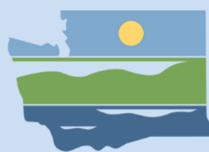

Standard Operating Procedure MEL 730136, Version 2.0

Extraction and Analysis of 6PPD-Quinone by EPA 1634

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Purpose of this Document

The Washington State Department of Ecology develops Standard Operating Procedures (SOPs) to document agency practices related to sampling, field and laboratory analysis, and other aspects of the agency's technical operations.

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Contact Information

Manchester Environmental Laboratory
Environmental Assessment Program
Washington State Department of Ecology
7411 Beach Dr. E.
Port Orchard, WA 98366
Phone: (360) 871-8800

Washington State Department of Ecology – https://ecology.wa.gov

- Headquarters, Olympia 360-407-6000
- Northwest Regional Office, Shoreline 206-594-0000
- Southwest Regional Office, Olympia 360-407-6300
- Central Regional Office, Union Gap 509-575-2490
- Eastern Regional Office, Spokane 509-329-3400

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Environmental Assessment Program

Standard Operating Procedure MEL730136
Version 2.0

Author – Joan Protasio
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QA Approval – Christina Frans
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Laboratory Director Approval – Rob Waldrop
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SOP Revision History

Revision Date	Revision History	Summary of Changes	Sections	Reviser(s)
12/07/2022	New	Not Applicable	All	Joan Protasio
3/17/2023	1.1	The following changes were made: Section 5.3.5 – added a stipulation that none of the components of a standard solution can be expired Section 6.6.2.1 - added “A minimum frequency of annually.” CAS Registry number added to table A01.	5.3.5 6.6.2.1 Table A01	Christina Frans
5/31/2023	1.2	Added explanation for diluted sample concentration calculation and added on column concentration equations.	7.3.2.3	Christina Frans
5/30/2024	2	Modifications to follow EPA Draft Method 1634 (January 2024)	All	Joan Protasio, Jen Pereira

1.0 Purpose and Scope

1.1 This document is Manchester Environmental Laboratory (MEL) Standard Operating Procedure (SOP) for the preparation and analysis of 6PPD-Quinone in water by EPA Draft Method 1634 (January 2024).

1.2 EPA Draft method 1634 is "performance-based," which means that modifications can be made provided that all performance criteria in the method are met.

2.0 Applicability

2.1 This SOP is applicable for 6PPD-Quinone in water. Other analytes and matrices may be added if they meet the minimum QC requirements as outlined in this document.

2.2 Analyte identifications are confirmed by retention time, a precursor ion, a product quantifier ion, at least 1 product qualifier ion, and the ratio between these two product ions.

3.0 Definitions

3.1 Acronyms

- 3.1.1 Ecology — Washington State Department of Ecology
- 3.1.2 EPA — U.S. Environmental Protection Agency
- 3.1.3 MEL — Manchester Environmental Laboratory
- 3.1.4 CAS — Chemical Abstracts Service Number
- 3.1.5 LIMS — Laboratory Information Management System
- 3.1.6 LLOQ — Lower Level of Quantitation
- 3.1.7 MRL — Method Reporting Limit
- 3.1.8 RPD — Relative Percent Difference
- 3.1.9 RSD — Relative Standard Deviation
- 3.1.10 RF — Response Factor
- 3.1.11 SS — Surrogate Standard
- 3.1.12 EIS — Extracted Internal Standard
- 3.1.13 NIS — Non-extracted Internal Standard
- 3.1.14 LC/HPLC — Liquid Chromatography/High Performance Liquid Chromatograph
- 3.1.15 MS/MS — Mass Spectrometer/Mass Spectrometer (also known as Triple Quadrupole Mass Spectrometer)
- 3.1.16 SPE — Solid Phase Extraction

3.2 Definitions

3.2.1 Analyte: An element, ion, compound, or chemical moiety (pH, alkalinity) which is to be determined. The definition can be expanded to include organisms, e.g., fecal coliform or Klebsiella.

3.2.2 Calibration: The process of establishing the relationship between the response of a measurement system and the concentration of the parameter being measured.

3.2.3 Continuing Calibration Verification Standard (CCV): A quality control (QC) sample analyzed prior to samples to check for acceptable bias in the measurement system. The CCV is usually a midpoint calibration standard that is re-run at an established frequency during the course of an analytical run.

3.2.4 Control limits: Statistical warning and action limits calculated based on control charts. Warning limits are generally set at +/- 2 standard deviations from the mean, action limits at +/- 3 standard deviations from the mean.

3.2.5 Duplicate samples (DUP): Two samples taken from and representative of the same population. The sample and its duplicate are carried through the steps of sampling and analytical procedures in an identical manner. Duplicate samples are used to assess variability of all method activities including sampling and analysis.

3.2.6 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.

3.2.7 Initial Calibration Verification Standard (ICV): A QC sample prepared independently of calibration standards and analyzed along with the samples to check for acceptable bias in the measurement system. The ICV is analyzed prior to the analysis of any samples and is obtained from a second source whenever available.

3.2.8 Initial precision and recovery (IPR): 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.

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

3.2.10 Lower Limit of Quantitation (LLOQ): The lowest point of quantitation, which, in most cases, is the lowest concentration in the calibration curve.

3.2.11 Matrix Spike (MS): A QC sample prepared by adding a known amount of the target analyte(s) to an aliquot of a sample to check for bias due to interference or matrix effects (Ecology, 2004).

3.2.12 Matrix Spike Duplicate (MSD): An additional replicate of the matrix spike sample following the same sample preparation and analytical testing as the original sample. MSDs are used to document the precision and bias of a method for a specific sample matrix.

3.2.13 Method: A formalized group of procedures and techniques for performing an activity (e.g., sampling, chemical analysis, data analysis), systematically presented in the order in which they are to be executed.

3.2.14 Method blank (MB): A blank prepared to represent the sample matrix, prepared and analyzed with a batch of samples. A method blank must contain all reagents used in the preparation of a sample, and the same preparation process is used for the method blank and samples.

3.2.15 Method Detection Limit (MDL): The MDL is defined in 40CFR-136-B as the minimum measured concentration of a substance that can be reported with 99% confidence that the measured concentration is distinguishable from method blank results.

3.2.16 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.

3.2.17 Precision: The extent of random variability among replicate measurements of the same property; a data quality indicator.

3.2.18 Quality assurance (QA): A set of activities designed to establish and document the reliability and usability of measurement data.

3.2.19 Quality Assurance Project Plan (QAPP): A document that describes the objectives of a project, and the processes and activities necessary to develop data that support those objectives.

3.2.20 Quality control (QC): The routine application of measurement and statistical procedures to assess the accuracy of measurement data.

3.2.21 Standard Operating Procedure (SOP): A document which describes in detail a reproducible and repeatable organized activity.

3.2.22 Surrogate: For environmental chemistry, a surrogate is a substance with properties similar to those of the target analyte(s). Surrogates are unlikely to be native to environmental samples. They are added to environmental samples for quality control purposes, to track extraction efficiency and/or measure analyte recovery. Deuterated organic compounds are examples of surrogates commonly used in organic compound analysis.

4.0 Personnel Qualifications/Responsibilities

4.1 The analysis in this method is restricted to use by or under the supervision of chemists experienced in the use of liquid chromatography mass spectrometry/mass spectrometry (LC/MS/MS) and the interpretation of chromatograms and mass spectra.

4.2 Training in this procedure with experienced personnel and completion of the training checklist and IDCs are recommended.

4.3 This analysis is typically performed by a Chemist 3 or Chemist 4.

5.0 Equipment, Reagents, and Supplies

5.1 Equipment

5.1.1 Liquid chromatography triple quadrupole mass spectrometer system (LC-QQQ). This system contains an octopole guide to focus the ions toward quadrupole 1 which is MS1, this is for the precursor ions. The second quadrupole is really a hexapole used as a collision cell. A hexapole is used here because it improves focusing like a quadrupole and ion transmission like an octopole. The third quadrupole is MS2; this is for the product ions.

5.1.2 LC - a system with gradient programming, injection control and interface to a mass spectrometer. Agilent model 1260 or 1290 HPLC system capable of performing gradient adjustments at a constant flow rate or equivalent.

5.1.3 Agilent model 6460 or Ultivo Triple Quadrupole Mass Spectrometer (LC-QQQ) with an electrospray Ion Source using jet stream technology (ESIJT) - capable of scanning from 50 to 300 m/z every 0.5 sec or less or equivalent.

5.1.4 Agilent MassHunter data acquisition and processing system - capable of controlling the LC-QQQ and the continuous acquisition of all mass spectra and ions obtained throughout the duration of the chromatographic program.

5.1.5 Analytical column – Reverse phase LC column 100 mm x 2.1 mm ID with 2.6 um Biphenyl 100 Å packing capable of baseline separation of the target compounds (Phenomenex 00D-4622-AN or equivalent).

5.1.6 Guard column (optional) – ZORBAX Extend C-18 4.6 mm, 1.8 um, UHPLC guard column (Agilent 820750-906)

5.2 Reagents

5.2.1 Milli-Q water – 18 megohms or better, free of organic contaminants.

5.2.2 Methanol - HPLC grade or equivalent.

5.2.3 Acetonitrile- HPLC grade or equivalent.

5.2.4 Hexane- Pesticide grade or equivalent.

5.2.5 Formic Acid – ACS grade or equivalent.

5.2.6 Organic reagent (Acetonitrile with 0.1% Formic Acid) – Add 1mL Formic Acid to a final volume of 1L of Acetonitrile. Reagent can be purchased premade.

5.2.7 Aqueous reagent (Water with 0.1% Formic Acid) – Add 1mL Formic Acid to a final volume of 1L of Milli-Q water. Reagent can be purchased premade.

5.3 Standards

5.3.1 Isotopically Labeled Standards:

5.3.1.1 D5-6PPD-Quinone: HPC Standards 688151 or Cambridge Isotopes DLM-11618. Store according to vendor specifications. This is used as the Non-extracted Internal Standard (NIS).

5.3.1.2 13C6-6PPD-Quinone: Cambridge Isotopes CLM-12293 or equivalent. Store according to vendor specifications. This is used as the Extracted Internal Standard (EIS).

5.3.1.3 13C12-6PPD-Quinone: Cambridge Isotopes CLM-11290 or equivalent. Store according to vendor specifications. This is used as sampling Recovery Standard when requested.

5.3.1.4 EIS Spike: Dilute EIS to 200 ng/mL with Acetonitrile. 100 uL of EIS Spike is added to a sample with a final extract volume of 10 mL.

5.3.1.5 NIS Spike: Dilute NIS to 20 ng/mL with Acetonitrile. 1 uL of IIS Spike is added by the LC autosampler for 10 uL of sample.

5.3.2 6PPD-Quinone Stock: Certified standard stock solutions from certified standard vendors (HPC Standards 688152, Cambridge Isotopes ULM-12288-S, or equivalent). Store according to vendor specifications.

5.3.2.1 6PPD-Quinone Intermediate Stock: Dilute 6PPD-Quinone Stock to 1000 ng/mL with Acetonitrile.

5.3.2.2 Matrix Spike: Dilute 6PPD-Quinone Stock to 200 ng/mL with Acetonitrile.

5.3.2.3 LLOQ Spike: Dilute Matrix Spike to 2.5 ng/mL with Acetonitrile.

5.3.2.4 ICAL Standards: Dilute in acetonitrile the 6PPD-Quinone Intermediate Stock, Matrix Spike, or LLOQ spike to the calibration concentrations and add EIS Spike to a final concentration of 2 ng/mL. The suggested ICAL concentrations are 0.025, 0.05, 0.5, 1, 2, 5, 10, 25, 50, and 100 ng/mL.

5.3.2.5 CCV: Use the equivalent ICAL standard. Suggested concentration is 2 ng/mL.

5.3.2.6 ICV: Prepared the same as the ICAL standard but with a different vendor. Suggested concentration is 2 ng/mL.

5.3.2.7 Instrument Sensitivity Check (ISC): The ISC is the lowest ICAL standard within the quantitation range. Currently, the concentration is at 0.05 ng/mL.

5.3.2.8 Instrument Blank: Acetonitrile with EIS at the concentration of 2 ng/mL.

5.3.3 Standard concentrations can differ from those stated in this SOP. Document all standard preparations in the standards section of the LIMS.

5.3.4 Store certified standard stocks as recommended by the vendor.

5.3.5 All intermediates, spikes, ICAL, ICV, CCV, and ISC standards are stored refrigerated. The maximum expiration is 6 months from the date of preparation or the expiration of the components whichever is shorter.

5.4 Supplies

5.4.1 SPE Cartridge: Waters Oasis HLB 6cc (200mg) SPE cartridge (WAT 106202) or Bakerbond Speedisk H2O-Philic DVB (8072-07) or Phenomenex Strata-XL 100um Polymeric Reversed Phase 100mg /6mL SPE Cartridge (8B-S043) or equivalent

5.4.2 Vacuum manifold: 12 or 24 port Supelco Visiprep or 6 port vacuum manifold & reservoir apparatus for Speedisk or equivalent.

5.4.3 Transfer tubing for HLB 6cc SPE cartridges.

5.4.4 Syringes – assorted sizes for the preparation of standards and spiking to samples.

5.4.5 2mL autosampler vials with crimp-top caps or screw-caps.

5.4.6 15 mL sample vials

5.4.7 Class A volumetric flasks of various sizes.

5.4.8 Filter Aid (optional) - Empore™ Filter Aid 400 (66897-U) or equivalent

5.4.9 Silica Cleanup Cartridges (optional) - Thermo Scientific HyperSep Silica Cartridges 100 mg (03-251-260) or equivalent

6.0 Summary of Method

6.1 This SOP describes procedures for the extraction and the qualitative and quantitative analysis of 6PPD-Quinone by triple quadrupole mass spectrometry.

6.2 This method uses reverse phase high performance liquid chromatographic, electrospray ionization with jet stream technology (ESIJT), and triple quadrupole mass spectrometric (LC-QQQ) conditions. Detection is achieved using positive ESIJT and a triple quadrupole mass spectrometer. Quantitative analysis is performed using Isotopic Dilution.

6.3 250 mL water samples are spiked with isotopically labeled 6PPD-Quinone (EIS). The necessary QC samples are also spiked with the target analyte(s) at this time. The samples are then extracted using SPE.

6.4 Interferences

6.4.1 Method interferences may be caused by contaminants in solvents, reagents, glassware and other sample processing apparatus that lead to discrete artifacts or elevated baselines in liquid chromatograms. All reagents and apparatus must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory method blanks. To minimize interference from sample matrix, this method is best utilized with samples of known matrix and interferences.

6.4.2 Raw LC-MS/MS data from all blanks, samples, and spikes must be evaluated for interferences. Determine if the source of interference is in the preparation and/or cleanup of the samples and take corrective action to eliminate the problem.

6.4.3 Cross contamination may occur when a sample containing a low concentration of analytes is analyzed immediately following a sample containing relatively high concentrations of analytes. After analysis of a sample containing high concentrations of analytes, one or more laboratory method blanks must be analyzed.

6.4.4 Matrix interference may be caused by contaminants that are present in the sample. The extent of matrix interference varies considerably from sample to sample, depending on the source sampled. Positive identifications must be confirmed by retention times, precursor ions, product ions, and product ion ratios. Samples can exhibit matrix suppression so extracting a subsample or dilution of the extract may be necessary to minimize the matrix interference.

6.5 Sample Collection, Preservation, Storage, and Holding Times

6.5.1 Water grab samples are collected in 250 mL amber glass bottles. Conventional sampling practices should be followed.

6.5.2 At this time, no preservative has been established for 6PPD-Quinone. For now, unpreserved samples are used.

6.5.3 Water samples must be stored at a temperature above freezing and up to 6°C from collection until sample preparation.

6.5.4 Water samples must be extracted within 14 days from sample collection.

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

6.6 Calibration and Standardization

6.6.1 Instrument Tune

6.6.1.1 Perform a check tune prior to an initial calibration to monitor the instrument status. The check tune requirements are set by the manufacturer and are noted on the check tune report.

6.6.1.2 If there are Mass Calibration results are out of criteria in the check tune report, check the tune solution and spray nozzle and/or adjust the failing tune parameter in manual tune. Perform another check tune. If the criteria is still not met, then instrument maintenance and/or a full autotune are required.

6.6.1.3 The autotune is performed at least annually or as recommended by the instrument manufacturer, whichever is more frequent.

6.6.1.4 All check tunes are accessible via the MassHunter acquisition software.

6.6.2 Initial Calibration (ICAL)

6.6.2.1 Prepare calibration standards at a minimum of six concentration levels for each analyte of interest (See Section 5.3). Seven calibration standards are needed for quadratic calibrations. The lowest standard represents analyte concentrations at or below the LOQ.

6.6.2.1.1 Initial calibrations are preformed prior to analyzing samples and are repeated as needed when calibration verification (CCV) or Instrument Sensitivity Check (ISC) is no longer within criteria or at a minimum frequency of annually.

6.6.2.2 Analyze each calibration standard using the MassHunter Software. Calculations are performed by the instrument's software. MassHunter Software has many options for calibration curves which may be used. The native unlabeled analytes are calibrated by isotope dilution where the EIS is the associated internal standard. The NIS is the associated internal standard for the EIS. See Appendix B for the internal standard associations.

6.6.2.3 All analytes must meet or exceed one of the following calibration model criteria:

6.6.2.3.1 Average Response Factor:
Minimum 6 ICAL points and %RSD ≤ 20%

Average Response Factor equation: $y = x/RF$

Where y = Instrument Target Concentration/ Instrument IS Concentration
x = Target Response/ IS Response
RF = Average Response Factor

6.6.2.3.2 Linear curve:
Minimum 6 ICAL points and %RSE ≤ 20%

Linear Equation: $y = ax + b$

Where y = Instrument Target Concentration/ Instrument IS Concentration
x = Target Response/ IS Response
a = Slope of the regression line
b = y-intercept of the regression line

6.6.2.3.3 Quadratic curve:
Minimum 7 ICAL points and %RSE ≤ 20%

Quadratic Equation: $y = ax^2 + bx + c$

Where y = Instrument Target Concentration/ Instrument IS Concentration
x = Target Response/ IS Response
a, b, c = quadratic coefficients

6.6.2.4 If the instrument sensitivity or the instrument linearity criteria for the initial calibration are not met, inspect the system for problems and take corrective actions to achieve the criteria. Instrument maintenance may need to be performed or new calibration standards may need to be prepared.

6.6.3 Initial Calibration Verification (ICV)

6.6.3.1 As of EPA Draft Method 1634 (January 2024), the Initial Calibration Verification (ICV) is not a requirement, but it is useful to confirm the standards of the primary source used for calibrations. If a secondary source is available, MEL must evaluate an ICV.

6.6.3.2 The initial calibration curve for each target analyte is verified with a standard from a source different from that used for the initial calibration. This standard must be made using stock standards prepared independently from those used for calibration. Preferably an alternate vendor is used. If an alternate vendor is not available, a different lot number from the same vendor may be used.

6.6.3.3 Analyze the ICV standard directly after calibration.

6.6.3.4 The analyte recoveries should be within 70-130% of their expected concentration. Recoveries outside of those criteria may be cause for concern. Use professional judgement to determine appropriate corrective action for ICV outlier(s).

6.6.3.5 Qualify data as appropriate based on the MEL SOP 730121 for Data Qualification.

6.6.4 Back Calculation (Residuals)

6.6.4.1 Although not explicitly required by EPA Draft Method 1634 (January 2024), back calculations are a useful tool to assess the fit of a curve by comparing the actual responses for each analyte in the standard to calculated response.

6.6.4.2 Re-calculate each ICAL concentration level using the updated calibration curve. The percent difference between the calculated concentration and the expected concentration met for each analyte at that level should not be more than ±30% or ± 50% for the lowest standard used in the curve. Higher percent differences may be cause for concern. Use professional judgement to determine appropriate corrective action for compound concentrations that do not meet the acceptance limits.

6.6.4.3 Qualify data as appropriate based on the MEL SOP 730121 for Data.

6.6.5 Instrument Sensitivity Check (ISC)

6.6.5.1 The instrument sensitivity check solution (See section 5.3) is used to check instrument sensitivity.

6.6.5.2 The signal to noise ratio must be greater than or equal to 3:1 for the quantitation ions and meet ion ratio requirements.

6.6.5.3 The recovery must be within 50-150% of the expected concentration.

6.6.6 Continuing Calibration Verification (CCV or VER).

6.6.6.1 The CCV is a mid-level calibration standard with the same concentration used during the initial calibration.

6.6.6.2 Analyze the CCV standard prior to the analysis of samples and blanks, after every 10 field samples or less, and at the end of an analytical sequence containing samples.

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

6.6.6.4 If a CCV does not meet quality criteria then instrument maintenance, repreparation of the standard, and/or recalibration of the instrument may be needed. Reanalyze any extracts with the failing bracketing CCVs. If the CCV failed with a high recovery and the analyte was not detected, the sample extract does not need to be reanalyzed. On a case-by-case basis, samples associated with CCV(s) not meeting acceptance limits can be reported so long as they are qualified appropriately as per the MEL SOP 730121 for Data Qualification.

7.0 Procedure

7.1 Sample Preparation:

7.1.1 Spike samples and QC samples with EIS spike and matrix spike as needed.

7.1.2 Place a SPE cartridge on the vacuum manifold for each sample and QC.

7.1.3 Filter aid (Optional)

7.1.3.1 Add about 2 grams of filter aid to the SPE cartridge. Filteraid helps prevent clogging of the SPE cartridge. If filter aid is used for a sample, all QC samples in the batch must do the same.

7.1.4 Condition the SPE cartridges by adding about 5 mL of Acetonitrile to each and allow it to flow through at a vacuum flow rate of 2.5 – 3.0 mL/minute.

7.1.5 Then condition with about 10 mL of Milli-Q water and allow it to pass through. Before the cartridge goes dry, load the sample at a vacuum flow rate of 2.5 – 3.0 mL/minute.

7.1.6 Rinse the sample bottle with about 10 mL of Milli-Q water and load the rinse through the SPE cartridge.

7.1.7 (Optional) Rinse the SPE cartridge with about 5 mL of 1:1 Methanol:Water and then 5 mL of Hexane.

7.1.8 Increase the vacuum to maximum for at least 5 minutes to dry the SPE cartridge.

7.1.9 Remove from vacuum and add a 15mL vial under each SPE cartridge to collect eluent.

7.1.10 Add 5mL of Acetonitrile to the sample bottle. Cap and shake well to extract any analytes from the inside glass surface. Add this to the top of the SPE cartridge and elute.

7.1.11 Elute with an additional 5 mL of Acetonitrile.

7.1.12 Bring to a final volume of 10 mL.

7.1.13 Cleanup (optional)

7.1.13.1 After extraction, a cleanup can be used to clean out any matrix from the sample extract before running on the instrument. If a cleanup is done for a sample, all QC samples in the batch must do the same.

7.1.13.2 Silica Gel Cartridges: Place a cleanup cartridge onto an autosampler vial. Load 1 mL of the sample extract through the cleanup cartridge. Discard the cartridge and cap the vial.

7.2 Sample Analysis:

7.2.1 Instrument run setup.

7.2.1.1 Starting the instrument.

7.2.1.1.1 If the system has been turned off, turn on the computer, mass detector, autosampler, pump and degas unit.

7.2.1.1.2 Start Triple Quadrupole (MassHunter) software. Ensure that all systems are communicating and status lights are yellow or green.

7.2.1.1.3 Load the current analysis method.

7.2.1.1.4 If needed, perform routine maintenance. See Appendix D for maintenance information.

7.2.1.2 Run a check tune if running an initial calibration.

7.2.1.2.1 Prior to running an autotune or check tune, let the pump equilibrate for approximately 20 minutes. Check background spectra in tune. Check abundance of ions in the tune. See Section 6.6.1 for more information.

7.2.1.3 Prepare the sample vials for the sequence.

7.2.1.3.1 Transfer samples, batch QC, and necessary QC standards into autosampler vials.

7.2.1.3.2 The NIS Spike standard is added by the autosampler program during the injection sequence. Fill the vial that holds the NIS spike solution with a fresh aliquot as needed.

7.2.1.3.3 Load vials for analysis onto the autosampler tray.

7.2.1.4 Setting up a Worklist.

7.2.1.4.1 Go to the Worklist tab to show the worklist spreadsheet.

7.2.1.4.2 Enter Sample name, Sample position, Comment, Method, and Data file. Other settings in the worklist can just stay at the default setting.

7.2.1.4.3 If the instrument has been idle, add at least 3 conditioning runs to the beginning of the sequence. This helps the retention times stabilize.

7.2.1.4.4 Typical ICAL sequence run:

1. If instrument has been idle, minimum 3 conditioning injections
2. Instrument Blank
3. ICAL Standards (See section 6.6.2)
4. ICV (See section 6.6.3.)

7.2.1.4.5 Typical Sample sequence run:

1. If instrument has been idle, minimum 3 conditioning injections
2. Instrument Blank
3. Instrument Sensitivity Check
4. CCV
5. Method Blank (MB)
6. OPR
7. Samples (Up to 10 injections of sample extracts, diluted extracts, laboratory duplicate extracts and MS/MSD extracts)
8. CCV
9. Instrument Blank
10. Samples (Up to 10 injections of sample extracts, diluted extracts, laboratory duplicate extracts and MS/MSD extracts)

11. CCV

7.2.1.4.6 (Recommended) At the end of the sequence, add 2 solvent rinse runs.

7.2.1.4.7 Run the Worklist.

7.2.2 Process the sample results using the MassHunter Quantitative Analysis.

7.2.2.1 Any samples outside of the criteria outlined in Section 6.6 (Calibration and Standardization) and Section 9.0 (Quality Control and Quality Assurance) may need to be rerun and reanalyzed.

7.2.2.2 Sample Dilutions

7.2.2.2.1 Dilute samples with concentrations exceeding the calibration range to bring the area of the quantitation ion to within the calibration range.

7.2.2.2.2 Since the EIS is also diluted and is used to quantify the target compound, the EIS must meet the qualitative requirements in section 7.2.3.1 and the EIS recovery limits in section 9.10.

7.2.2.2.3 If the EIS response is outside of the qualitative and recovery criteria in the dilution, the target compound cannot be calculated by isotope dilution. If additional sample is available, re-extract the sample using a smaller initial volume. If the sample cannot be re-extracted, the results must be qualified and reported as an estimated value.

7.2.2.2.4 Screening samples: If high concentrations are expected, it may be beneficial to analyze a direct injection or dilution of the sample prior to sample preparation. Depending on the result, a smaller initial volume of sample can be extracted.

7.2.3 Calculations

7.2.3.1 Qualitative Identification of Target Compounds

7.2.3.1.1 Target compound identification is made by precursor and product ions as well as retention time matching. The precursor ions are mass filtered in MS1 then they enter the collision cell where the ions collide. The ions are filtered again in MS2 and then product ions are detected. This process eliminates much interference which aides in compound identification since we are looking for compounds that begin at one mass and are then broken into certain ions with a specific ratio. Sample compound and a current laboratory-generated standard must be present and compared.

7.2.3.1.2 Using available software, search for each target compound in the established retention time window. Examine chromatograms and determine if a positive identification is present.

7.2.3.1.3 Examine baseline and peak integration to insure proper area integration. If the compound is present but not properly integrated, then manually integrate the peak. See SOP 730127 Proper Manual Peak Integration.

7.2.3.1.4 Examine transition and all product ions for confirmation ions to further validate the compound identification.

7.2.3.1.5 If there is evidence of retention time shift, use relative retention to the surrogate or internal standard along with confirming ions to validate the identification.

7.2.3.1.6 Technical Acceptance Criteria are determined by qualitative analysis of ion retention times, transition ions (precursor and product ions), chromatography, and ion abundance ratios.

7.2.3.1.7 Signal-to-Noise:

Peak responses for target analytes must be at least three times the background noise level (signal-to-noise ratio S/N ≥3:1) and the EIS and NIS response must have S/N of at least 10:1.

7.2.3.1.8 Retention Time:

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 CCV, whichever was used to establish the RT window position for the analytical batch.

7.2.3.1.9 Relative Retention Time:

For all target analytes with exact corresponding isotopically labeled analogs, target analytes must elute within 0.1 minutes of the associated EIS compound.

Use professional judgment when there are retention time shifts. Document when reporting results outside of criteria including rationale.

7.2.3.1.10 Ion Abundance Ratio (IAR):

The Ion Abundance Ratio is calculated according to the equation below.

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

Where:

IAR = Ion Abundance Ratio
$Area_{Q1}$ = Area of Q1 (quantitation ion)
$Area_{Q2}$ = Area of Q2 (qualifier ion)

The acceptance window for the IAR of each target is 50%-150% of the IAR of the mid-point calibration standard.

7.2.3.2 Quantitative analysis of target analytes:

7.2.3.2.1 When a compound has been identified, the quantification of that compound is based on the integrated abundance from the primary product ion (also called the quantifying ion). The initial calibration (see Section 6.6.2) is used for the determination of the extract concentration.

7.2.3.2.2 Sample Dilutions

As this is an isotope dilution method, calculation of the on column concentration when a sample is diluted is taken into account by the response of the extracted internal standard. The EIS is added to the sample prior to extraction therefore, it is also diluted by the same factor as all other analytes. A separate dilution factor is not required in the calculation of the target analyte, 6PPD-Q (see equation in Section 7.2.3.2.3). The surrogate compound is calculated using the injected internal standard (IIS) and is not calculated in the same way as 6PPD-Q (see equation in Section 7.2.3.2.4)

If the area of Q1 exceeds the calibration range, dilute the sample extract to bring the concentration to within the calibration range. The EIS must meet the S/N criteria in 7.2.3.1 in the diluted extract.

If the needed dilution causes the EIS to not meet S/N criteria or become not detected, the target analyte cannot be calculated reliably in the diluted extract. If there is additional sample available, re-extract the sample with a lower initial volume. If the sample cannot be re-extracted, dilute to a level where the EIS meets the S/N criteria, and report the results as an estimate. Use professional judgment and document when reporting results outside of criteria.

7.2.3.2.3 For 6PPD-Q:

$$C_I = \frac{(\mathrm{Area_n})(M_{EIS})}{(\mathrm{Area_{EIS}})(\overline{RF})}$$

Where:
C_I = On column Concentration (ng/mL)
$\mathrm{Area_n}$ = The measured area of 6PPD-Q
$\mathrm{Area_{EIS}}$ = The measured area for the EIS
M_{EIS} = The Concentration of the EIS added (ng/mL)
$\overline{RF}$ = Average response factor

7.2.3.2.4 And for the SS analyte:

$$C_I = \frac{(Area_{SS})(M_{IIS})}{(Area_{IIS})(\overline{RF_s})}$$

Where:
C_I = Final Concentration (ng/mL)
$Area_{SS}$ = The measured area of D5-6PPD-Q
$Area_{IIS}$ = The measured area of 13C6-6PPD-Q
M_{IIS} = The concentration of the IIS added (ng/mL)
$\overline{RF_s}$ = Average response factor

7.2.3.3 Calculate the concentration of each identified analyte in the sample as follows:

$$C_F = \frac{C_I(v_F)(D)}{v_I}$$

Where:
C_F = Final Concentration (ng/L)
C_I = On Column Concentration (ng/mL)

V_F = Final Volume of Extract (mL)
D = Dilution Factor (only used for surrogate)
V_I = Initial Volume of Sample (mL)

Results are reported as nanograms/liter (ng/L).

7.2.3.4 Ongoing Precision and Recovery (OPR) recoveries are calculated as follows:

$$Recovery(\%) = \frac{\text{MCSS}}{\text{SCA}} \times 100$$

Where: MCSS = Measured Concentration of Spiked Sample

SCA = Spike Concentration Added

7.2.3.5 If an Ongoing Precision and Recovery (OPR) and an Ongoing Precision and Recovery Duplicate pair was analyzed, calculate the Relative Percent Difference (RPD) of each compound as follows:

$$\text{RPD}=\left[\frac{|\text{OPR-OPRD}|}{(\text{OPR+OPRD})/2}\right]\times 100$$

Where: OPR = OPR Recovery

OPRD = OPR Duplicate Recovery

7.2.3.6 Matrix Spike (MS) recoveries are calculated as follows:

$$\text{MSR}=\left[\frac{\text{MCSS-MSSC}}{\text{SCA}}\right]\times 100$$

Where: MCSS = Measured Concentration of Spiked Sample

MSSC = Measured Source Sample Concentration

SCA = Spike Concentration Added

MSR% = Matrix Spike Recovery %

7.2.3.7 If a Matrix Spike and Matrix Spike Duplicate (MS/MSD) pair was analyzed, calculate the RPD of each compound as follows:

$$\text{RPD}=\left[\frac{|\text{MSR-MSDR}|}{(\text{MSR+MSDR})/2}\right]\times 100$$

Where: MSR = Matrix Spike Recovery

MSDR = Matrix Spike Duplicate Recovery

8.0 Records Management

8.1 Retain raw data for 7 years following reporting. The data PDF reports are stored in Element. Raw data are also stored on the instrument computer or in a designated area for 7 years.

8.2 Instrument and/or sample preparation logbooks are kept next to the instrument or with the Chemist performing the analysis.

8.2.1 When the logbooks are full, they are given to the MEL QA Coordinator for filing and secure storage.

8.2.2 Logbooks used to document instrument maintenance or routine documentation of a single piece of equipment are retained for 10 years after the retirement of the instrument/equipment.

8.2.3 Logbooks used to document procedures, such as preparation/extraction, preservation, etc. not tied to specific to equipment, or that are used to document quality control of more than one piece of equipment, are retained for 10 years after submission to QAC for secure storage.

8.3 The LCMSMS Data Review Checklist can be found in MEL's SharePoint page under Organics – Documents – Data Review. The checklist indicates what reports and data must be included with the work order package.

8.4 MassHunter generates the following reports: Sequence Logs, Tune Reports, ICAL Reports, and Quantitation Reports.

8.5 Element generates the following reports: Sample Preparation Batch, Sequence Report, Review Reports, and Final Reports.

8.6 If necessary, the Corrective Action Form (CAF) can be found in MEL's SharePoint page under Organics – Forms.

9.0 Quality Control and Quality Assurance

9.1 Refer to client's QAPP for special QA/QC protocols.

9.2 Samples are qualified following data qualification SOP 730121 guidelines.

9.3 Non-extracted internal standard (NIS):

9.3.1 Each sample run is spiked with the NIS to a concentration of 2 ng/mL by the instrument.

9.3.2 The NIS peak area of the QC and field samples must be within 50-200% compared to the average NIS area of the initial calibration.

9.3.3 Reanalysis is necessary for any sample outside of NIS criteria. If reanalysis confirms this variance in signal, all the analytes associated with that internal standard must be qualified following data qualification SOP 730121 guidelines.

9.3.4 Sample Dilution: Instead of reanalysis at the original LLOQ, reanalysis of the sample at a dilution may minimize the NIS failure by lessening matrix interference. Use professional judgment to decide the best way to report the results.

9.4 Extracted Internal Standard (EIS):

9.4.1 The EIS is added during preparation of the samples and calibration standards.

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

9.5 Instrument Blank:

9.5.1 The instrument blank is analyzed to ensure that no instrument contamination has occurred.

9.5.2 The instrument blank is analyzed prior to the start of the analytical sequence and after any time a sample with high concentrations is expected.

9.5.3 If detections in the instrument blank are found, use professional judgement to determine if instrument maintenance and/or reanalysis of the affected samples is necessary.

9.6 Method Blank:

9.6.1 A Method Blank (MB) must be prepared with each extraction batch of 20 or fewer samples.

9.6.2 The blanks must be free from contamination at a concentration at or below ½ the LLOQ.

9.6.2.1 If the MB fails to meet quality criteria, the analyst determines whether to qualify the data, reanalyze, or re-extract the samples depending on severity of contamination and project objectives. At a minimum, the reanalysis includes the MB and the affected samples.

9.6.2.2 If low reporting limits are not required, the RL may be raised, per client approval.

9.6.2.3 On a case-by-case basis, per client or supervisor approval, samples associated with a MB not meeting acceptance limits can be reported so long as they are addressed in the case narrative and qualified following data qualification SOP 730121 guidelines.

9.7 Laboratory Control Sample (LCS) or Ongoing Precision and Recovery (OPR):

9.7.1 One Ongoing Precision and Recovery (OPR) must be prepared with each extraction batch of 20 or fewer samples.

9.7.2 The OPR recoveries must fall within 70%-130% recovery until statistical control charting the limits.

9.7.3 As of EPA Draft Method 1634 (January 2024), laboratory spike duplicates are not required. If a duplicate was performed, the duplicate RPD should be less than or equal to 40%.

9.7.4 OPR recoveries outside criteria are typically reanalyzed to confirm results. The associated samples may need to be re-extracted if hold time and extra sample volume permits.

9.7.5 On a case-by-case basis, per client or supervisor approval, samples associated with an OPR not meeting acceptance limits can be reported so long as they are addressed in the case narrative and qualified following data qualification SOP 730121 guidelines.

9.8 Matrix Spike/Matrix Spike Duplicate (MS/MSD):

9.8.1 If requested by the client, Matrix Spike Sample and Matrix Spike Sample Duplicate (MS/MSD) are prepared with an extraction batch of 20 or fewer samples.

9.8.2 MS/MSDs generally are not required for isotope dilution methods because any deleterious effects of the matrix are generally evident in the recoveries of the labeled compounds spiked into every sample.

9.8.3 The MS/MSD recoveries should fall within 50%-150% recovery until statistical control charting the limits.

9.8.4 The duplicate RPD should be less than or equal to 40%.

9.8.5 MS/MSD samples are typically not re-prepared or re-analyzed unless obvious preparation or analysis errors occurred or the results are grossly outside criteria.

9.8.6 For results outside of the acceptance limit, qualify the source sample analytes as estimates following data qualification SOP 730121 guidelines. All other anomalies are dealt with on a case-by-case basis and referred to the supervisor.

9.9 Sample Duplicate:

9.9.1 A DUP is analyzed if requested by the client.

9.9.2 The duplicate RPD should be less than or equal to 40%.

9.9.3 DUP samples are typically not re-prepared or re-analyzed unless obvious preparation or analysis errors occurred.

9.9.4 If the RPD fails due to heterogeneity or matrix interference, qualify the failing analytes in the source sample following data qualification SOP 730121 guidelines. All other anomalies are dealt with on a case-by-case basis and referred to the supervisor.

9.10 Surrogates:

9.10.1 The EIS is used as the surrogate. The recovery limits are 25-200%.

9.11 Investigate samples not meeting control limits to determine the root cause of QC failure(s) by checking calculation errors, standard solution degradation, contamination, and instrument performance. If applicable, make the necessary adjustments and reanalyze the sample. If the limits are met, report results from the reanalyzed sample. If the limits are still not met, re-extract if hold time and extra sample volume permits; otherwise, qualify that sample data following data qualification SOP 730121 guidelines.

9.12 Lower Level of Quantitation:

9.12.1 LLOQs are analyzed annually.

9.12.2 See SOP 770044 Method Detection Limits and Lower Limits of Quantitation/Reporting Limits.

9.13 Method Detection Limits

9.13.1 Perform an MDL study for all projects supporting the Clean Water Act or if needed for client specific projects as stated in its QAPP.

9.13.2 See SOP 770044: Method Detection Limits and Lower Limits of Quantitation/Reporting Limits.

9.14 Initial Demonstration of Capability (IDC) or Initial precision and recovery (IPR)

9.14.1 See SOP: 770032 Personnel Training.

9.14.2 IDCs are performed when:

9.14.2.1 There are new personnel responsible for analysis or sample preparation.

9.14.2.2 There is a major change in hardware.

9.14.2.3 There is a major change in sample preparation.

9.14.2.4 There is a major change to the instrument method.

9.14.2.5 New analytes are added to the method.

9.14.3 Blind Sample

9.14.3.1 Performed annually.

9.14.3.2 Another chemist (not the primary chemist for the analysis) prepares an unknown spike sample and sends the concentration information to the QAC.

9.14.3.3 The primary chemist analyzes this spiked sample.

9.14.3.4 The blind sample measured concentration must be within LCS control limits.

9.15 Document the preparation of standards in Element standard preparation module.

9.16 Document the preparation of samples in Element and the preparation logbook.

9.17 Document all instrument problems in the instrument logbook.

9.18 Print and store the sequence in the instrument logbook.

10.0 Safety

10.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound must be treated as a potential health hazard. Accordingly, exposure to these chemicals must be reduced to the lowest possible level.

10.2 The analysts must be familiar with the location and proper use of the fume hoods, eye washes, safety showers, and fire extinguishers. In addition, the analysts must wear protective clothing at all times, including safety glasses, goggles, or a face shield.

10.3 Fume hoods must be utilized whenever possible to avoid potential exposure to organic solvents.

10.4 Work with solvents or chemicals may be performed only when at least one other person is in the area.

10.5 Follow all safety guidelines outlined in the Laboratory Health and Safety Manual and Chemical Hygiene Plan.

10.6 Waste Management/Pollution Prevention

10.6.1 Dispose of laboratory-generated waste and waste sample in accordance with the Manchester Laboratory Dangerous Waste Disposal Manual.

10.7 Ecology [Washington State Department of Ecology]. 2019. Environmental Assessment Program Safety Plan. Washington State Department of Ecology, Olympia.

10.8 MEL [Manchester Environmental Laboratory]. 2016. Lab Users Manual, Ninth Edition. Manchester Environmental Laboratory, Washington State Department of Ecology, Manchester, WA.

10.9 Ecology [Washington State Department of Ecology]. 2019. Quality Assurance at Ecology. Environmental Assessment Program, Washington State Department of Ecology, Olympia. https://ecology.wa.gov/Quality.

11.0 References

11.1 EPA Draft Method 1634: Determination of 6PPD-Quinone in Aqueous Matrices Using Liquid Chromatography with Tandem Mass Spectrometry (LC/MS/MS), January 2024

11.2 EPA SW-846 Update IV Method 8000D: Determinative Chromatographic Separations, Revision 5 March 2018

11.3 40 CFR Part 136, Appendix B, "Definition and Procedure for the Determination of Method Detection Limit", Revision 2, 8/28/17

11.4 40 CFR Part 136.6: Method modifications and analytical requirements.

11.5 40 CFR Part 136.7: Quality assurance and quality control.

11.6 Tian, et al. A Ubiquitous Tire Rubber–Derived Chemical Induces Acute Mortality in Coho Salmon. Science 2021, 371(6525), 185–189.

11.7 Quantitation of Toxic Tire Degradant 6PPD-Quinone in Surface Water, Agilent Technologies, Inc. 2021, 5994-3754EN

11.8 Agilent 6400Series QQQ LC/MS Techniques and Operation Course Number R1893A Volume I Student Manual, Data Acquisition B.02.01; Qual B.2 SP3; Quant B.03.01. 2009 Agilent Technologies, Inc.

11.9 Maintaining Your Agilent LC and LC/MS Systems. Agilent.

11.10 Manchester Environmental Laboratory Quality Assurance Manual, Washington State Department of Ecology.

11.11 Chemical Hygiene Plan, US EPA Region 10 Laboratory.

11.12 Dangerous Waste Disposal Manual, US EPA Region 10 Laboratory and Washington State Dept. of Ecology.

11.13 Laboratory Health and Safety Manual for US EPA Region 10 Laboratory and Washington Department of Ecology Laboratory.

11.14 MEL SOP 730121: Data Qualification of Organic Sample Results.

11.15 MEL SOP 730127: Proper Manual Peak Integration

11.16 MEL SOP 770044: Method Detection Limits and Lower Limits of Quantitation/Reporting Limits

11.17 MEL SOP 770032 SOP for Personnel Training

Appendix A: Compound List and Transitions

Table A01

Analyte	CAS	Quantitation Transition (Q1)	Qualifier Transition (Q2)	Ion Polarity
6PPD-quinone	2754428-18-5	299.1 → 215.1	299.1 → 241.1	Positive
13C6-6PPD-Quinone (EIS/Surrogate)	NULL	305.1 → 221.1	305.1 → 247.1	Positive
D5-6PPD-quinone (NIS)	NULL	304.1 → 220.1	304.1 → 246.1	Positive
13C12-6PPD-Quinone (Recovery Standard)	NULL	311.1 → 253.1	311.1 → 227.1	Positive

Note 1: This table has the current compound list for this method. Depending on demand, compounds may be added or removed. Additional compounds require further requirements (see Section 9).
Note 2: This table has the current transitions used for this analysis. Alternate transitions may be used as long as they are consistent with the ICAL used for calculations.

Appendix B: Retention Times and IS Associations

Table B01

Analyte	Retention Time	Associated IS
6PPD-quinone	6.27	13C6-6PPD-quinone (EIS)
13C6-6PPD-Quinone (EIS/Surrogate)	6.27	D5-6PPD-quinone (NIS)
D5-6PPD-quinone (NIS)	6.27	NA
13C12-6PPD-Quinone	6.27	13C6-6PPD-quinone (EIS)

Note 1: Retention Times are approximate and can change depending on instrument conditions.

Appendix C1: Instrument Method (6460 Model)

Method Name: 6PPDQ_2022A.m

Method Path: C:\MassHunter\methods\CURRENT METHODS\6PPDQ_2022A.m

MS QQQ Mass Spectrometer Model G6460A Settings:

Table C1-01: MS Settings

Parameter	Setting
Ion Source	AJS ESI
Stop Mode	No Limit/As Pump
Time Filter	On
LC->Waste Pre Row	N/A
Tune File	C:\MassHunter\Tune\QQQ\G6460A\atunes.TUNE.XML
Stop Time (min)	No limit
Time Filter Width (min)	0.05
LC->Waste Post Row	N/A

Table C1-02: MS Time Segments

Index	Start Time (min)	Scan Type	Ion Mode	Div Valve	Delta EMV (+)	Store	Cycle Time (ms)	Triggered?	MRM Repeats
1	0.4	Dynamic MRM	ESI+ Agilent Jet Stream	To MS	400	Yes	500	No	3

Table C1-03: MS Scan Segments

Cpd Name	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
6PPD-quinone	299.1	Unit/Enh (6490)	256.1	Unit/Enh (6490)	140	20	4	7.3	3	Positive
6PPD-quinone	299.1	Unit/Enh (6490)	241.1	Unit/Enh (6490)	105	32	4	7.3	3	Positive
6PPD-quinone	299.1	Unit/Enh (6490)	215.1	Unit/Enh (6490)	105	16	4	7.3	3	Positive
6PPD-quinone	299.1	Unit/Enh (6490)	187.1	Unit/Enh (6490)	105	32	4	7.3	3	Positive
6PPD-quinone	299.1	Unit/Enh (6490)	170.1	Unit/Enh (6490)	120	30	4	7.3	3	Positive
D5-6PPDQuinone	304.1	Unit/Enh (6490)	246.1	Unit/Enh (6490)	110	36	4	7.3	3	Positive
D5-6PPDQuinone	304.1	Unit/Enh (6490)	220.1	Unit/Enh (6490)	110	20	4	7.3	3	Positive
13C6-6PPDQuinone	305.1	Unit/Enh (6490)	247.1	Unit/Enh (6490)	110	36	4	7.3	3	Positive
13C6-6PPDQuinone	305.1	Unit/Enh (6490)	221.1	Unit/Enh (6490)	110	20	4	7.3	3	Positive

Table C1-04: MS Scan Parameters

Data Stg	Threshold
Centroid	0

Table C1-05: MS Source Parameters

Parameter	Value (+)	Value (-)
Gas Temp (°C)	300	300
Gas Flow (l/min)	10	10
Nebulizer (psi)	40	40
Sheath Gas Heater	375	375
Sheath Gas Flow	11	11
Capillary (V)	2500	0
V Charging	0	0

Table C1-06: MS Chromatograms

Chrom Type	Label	Offset	Y-Range
TIC	TIC	0	1500000

Sampler Model G1329B:

Table C1-07: Sampler Settings

Parameter	Setting
Auxiliary: Draw Speed	200 µL/min
Auxiliary: Eject Speed	100 µL/min
Auxiliary: Draw Position Offset	5.0 mm
Injection Mode	Standard injection
Injection Volume	5.00 µL
Enable Overlapped Injection	No
Stoptime Mode	As pump/No limit
Posttime Mode	Off
Pretreatment Step 1: Wash	Wash needle in location "Vial 92" 1 times
Pretreatment Step 2: Draw	Draw 1 µL from location "Vial 91" with default speed using default offset
Pretreatment Step 3: Wash	Wash needle in location "Vial 92" 1 times
Pretreatment Step 4: Draw	Draw 10 µL from sample with default speed using default offset
Pretreatment Step 5: Inject	Inject

Note 1: A vial of Methanol is in location “Vial 92” of the sample tray.
Note 2: A vial of the IIS solution is in location “Vial 91” of the sample tray.

Table C1-08: Column Comp. Settings

Parameter	Setting
Valve Position	Position 1 (Port 1 -> 2)
Left Temperature Control Mode	Temperature Set
Left Temperature	40.0 °C
Enable Analysis Left Temperature On	Yes
Enable Analysis Left Temperature Value	0.8 °C
Right Temperature Control Mode	Combined
Enable Analysis Right Temperature On	Yes
Enable Analysis Right Temperature Value	0.8 °C
Stop Time Mode	As pump/injector
Post Time Mode	Off

Binary Pump Model G1312B:

Table C1-09: Binary Pump Settings

Parameter	Setting
Flow	0.400 mL/min
Use Solvent Types	No
Low Pressure Limit	0.00 bar
High Pressure Limit	590.00 bar
Maximum Flow Gradient	100.000 mL/min²
Automatic Stroke Calculation A	Yes
Automatic Stroke Calculation B	Yes
Compressibility Mode A	Compressibility Value Set
Compressibility A	50 10e-6/bar
Compressibility Mode B	Compressibility Value Set
Compressibility B	115 10e-6/bar
Stop Time Mode	Time set
Stop Time	10.5 min
Post Time Mode	Time set
Post Time	4.00 min

Table C1-10: Binary Pump Solvent Composition

Solvent Composition	Channel	Name 1	Selected	Used	Percent
1	A	H2O (0.1% formic)	Ch. 1	Yes	90.0 %
2	B	ACN (0.1% formic)	Ch. 1	Yes	10.0 %

Table C1-11: Binary Pump Timetable

Timetable	Time	A	B	Flow	Pressure
1	0.50 min	90.0 %	10.0 %	0.400 mL/min	590.00 bar
2	5.00 min	15.0 %	85.0 %	0.400 mL/min	590.00 bar
3	10.00 min	0.0 %	100.0 %	0.400 mL/min	590.00 bar
4	10.50 min	0.0 %	100.0 %	0.400 mL/min	590.00 bar

Appendix C2: Instrument Method (Ultivo Model)

Method Name: 2024 6PPDQ Acquisition 13C6 Double Vol.m
Method Path: C:\MassHunter\Methods\10.0\Acquisition\2024 6PPDQ Acquisition 13C6 Double Vol.m

Table C2-01: Multisampler G7167B Settings

Parameter	Setting
Sampling Speed	
Draw Speed	100.0 µL/min
Eject Speed	400.0 µL/min
Wait Time After Drawing	1.2 s
Injection	
Needle Wash Mode	Standard Wash
Injection Volume	5.00 µL
Standard Needle Wash	
Needle Wash Mode	Flush Port
Duration	3 s
High Throughput	
Injection Valve to Bypass for Delay Volume Reduction	No
Sample Flush-Out Factor	5.0
Overlap Injection Enabled	No
Needle Height Position	
Draw Position Offset	0.0 mm
Use Vial/Well Bottom Sensing	Yes
Stoptime Mode	As Pump/No Limit
Posttime Mode	Off
Pretreatment	
Wash	Wash needle in "Vial 1" 1 times
Draw	Draw 1.00 µL from location "Vial 4" with default speed using default offset
Wash	Wash needle in flushport with "S1" for 3 s
Draw	Draw 10.00 µL from sample with default speed using default offset
Inject	Inject

Table C2-02: Binary Pump G7120A Settings

Parameter	Setting
Flow	0.300 mL/min
Use Solvent Types	Yes
Stroke Mode	Synchronized
Low Pressure Limit	0.00 bar
High Pressure Limit	590.00 bar
Max. Flow Ramp Up	100.000 mL/min²
Max. Flow Ramp Down	100.000 mL/min²
Expected Mixer	No check
Automatic Stroke Calculation A	Yes
Stoptime Mode	Time set
Stoptime	7.50 min
Posttime Mode	Time set
Posttime	2.50 min

Table C2-03: Binary Pump Solvent Composition

Solvent Composition	Channel	Name	Percent
1	A	0.1% Formic Acid/Water	95.00 %
2	B	0.1% Formic Acid/ACN	5.00 %

Table C2-04: Binary Pump Timetable

Timetable	Time	A	B	Flow	Pressure
1	Start. Cond. min	95.00 %	5.00 %	0.300 mL/min	590.00 bar
2	1.00 min	95.00 %	5.00 %	0.300 mL/min	590.00 bar
3	4.00 min	50.00 %	50.00 %	0.300 mL/min	590.00 bar
4	7.00 min	0.00 %	100.00 %	0.300 mL/min	590.00 bar
5	8.00 min	0.00 %	100.00 %	0.300 mL/min	590.00 bar

Table C2-05: Column Comp. G7116B Settings

Parameter	Setting
Left Temperature Control	
Temperature Control Mode	Temperature Set
Temperature	40.0 °C
Enable Analysis Left Temperature	
Enable Analysis Left Temperature On	Yes
Enable Analysis Left Temperature Value	0.8 °C
Left Temp. Equilibration Time	0.0 min
Right temperature Control Mode	Combined
Enable Analysis Right Temperature	
Enable Analysis Right Temperature On	Yes
Enable Analysis Right Temperature Value	0.8 °C
Right Temp. Equilibration Time	0.0 min
Enforce column for run enabled	No
Stoptime Mode	As Pump/Injector
Posttime Mode	Off
Timetable	
Ready when front door open	Yes
Position Switch After Run	Do not switch

Table C2-06: QQQ Mass Spectrometer Ultivo Settings

Parameter	Setting
General	
Tune File	atunes.tune
Ion Source	AJS ESI
Time Filter Enabled	On
Time filter window	0.05 min
Stop Mode	By Pump Time
Current Time Segment	
Start Time (min)	0
Scan Type	dMRM

Table C2-07: QQQ Mass Spectrometer Time Segment

Start Time (min)	Scan Type	Ion Mode	Cycle Time (ms)
0	dMRM	AJS ESI	500

Table C2-08: QQQ Mass Spectrometer dMRM Settings

Compound name	Precursor (m/z)	MS1 res	Product (m/z)	MS2 res	RT (min)	RT Window (min)	Fragmentor (V)	CE (V)	Polarity
13C12-6PPD-Quinone	311.1	Unit	268.1	Unit	6.2	3	140	23	+
13C12-6PPD-Quinone	311.1	Unit	253.1	Unit	6.2	3	110	32	+
13C12-6PPD-Quinone	311.1	Unit	227.1	Unit	6.2	3	110	19	+
13C6-6PPD-Quinone	305.1	Unit	247.1	Unit	6.2	3	110	33	+
13C6-6PPD-Quinone	305.1	Unit	221.1	Unit	6.2	3	110	20	+
D5-6PPD-Quinone	304.1	Unit	246.1	Unit	6.2	3	110	33	+
D5-6PPD-Quinone	304.1	Unit	220.1	Unit	6.2	3	110	19	+
6PPD-Quinone	299.1	Unit	256.1	Unit	6.2	3	140	23	+
6PPD-Quinone	299.1	Unit	241.1	Unit	6.2	3	105	33	+
6PPD-Quinone	299.1	Unit	215.1	Unit	6.2	3	105	18	+
6PPD-Quinone	299.1	Unit	187.1	Unit	6.2	3	105	29	+
6PPD-Quinone	299.1	Unit	170.1	Unit	6.2	3	120	38	+

Table C2-09: QQQ Mass Spectrometer Source Settings

Parameter	Positive Value	Negative Value
Gas Temperature (°C)	300	300
Gas Flow (L/min)	10	10
Nebulizer (psi)	40	40
Sheath Gas Temperature (°C)	375	375
Sheath Gas Flow (L/min)	11	11
Capillary Voltage (V)	2500	0
Nozzle Voltage (V)	0	0

Table C2-10: QQQ Mass Spectrometer Timetable Settings

Start Time (min)	Timetable Type	Timetable Value
0 min	Diverter Valve	To MS
8.5 min	Diverter Valve	To Waste

Appendix D: Routine Maintenance

Routine Maintenance Schedule:

Daily Maintenance:
1. Change the wash solvents.
2. Replace NIS vial.
3. Check solvent eluent levels.
4. Check seal wash and needle wash levels.
5. Check column pressure. If it has significantly changed for no reason, reload the method, check for leaks, line kinks, pump bypass valve closure, and solvent eluent levels.

Weekly:
1. Check and drain rough pump reservoir mist filter (Model 6460 only).
2. Run a check tune.
3. Check LC waste buckets and empty as needed.

Monitor:
1. Rough Vac number: (1.62 torr is normal)
2. High Vac number (2.6 to 2.8 X 10^{-5} torr is normal)

As Required:
1. Clean the source and capillary inlet:
    a. If instrument has been on, then set to standby, turn source gas and sheath gas to 0, and cool source before cleaning.
    b. Open ESIJT source door cover, rinse and wipe down interior of the spray chamber with isopropyl alcohol or methanol.
    c. If several analytes lose sensitivity, check capillary cover for discolor, polish the capillary cover with aluminum oxide power and then sonicate in water or a mixture of water and acetonitrile or methanol or isopropyl alcohol.

2. Solvent Eluents:
    a. If necessary, Refill or Change the eluent.
    b. Prime the pumps when eluent is refilled, changed, or the system has been idle.
        i. Open the pump bypass valve and increase flow.
        ii. Increase the % of the solvent bottle being primed. Allow the solvent to flow until no bubbles can be seen going through the lines.
        iii. Decrease flow and close valve after pump is primed.

3. Reboot PC.

4. Check Software Center for computer updates.