be documented and reported (e.g., as a qualifier on results), unless the failure is not required to be reported as determined by the regulatory/control authority. Results associated with a QC failure cannot be used to demonstrate Clean Water Act regulatory compliance. QC failures do not relieve a discharger or permittee of reporting timely results. If the holding time would be exceeded

for a reanalysis of the sample, the regulatory/control authority should be consulted for disposition.

15.0 Method Performance

Routine method performance is validated through analysis of matrix-specific reference samples, including IPRs, MDLs, and certified reference materials. Ongoing method performance is monitored through QC samples analyzed alongside samples. The parameters monitored include percent recovery of isotopically labeled compounds, blank concentrations, and native compound recoveries.

Data provided in Section 19 are derived from the single-laboratory validation study (Reference 12) and are only provided as examples for this draft method. These are not requirements and performance will likely vary by laboratory. These data will be updated to reflect the interlaboratory study results in a subsequent revision. Therefore, the interim criteria will change after interlaboratory validation.

Every laboratory performing analyses in support of Clean Water Act compliance monitoring must have an effective quality management system in place (see Section 9.1). Such systems must include assessment of all results against the various QC acceptance limits in each analytical method, but also should include procedures for longer-term internal evaluations of laboratory performance. EPA expects that responsible laboratories will perform such evaluations and develop and apply in-house acceptance criteria, which may differ from the limits listed in this method.

Example method performance data from the single-laboratory validation study are in Tables 5 – 8.

16.0 Pollution Prevention

16.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Many opportunities for pollution prevention exist in laboratory operations. EPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address waste generation. When wastes cannot be reduced feasibly at the source, the Agency recommends recycling as the next best option.

16.2 6PPD-Q and the labeled compounds are used in extremely small amounts and pose little threat to the environment when managed properly. Standards should be prepared in volumes consistent with laboratory use to minimize the disposal of excess volumes of expired standards.

16.3 For information about pollution prevention that may be applied to laboratories and research institutions, consult *Less is Better: Laboratory Chemical Management for Waste Reduction* (Reference 7).

17.0 Waste Management

17.1 The laboratory is responsible for complying with all Federal, State, and local regulations governing waste management, particularly the hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance is also required with any sewage discharge permits and regulations. An overview of requirements can be found in *Environmental Management Guide for Small Laboratories* (Reference 8).

17.2 This method generates solvent waste containing methanol and acetonitrile that must be disposed of in accordance with all federal, state and local regulations. The LC/MS/MS also generates aqueous waste contaminated with methanol and acetonitrile that must be disposed of properly.

17.3 For further information on waste management, consult Less is Better: Guide to Minimizing Waste in Laboratories (Reference 7) and Prudent Practices in the Laboratory: Handling and Management of Chemical Hazards (Reference 9).

18.0 References

1. "NIOSH Pocket Guide to Chemical Hazards," Department of Health and Human Services, Centers for Disease Control, NIOSH, Publication 2005-149, September 2007, NTIS PB-2005-108099.

2. "OSHA Safety and Health Standards, General Industry," OSHA 2206, 29 CFR 1910.

3. "Safety in Academic Chemistry Laboratories," ACS Committee on Chemical Safety, 8th Edition, 2017.

4. "Standard Methods for the Examination of Water and Wastewater," 18th edition and later, American Public Health Association, 1015 15th St, NW, Washington, DC 20005, 1-35: Section 1090 (Safety), 1992 – 2017.

5. https://blog.ansi.org/2020/04/ansi-isea-z87-1-2020-safety-glasses-eye-face/#gref
6. "Guidance for Developing Quality Systems for Environmental Programs," USEPA Office of Environmental Information, Washington DC, 20460, EPA QA/G-1, November 2002.

7. "Less is Better: Guide to Minimizing Waste in Laboratories," American Chemical Society, 2002. Available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street NW, Washington, DC 20036.

8. "Environmental Management Guide for Small Laboratories," USEPA, Small Business Division, Washington DC, EPA 233-B-00-001, May 2000.

9. "Prudent Practices in the Laboratory: Handling and Management of Chemical Hazards," National Research Council (US) Committee on Prudent Practices in the Laboratory, 2011. Available from the National Academies Press, 500 Fifth Street, NW, Washington, DC 20001.

10. "Definition and Procedure for the Determination of the Method Detection Limit," USEPA, Office of Water, Engineering and Analysis Division, Washington DC, EPA 821-R-16-006, December 2016.

11. "Research Summary for the Single-laboratory Validation Study of a Draft EPA LC-MS-MS Isotope Dilution Method for 6PPD-Quinone," Eurofins Environment Testing, 800 Riverside Parkway, West Sacramento, CA, November 2023.

19.0 Tables

Table 1. Analyte Name, Synonyms, and Chemical Abstracts Registry Number

Abbreviation	Synonyms	CAS Number	Aqueous ML[1]
6PPD-Q	2-anilo-5-[(4-methylpentan-2-yl)amino]cyclohexa-2,5-diene-1,4-dione N-(1,3-Dimethylbutyl)-N′-phenyl-*p*-phenylene diamine-quinone 2-[(1,3-Dimethylbutyl)amino]-5-(phenylamino) 2,5-cyclohexadiene-1,4-dione	2754428-18-5	2 ng/L

[1] Minimum level

Table 2. Example Initial Calibration Standard Concentrations

Solution Used	Volume (µL) of solution diluted to 10 mL in Acetonitrile[1]						
	V1	V2	V3	V4	V5	V6	V7
6PPD-Q IM (20 ng/mL)	12.5	25	50	250	500	2500	5000
$^{13}C_6$-6PPD-Q IM (20 ng/mL)	500	500	500	500	500	500	500
D_5-6PPD-Q IM (20 ng/mL)	500	500	500	500	500	500	500
	Concentration of Standard (ng/mL)						
	C1	C2	C3	C4	C5	C6	C7
6PPD-Q	0.025	0.050	0.10	0.50	1.0	5.0	10.0
$^{13}C_6$-6PPD-Q	1.0	1.0	1.0	1.0	1.0	1.0	1.0
D_5-6PPD-Q	1.0	1.0	1.0	1.0	1.0	1.0	1.0

[1] Other volumes and intermediate standard concentrations may be used

Table 3. MRM Transitions

Compound	Reaction (MRM)
6PPD-Q	299.2 > 215.1
6PPD-Q	299.2 > 241.1
$^{13}C_6$-6PPD-Q	305.2 > 221.1
D_5-6PPD-Q	304.2 > 220.1

Table 4. Analyte Ions Monitored, Extracted Internal Standard, and Non-extracted Internal Standard Used for Quantification

Abbreviation	Example Retention Time [1]	Parent Ion Mass	Quantification Ion Mass	Confirmation Ion Mass	Typical Ion Ratio	Quantification Reference Compound
Target Analyte						
6PPD-Q	7.53	299	215	241	1.26	$^{13}C_6$-6PPD-Q
Extracted Internal Standard						
$^{13}C_6$-6PPD-Q	7.52	305	221	NA	NA	D_5-6PPD-Q
Non-Extracted Internal Standard						
D_5-6PPD-Q	7.51	304	220	NA	NA	NA

[1] Times shown are in decimal minute units. Example retention times are based on the instrument operating conditions and column specified in Section 10.2.

Table 5. Example Single-Laboratory IPR Performance Data[1]

IPR Study #	Mean Recovery (%)	RSD (%)
1	89.8	2.0
2	90.0	3.4
3	89.3	3.0
4	88.8	1.8
5	88.8	2.2

[1] See Reference 11 for the derivation of the IPR results. Pending completion of a multi-laboratory validation study, as noted in Section 9.2.1.3, the interim acceptance criteria are a mean recovery of 70-130% and an RSD of less than 20%.

Data for this table are derived from the single-laboratory validation study and are only provided as examples for this draft method. The data will be updated to reflect the interlaboratory study results in a subsequent revision.

Table 6. Example Single-Laboratory OPR Performance Data[1]

n	Mean Recovery (%)	RSD (%)	Range (%)
20	89.3	2.7	85 - 92

[1] See Reference 11 for the derivation of the OPR results. Pending completion of a multi-laboratory validation study, as noted in Section 13.4.3, the interim acceptance criterion is a recovery of 70-130%.

Data for this table are derived from the single-laboratory validation study and are only provided as examples for this draft method. The data will be updated to reflect the interlaboratory study results in a subsequent revision.

Table 7. Example Single-Laboratory Validation Performance Summary for Extracted Internal Standards and Non-Extracted Internal Standards in Stormwater and Surface Water Samples

n	Standard Type	Rec. (%)[1]	RSD (%)	Range (%)[2]
60	EIS	90	5.3	78 - 96
60	NIS	106	6.9	89 - 123

[1] For the NIS data, sample area counts were compared to the average area for the NIS from the initial calibration.
[2] Range reflects the lowest and highest values during the study. Pending completion of a multi-laboratory validation study, as noted in Sections 13.5 and 13.8, the interim acceptance criteria are an EIS recovery of 70-130% and an NIS recovery of 50-200%.

Data for this table are derived from the single-laboratory validation study and are only provided as examples for this draft method. These are not requirements and will likely vary by laboratory. The data will be updated to reflect the interlaboratory study results in a subsequent revision.

Table 8. Pooled MDL_s and ML values from the Single-laboratory Validation Study for 6PPD-Q in Aqueous Samples (ng/L)

Pooled MDL_s[1]	ML[2]
0.43	2.0

[1] Based on three MDL studies.
[2] ML was set at the concentration of the second calibration standard.

Data for this table are derived from the single-laboratory validation study, and are only provided as examples for this draft method. The data will be updated with the pooled MDLs from the interlaboratory study results in a subsequent revision.

Table 9. Summary of Quality Control

Method Reference	Requirement	Specification and Frequency
10.1	Mass Calibration	Annually and on as-needed basis
10.2.5	Mass Calibration Verification	After mass calibration
10.4	Initial Calibration (ICAL)	Minimum 6 calibration standards for linear model and 7 calibration standards for non-linear models
10.3.3 and 13.3	Retention Time (RT) window	After ICAL and at the beginning of analytical sequence
7.2.4, 11.1.2, 13.5	Extracted Internal Standard (EIS)	All CAL standards, batch QC and field samples
7.2.5, 11.3.1, and 13.8	Non-extracted Internal Standard (NIS)	All CAL standards, batch QC and field samples
13.1	Instrument Sensitivity Check (ISC)	At the beginning of analytical sequence
13.2	Calibration Verification (VER)	At the beginning and every 10 samples
13.6	Instrument Blank	At the beginning of analytical sequence and after high standards and high samples
9.5 and 13.7	Method Blank (MB)	One per preparation batch
13.4	Ongoing Precision Recovery (OPR)	One per preparation batch
9.9 and 11.1.2	Matrix Spike (MS/MSD)	One per preparation batch (if requested)

20.0 Glossary

The following definitions and purposes are specific to this method but have been conformed to common usage to the extent possible.

20.1 Units of weight and measure and their abbreviations

20.1.1 Symbols

ºC	degrees Celsius
µg	microgram
µL	microliter
<	less than
≤	less than or equal
>	greater than
%	percent

20.1.2 Alphabetical abbreviations

ESI	Electrospray Ionization
eV	electron Volt
g	gram
L	liter
M	molar
mg	milligram
mL	milliliter
mm	millimeter
m/z	mass-to-charge ratio
ng	nanogram
ppm	parts per million
psi	pounds per square inch
Q1	quantitation ion
Q2	confirmation ion

20.2 Definitions and acronyms (in alphabetical order)

6PPD-Q – N-(1,3-Dimethylbutyl)-N'-phenyl-p-phenylenediamine-quinone or 6PPD-Quinone (also see Table 1)

Analyte – 6PPD-Q, $^{13}C_6$-6PPD-Q, and D_5-6PPD-Q, tested for in this method.

American National Standards Institute (ANSI) – A private nonprofit organization that oversees the development of voluntary consensus standards for products, services, processes, systems, and personnel in the United States.

Calibration standard (CS) – A solution prepared from a secondary standard and/or stock solutions and used to calibrate the response of the instrument.

Calibration verification standard (VER) – The mid-point calibration standard (CS-4) that is used to verify calibration. See Section 10.5

CFR – Code of Federal Regulations

Class A glassware – Volumetric glassware that provides the highest accuracy. Class A volumetric glassware complies with the Class A tolerances defined in ASTM E694, must be permanently labeled as Class A, and is supplied with a serialized certificate of precision.

CWA – Clean Water Act

Extracted Internal Standard (EIS) – An isotopically labeled analog of a target analyte that is structurally identical to a native (unlabeled) analyte. The EIS is added to the sample at the beginning of the sample preparation process and are used to quantify the native target analyte. In this method, the labeled compound $^{13}C_6$-6PPD-Q used to quantify 6PPD-Q by isotope dilution.

IPR – Initial precision and recovery; four aliquots of a reference matrix spiked with the analytes of interest and labeled compounds and analyzed to establish the ability of the laboratory to generate acceptable precision and recovery. An IPR is performed prior to the first time this method is used and any time the method or instrumentation is modified.

Isotope dilution quantitation – A means of determining the concentration of a target analyte by reference to the same compound in which one or more atoms has been replaced with a stable isotope. In this method, 6 carbon atoms in a phenyl ring are replaced with carbon-13 atoms to produce $^{13}C_{12}$- labeled analog to 6PPD-Q. The $^{13}C_{12}$-labeled 6PPD-Q is spiked into each sample and allows identification and correction of the concentration of the native compounds in the analytical process. Likewise, the concentration of $^{13}C_{12}$-labeled 6PPD-Q is determined using a deuterium-labeled analog to 6PPD-Q, which is spiked into each sample just prior to sample analysis.

LC – Liquid Chromatography

LC/MS/MS – Liquid Chromatography Tandem Mass Spectrometry

Limit of Quantitation (LOQ) – The smallest concentration that produces a quantitative result with known and recorded precision and bias. The LOQ shall be set at or above the concentration of the lowest initial calibration standard (the lowest calibration standard must fall within the linear range).

Mass accuracy – A metric describing the difference between the measured mass/charge of an ion and the real or exact mass/charge of that ion.

Mass resolution – The ability of separating two narrow mass spectral peaks. This is also referred to as “tuning” or the “tune check” in this method

May – This action, activity, or procedural step is neither required nor prohibited.

May not – This action, activity, or procedural step is prohibited.

Method blank – An aliquot of reagent water that is treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and labeled compounds that are used with samples. The method blank is used to determine if analytes or interferences are present in the laboratory environment, the reagents, or the apparatus.

Method Detection Limit (MDL) – The minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is distinguishable from method blank results (40 CFR 136, Appendix B).

Minimum level of quantitation (ML) – The lowest level at which the entire analytical system must give a recognizable signal and acceptable calibration point for the analyte. The ML represents the lowest concentration at which an analyte can be measured with a known level of confidence. It may be equivalent to the concentration of the lowest calibration standard, assuming that all method-specified sample weights, volumes, and cleanup procedures have been employed. Alternatively, the ML may be established by multiplying the MDL (pooled or unpooled, as appropriate) by 3.18 and rounding the result to the number nearest to 1, 2, or 5 x 10n, where n is zero or an integer (see 68 FR 11770). For the purposes of NPDES compliance monitoring, EPA considers the following terms to be synonymous: "quantitation limit," "reporting limit," and "minimum level."

MS – Mass spectrometer or mass spectrometry

Must – This action, activity, or procedural step is required.

Non-extracted internal standard (NIS) – A labeled compound spiked into the sample extract immediately prior to injection into the instrument and used to quantify the EIS compound. In this method the labeled compound D_5-6PPD-Q is used as the NIS.

OPR – Ongoing precision and recovery standard (OPR); a method blank spiked with known quantities of analytes. The OPR is analyzed exactly like a sample. Its purpose is to assure that the results produced by the laboratory remain within the limits specified in this method for precision and recovery.

Reagent water – Water demonstrated to be free from the analytes of interest and potentially interfering substances at the method detection limit for the analyte.

Relative standard deviation (RSD) – The standard deviation multiplied by a 100 and divided by the mean. Also termed "coefficient of variation."

Relative standard error (RSE) – The standard error multiplied by a 100 and divided by the mean. RSE may be used as an alternative approach to evaluating the calibration model.

RF – Response factor. See Section 10.4.3.1

RR – Relative response. See Section 10.4.3.1

RT – Retention time**;** the time it takes for an analyte or labeled compound to elute from the GC column. Usually measured from the point of injection to the apex of the peak. For asymmetric peaks, it is measured to the center of the mass of the peak.

Should – This action, activity, or procedural step is suggested but not required.

Short-term exposure limit (STEL) – A measurement of exposure over a short period, usually 15 min.

Signal-to-noise ratio (S/N) – The height of the signal as measured from the mean (average) of the noise to the peak maximum divided by the width of the noise.

SPE – Solid-phase extraction; an extraction technique in which an analyte is extracted from an aqueous sample by passage over or through a material capable of reversibly adsorbing the analyte. Also termed liquid-solid extraction.

Stock solution – A solution containing an analyte that is prepared using a reference material traceable to EPA, NIST, or a source that will attest to the purity and authenticity of the reference material.

Tuning – see Mass resolution

Time-weighted average (TWA) – a measurement of average exposure over a certain time period, usually given as 8 hours.