Development of a Laboratory Procedure to Evaluate 6PPDQ in Stormwater Sediments

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Abstract

While several validated methods have been developed for analysis of tire-derived contaminants in aqueous matrices, no standardized methods currently exist for the quantification of 6PPD-quinone (6PPDQ) in soil/sediment matrices. A simple, sensitive, and robust analytical method was developed and validated for the quantification of 6PPDQ in soil/sediment matrices. Due to the redox-reactive and thermally labile nature of 6PPDQ, a single non-aggressive extraction procedure was optimized to avoid degradation. A laboratory-prepared reference material was created by uniformly spiking known concentrations of 6PPDQ into Bentonite clay to simulate environmentally relevant distribution in sediment. Extraction was performed using ultrasonic-assisted extraction combined with vortex mixing. The samples were then analyzed by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). Chromatographic separation was achieved using a C18 reverse-phase column under gradient elution conditions, with electrospray ionization (ESI) in positive ion mode and multiple reaction monitoring (MRM) used for compound detection. The method exhibited good linearity ($R^2 > 0.999$) across the constructed matrix-matched calibration range of 0.01–100 ng/mL. A method detection limit study was conducted at two fortification levels (5 ppb and 25 ppb), demonstrating acceptable precision and accuracy according to standard method validation criteria. The method detection limit (MDL) and limit of quantification (LOQ) were determined to be 1.1 ng/g and 5 ng/g, respectively. This method provides a reliable approach for quantifying 6PPDQ in complex soil/sediment matrices, supporting future environmental monitoring, fate, and transport studies, while ultimately contributing to the protection of urban watersheds and informing the development of effective removal strategies for 6PPDQ.

Project Objectives

The goal of this project was to develop and validate a laboratory procedure for the quantitative evaluation of 6PPDQ in stormwater sediment. This chemical, identified in 2020 as a key contributor to coho salmon pre-spawn mortality syndrome (PSM) fatalities, is primarily generated from the oxidation of 6PPD, an additive found in rubber products such as tires. As stormwater runoff carries tire wear particles into receiving water bodies, understanding the fate and transport of 6PPDQ in stormwater sediments is crucial for determining its environmental impact.

The primary objectives of this research project were to:

1. Develop a standard operating procedure (SOP) for the extraction and quantification of 6PPDQ from laboratory-prepared sediment samples, using appropriate standard reference materials (SRM).
2. Establish and validate recovery techniques for 6PPDQ in diverse sediment compositions, employing different extraction methods such as ultrasound-assisted extraction (UAE) and microwave assisted extraction (MAE), with and without clean-up technologies such as solid-phase extraction (SPE).
3. Quantify 6PPDQ in the prepared sediment matrices using LC-MS/MS and evaluate precision, accuracy, and quantitation limits.

The first objective was accomplished by developing an SOP for sediment preparation and 6PPDQ extraction, which involved creating an SRM using different medias. The second objective was addressed by conducting experiments using different extraction and cleanup methods, followed by quantification using LC-MS/MS. The third objective was achieved through method validation and iterative testing to ensure our method for quantifying 6PPDQ in stormwater sediments was robust. This report provides a summary of the methodology, data, and findings relevant to achieving these objectives.

Introduction and Background

6PPD-quinone is an environmental transformation product recently identified as the primary toxicant responsible for acute coho salmon (Oncorhynchus kisutch) pre-spawn mortality syndrome observed in urban streams across the Puget Sound region (Tian et al., 2021). This transformation product originates from the tire-derived antiozonant N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD), which is widely used in rubber products. While the parent compound 6PPD is intentionally added to tires to prevent ozone cracking and increase tire durability, 6PPDQ byproduct is formed when 6PPD reacts with ozone and other oxidants in the environment (Hu et al., 2022). The recent identification of 6PPDQ as a potent aquatic toxicant has spurred urgent interest from regulatory agencies, environmental scientists, and engineers. This discovery has brought renewed attention to the environmental fate, transport pathways, and mitigation strategies necessary to manage 6PPDQ. Given the widespread use of its parent compound 6PPD in vehicle tires, and the consistent detection of 6PPDQ in urban stormwater and receiving waters, understanding the behavior of this transformation product across environmental compartments has risen to the forefront of environmental research, monitoring, and stormwater management efforts.

The ecological ramifications of 6PPDQ contamination are particularly acute for coho salmon. These fish experience mortality at concentrations as low as 20 ng/L, with LC50 values estimated in the range of 95–125 ng/L for juvenile fish (Tian et al., 2021). The phenomenon of coho salmon dying shortly after entering urban streams to spawn despite seemingly suitable water quality conditions has been documented for decades and is now understood to be caused by 6PPDQ. The widespread use of 6PPD in tire manufacturing and its prevalence in roadway runoff result in 6PPDQ contamination of receiving waters during storm events, with concentrations reaching 100s to 1000s of ng/L in highly urbanized watersheds (Klöckner et al., 2021; Johannessen et al., 2021a). Following the discovery of 6PPDQ, research has accelerated to evaluate its occurrence in a variety of environmental compartments including surface waters, stormwater, sediment, road dust, and air particulate matter (Challis et al., 2021; Huang et al., 2021). Environmental detection of 6PPDQ in stormwater-impacted streams, roadside soils, and sediments suggests a complex environmental distribution driven by both hydrologic and atmospheric processes. 6PPDQ's chemical properties such as low solubility, tendency to precipitate, and strong sorption are critical to understanding its environmental persistence and behavior. Despite the recent advances in 6PPDQ detection and toxicity characterization, significant knowledge gaps remain. One critical gap pertains to the fate and transport of 6PPDQ in sediments. Understanding how 6PPDQ interacts with natural soils is essential for developing effective stormwater best management practices (BMPs).

Given the ubiquity of vehicle traffic and the increase of tire use due to urbanization, 6PPDQ is anticipated to be an emerging contaminant of global relevance. Urban runoff is a growing source of non-point pollution worldwide, and 6PPDQ adds to the complex suite of contaminants of concern that can affect aquatic ecosystems and drinking water sources. Regulatory agencies including the U.S. Environmental Protection Agency and state-level bodies such as the Washington Department of Ecology are beginning to assess the risks associated with 6PPDQ and explore mitigation strategies. However, due to the novelty of this contaminant, standardized guidelines for allowable environmental concentrations have yet to be established.

This study focuses on the development of a standard laboratory procedure for measuring 6PPDQ in sediment matrices. Method development and analysis was performed at the City of Tacoma Environmental Services Laboratory at the Center for Urban Waters (CUW). A laboratory-prepared reference material was created by spiking 6PPDQ into clay and thoroughly distributing it throughout the matrix to mimic its natural occurrence in environmental sediment samples. Alternative extraction techniques were ruled out due to the target analytes instability under high thermal conditions. Efforts were concentrated on optimizing a single, minimally invasive, and simple method that would preserve the native chemical structure of 6PPDQ. Quantitative analysis was performed using Agilent 6470 Liquid Chromatography coupled with Triple Quadrupole Mass Spectrometry (LC-MS/MS or LC-QQQ). The outcomes of this research are intended to inform future sediment monitoring efforts.

Materials and Methods

Chemicals

Commercial standard solutions of 6PPDQ and mass labeled injection extractable internal standard solution (EIS/SS) 13C6-6PPD-quinone (100 ug/mL) were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). Mass labeled injection internal standard (NIS/IIS) D5-6PPD-quinone (100 ug/mL) was purchased from HPC Standards (Atlanta, GA). HPLC grade Acetonitrile, Hexane, and Ethyl acetate were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Ultra LCMS grade ammonium acetate and formic acid > 96% purity were purchased from fisher scientific (Bridgewater, NJ).

Standard Reference Material

A major challenge in the development of a robust analytical method for 6PPDQ in environmental sediments was the lack of a suitable certified reference material (CRM). Conventional spiking protocols, in which the analyte is introduced to the matrix and extracted immediately, do not accurately reflect the physicochemical interactions that govern 6PPDQ behavior in natural sediments. Such approaches neglect important sorptive dynamics and fail to simulate real-world partitioning processes within particulate matrices. Due to the absence of a commercially available certified reference material (CRM/SRM) for 6PPDQ in sediment matrices, the laboratory developed one as part of this study. The goal was to create a representative and reproducible matrix that mimics environmental conditions under which 6PPDQ is typically present.

Several media types were initially evaluated to determine their suitability as a base matrix, including diatomaceous earth, Ottawa sand, blend of diatomaceous earth and Ottawa sand at various ratios, and bentonite clay. Full descriptions of recovery efficiencies and matrix ratios are provided in **Table 1.** Each medium was assessed for its physical properties and how closely the matrix represented real-world samples. Bentonite clay was selected due to its fine particle size, high surface area, and sorptive characteristics, which simulates those of real environmental sediment samples.

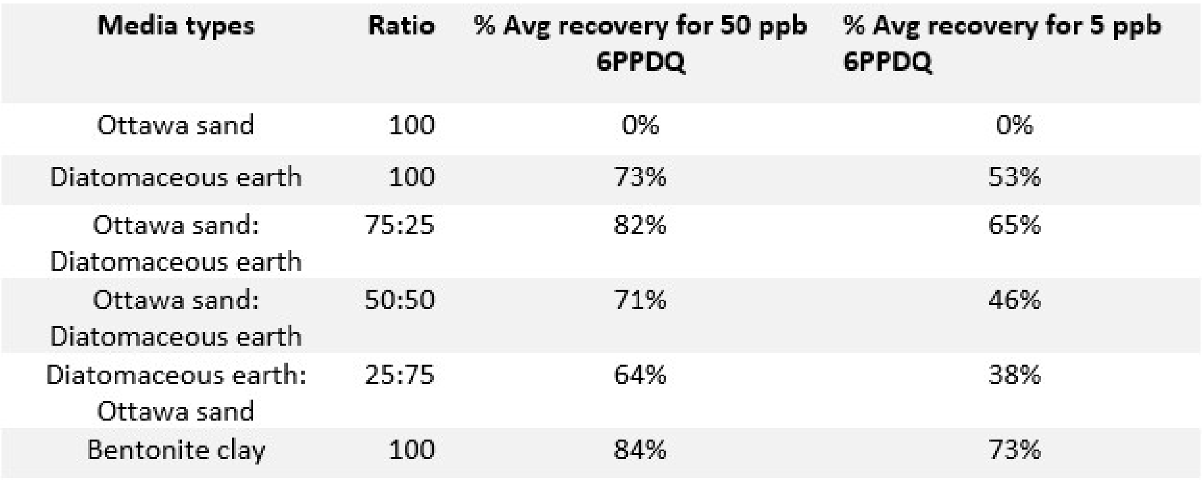


***Table 1**. Media types tested: Ottawa sand, diatomaceous earth, ratios of Ottawa sand & diatomaceous earth, and Bentonite clay*

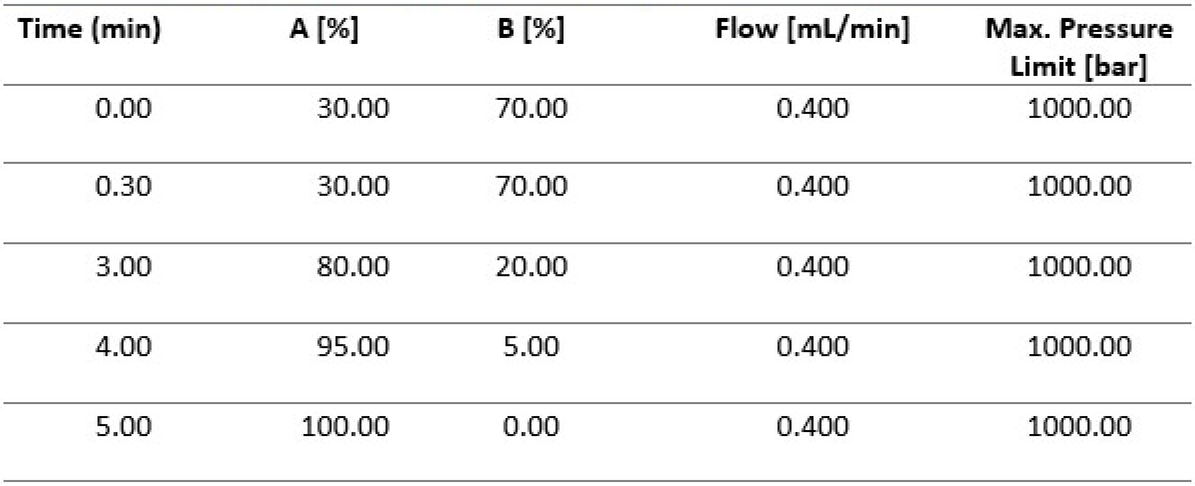
Media types	Ratio	% Avg recovery for 50 ppb 6PPDQ	% Avg recovery for 5 ppb 6PPDQ
Ottawa sand	100	0%	0%
Diatomaceous earth	100	73%	53%
Ottawa sand: Diatomaceous earth	75:25	82%	65%
Ottawa sand: Diatomaceous earth	50:50	71%	46%
Diatomaceous earth: Ottawa sand	25:75	64%	38%
Bentonite clay	100	84%	73%

To prepare the laboratory SRM, bentonite clay (clay) was spiked at a known concentration. The spiking process involved adding an aliquot of 6PPDQ standard solution to 1 g clay, followed by the addition of 2 mL solvent to facilitate uniform distribution of the analyte throughout the matrix. The SRM was vortexed for 15 secs and then dried under a gentle stream of nitrogen. Particular attention was paid to the physicochemical sensitivity of 6PPDQ, which is redox-reactive and prone to degradation under thermal and oxidative conditions. Initial attempts to dry the SRM in a conventional laboratory oven led to near-complete loss of the analyte. This observation highlighted its inherent instability under elevated temperatures. Therefore, thermal methods were excluded, and drying was carried out under nitrogen to preserve the chemical integrity and homogeneity throughout the preparation process. This designed method ensured reduced variability of subsample concentrations drawn from the SRM. From the homogenized mixture, 0.25 g of the SRM sample was weighed out for extraction. For our method detection limit (MDL) study, the method detection limit check sample (MRL) was prepared by spiking 1 g of clay at 5 ppb using the 1000 ppb stock solution. The laboratory control sample (LCS) was similarly prepared at 25 ppb.

Instrument Analysis

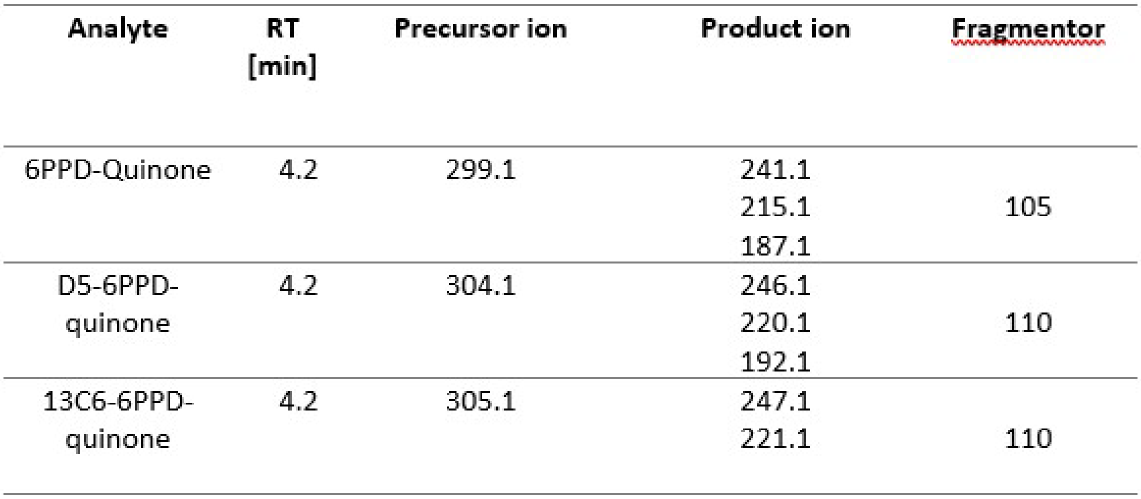
6PPDQ quantification was performed using Agilent's 6470 liquid chromatography triple quadrupole mass spectrometer (LC-MS/MS) with electrospray ionization (ESI) in positive mode. The chromatographic separation was achieved using an Agilent InfinityLab Poroshell 120 EC-C18 reverse-phase column (2.1 x 50 mm, 1.9 um) at 40°C, with an Agilent InfinityLab PFC Delay Column (4.6 × 30 mm) to prevent co-eluting salts and other impurities from contaminating the ion source. The mobile phases consisted of A: acetonitrile with 1% formic acid per 1 L and B: DI water with 1% formic acid and 0.154 g of ammonium acetate per 1 L, both at a flow rate of 0.4 mL/min. Gradient information can be found in **Table 2**. The quantification and confirmation of 6PPDQ was performed using multiple reaction monitoring (MRM) mode. Transitions per analyte and instrument parameters can be found in **Table 3**. We employed an 11-point calibration curve ranging 0.01 – 100 ug/g, with the lowest standard at or below the MRL. Calibration curve regression coefficients (R^2) were > 0.99. The MDL and minimum quantitation limit (MQL) were determined based on signal-to-noise (S/N) ratios of 3:1 and 10:1, respectively.

Table 2. *Gradient elution profile (time, flow rate, composition) and the pressure*



Time (min)	A [%]	B [%]	Flow [mL/min]	Max. Pressure Limit [bar]
0.00	30.00	70.00	0.400	1000.00
0.30	30.00	70.00	0.400	1000.00
3.00	80.00	20.00	0.400	1000.00
4.00	95.00	5.00	0.400	1000.00
5.00	100.00	0.00	0.400	1000.00

Table 3. *Retention time, transition ions, and fragmentor for 6PPDQ, D5-6PPD-quinone, and 13C6-6PPD-quinone*



Analyte	RT [min]	Precursor ion	Product ion	Fragmentor
6PPD-Quinone	4.2	299.1	241.1 215.1 187.1	105
D5-6PPD-quinone	4.2	304.1	246.1 220.1 192.1	110
13C6-6PPD-quinone	4.2	305.1	247.1 221.1	110

Extraction Development

Extraction steps are imperative when quantifying trace-level contaminants like 6PPDQ from sediments. Conventional extraction techniques such as Soxhlet extraction, microwave-assisted extraction (MAE), and supercritical fluid extraction (SFE) are widely used in environmental analysis due to their reliability and efficiency in extracting a wide range of analytes. However, these methods typically operate under elevated temperatures and/or pressures, making them incompatible for analytes that are thermally or chemically labile. 6PPDQ is known to be sensitive to oxidative and thermal degradation under prolonged exposure to heat or reactive supercritical conditions. Soxhlet extraction can require hours of solvent reflux at temperatures approaching the boiling point of the extraction solvent, while MAE and SFE often involve rapid heating or the use of reactive fluid environments that may alter or degrade quinone structures. For this reason, these aggressive techniques were not considered further in our method development, as preserving the chemical integrity of 6PPDQ was paramount. Instead, we employed ultrasound-assisted extraction (UAE), a milder and more selective approach that avoids excessive thermal stress while still offering high extraction efficiency. UAE utilizes ultrasonic waves to induce acoustic cavitation, which is the formation, growth, and violent collapse of microscopic bubbles in the liquid phase. The implosion of these cavitation bubbles generates localized high-pressure and high-temperature microenvironments, along with intense shock waves and liquid microjets. When cavitation occurs near the surface of solid particles, these forces act to disrupt the matrix, improve solvent penetration, and desorb analytes from particle surfaces. In sediment samples, where 6PPDQ is strongly adsorbed onto fine-grained mineral surfaces, sonication facilitates the rapid release of the analyte into the solvent phase without requiring elevated temperatures. The mechanical effects of cavitation enhance mass transfer, minimize extraction time, and reduce solvent consumption.

The efficiency of quinone extraction is governed by multiple factors, including the chemical compatibility of the solvent with the analyte, solvent volume, extraction time, temperature, matrix composition, and the chemical properties of the analyte itself. Among these, we found that the polarity of the solvent significantly affected the extraction efficiency. We found that an optimal solvent for 6PPDQ should balance polarity to match the moderately polar character of the quinone, offer high solubility for the target compound, and possess a low boiling point and latent heat of evaporation to allow post-extraction solvent removal at low temperatures. In this study, we identified that adding a moderately polar solvent to a non-polar solvent efficiently desorbs 6PPDQ from sediment matrices, such as montmorillonite-rich clays and real samples, while minimizing the co-extraction of polar constituents that contribute to matrix interferences in LC-MS/MS analysis.

Procedure

Extraction of 6PPD and 6PPDQ was performed using Ultrasound-Assisted Extraction (UAE). After drying and homogenizing the sediment sample, a 250 mg of aliquot was spiked with 10 uL of the extracted internal standard (EIS/SS) 13C6-6PPD-quinone. The sample was then sequentially extracted with (I) Acetonitrile (II) Acetone, (III) Hexane, and (IV) a ratio of 4.5 mL of

Hexane and 0.5 mL of Ethyl acetate under sonication for 30 minutes. The extracts were separated by centrifuging the mixture at 2000 RPM for 5 minutes. The resulting supernatant was concentrated to near dryness using nitrogen flow and reconstituted using 1 mL of acetonitrile. The 1 mL extract was then spiked with 10 uL of the non-extracted internal standard (IIS/NIS) D5-6PPD-quinone and subjected to analysis.

Solvent Choice

Acetonitrile was initially selected as the extraction solvent because 6PPD and 6PPDQ standards are prepared in it. However, extraction tests using acetonitrile produced inconsistent and low surrogate (EIS/SS) recoveries. While 6PPD and 6PPDQ are stable and soluble in Acetonitrile, it does not effectively penetrate the sediment matrix or disrupt interactions between the analyte and the sediment matrix. To improve extraction efficiency, we chose Acetone, a solvent with higher polarity and lower surface tension (23.0 mN/m for Acetone vs. 28.7 for Acetonitrile)

Acetone's lower surface tension allows it to penetrate fine-grained organic-rich sediments and the clay's porous and high surface area. Surrogate recoveries using Acetone were consistent and significantly higher across replicate control sample extractions. However, when applied to real sediment samples, Acetone co-extracted interfering matrix components, which adversely impacted the recovery of the IIS/NIS. To reduce such interferences, we focused on employing solid-phase extraction (SPE) as a potential solution to address matrix interference. Agilent HLB Bond Elut cartridges were tested in an attempt to remove co-extracted interferences and improve the recovery of IIS/NIS. However, SPE was ineffective in reducing observed matrix interferences, likely due to the high concentration of matrix components that could not be removed by the SPE procedure.

This led us to re-evaluate our approach, and we concluded that the extraction parameters, rather than the clean-up procedure, needed to be optimized. We hypothesized that selective extraction would more effectively reduce matrix effects and improve internal standard recovery. Given the non-polar nature of 6PPDQ, a non-polar solvent such as hexane was evaluated for its potential to selectively extract the target analyte. This modification resulted in a significant improvement in internal standard recovery and reduced ion suppression caused by co-extracted matrix components. The use of Hexane led to more consistent IIS/NIS recovery and reduced ion suppression, likely due to limited solubility of polar matrix constituents in the non-polar solvent.

While Hexane effectively extracts 6PPDQ from environmental sediment samples, its performance was suboptimal when applied to laboratory-prepared clay-based matrices, including LCSs, MRLs, and BLKs. The non-polar nature of Hexane is unable to disrupt the interactions between 6PPDQ and the clay-rich matrix, which is predominantly composed of bentonite, a clay mineral rich in montmorillonite. Montmorillonite has a high surface area and a polar surface charge which can adsorb compounds through electrostatic interactions. Hexane lacks the necessary intermolecular forces, such as hydrogen bonding or dipole interactions, to interact with the clay's polar surface. As a result, non-polar solvents cannot disrupt these adsorption interactions, impeding the extraction of non-polar analytes like 6PPDQ from the clay matrix.

To improve desorption of 6PPDQ from the clay matrix, a more polar co-solvent, Ethyl acetate, was introduced. Systematic optimization of solvent ratios led to the identification of a 45:5 (v/v) Hexane: Ethyl acetate mixture as the most effective combination. This ratio provided sufficient polarity to increase extraction from clay-rich matrices, while preserving the selectivity benefits of Hexane. The modified solvent system yielded consistent and improved recovery of both the target analyte and internal standards across both real sediment and laboratory-prepared samples. Moreover, the use of this binary solvent system improved quantitative precision and minimized variability in signal response across replicate injections during LC-MS/MS analysis.

Quality Control and Quality Assurance (QA\QC)

Our Method Detection Limit (MDL) study consisted of 7 replicates (over a 3-day period) of method blanks and MRLs, and 4 replicates of LCSs showed sufficient EIS/SS and NIS/IIS recoveries. Recovery of the MRLs and the LCSs were all within the 70-130% limits. In the absence of any commercially available Proficiency Tests (PTs), a blind to the analyst (BTTA) study was performed to validate the method. A chemist who did not perform the extraction or the analysis of the BTTA, prepared an LCS at a concentration known only by the chemist and reviewed by the Quality Assurance (QA) Manager. The BTTA sample was then provided for extraction and analysis as though it were a real-world sample. After analysis, the results were compared to known concentration by the QA manager and summarized in a report. The BTTA surrogate and 6PPDQ spiked recoveries were 92% and 91%, respectively.

Prior to the start of the analytical sequence, an instrument blank (IBL) consisting of acetonitrile spiked with EIS & NIS was analyzed to ensure no instrument contamination had occurred. In addition, an instrument blank was analyzed any time carryover contamination is suspected. To assess method performance on the sample matrix, all samples were spiked with the EIS/SS and the IIS/NIS spiking solution. The recovery limits for the EIS/SS are 25-200%. If the recovery of the EIS/SS falls outside of these limits, method performance is unacceptable for 6PPDQ in that sample. Additional cleanup procedures must then be employed to attempt to bring the recovery within the normal range or the extracts may be diluted. If after dilution and/or re-extraction any EIS/SS recovery is still outside limits and there is no further sample available for extraction or dilution, the sample is qualified. For target analyte detections where the recovery exceeds the upper limit, the results are qualified as estimated, “J”. Sample non-detects are not qualified. If the recovery is less than the lower recovery limit but greater than 10%, sample results are qualified as estimated “J" for detections, and “UJ” for non-detects. If the recovery is less than 10%, sample results are qualified as “J” for detections and unusable “R” for non-detects. Further information on data qualification can be found in our *5041_6PPD-Quinone Soil Extraction and Analysis by Triple Quadrupole LC-MS-MS SOP*. The IIS/NIS area in the field samples and QC samples must be within 50-200% of the most recent continuing calibration verification (CCV), the most recent CCV must be within 50- 200% of the mean area of the calibration. A CCV standard is analyzed prior to the analysis of samples and blanks, at the end of each an analytical sequence containing samples, and after every 10 field samples. A Low-Level continuing calibration verification (LCV) standard is analyzed prior to the analysis of a CCV. The acceptance range is +/- 50%, signal-to-noise ≥ 3:1 for quantitation and confirmation ions and an ion ratio of ± 50%. If the LCV does not meet quality criteria, analysis must be halted, and the sensitivity of the LC/MS/MS system is adjusted before analysis of field or QC samples.

Matrix Effects

Matrix components in complex sediment samples can interfere with the analytical signal of the target compound — a phenomenon known as matrix effect. Matrix effects were among the most significant analytical challenges we encountered during high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) analysis. These effects arise when co-eluting matrix components such as salts, lipids, organic acids, and other endogenous substances interfere with the ionization of the analyte during the MS/MS analysis. This interference can result in ion suppression, where the analyte signal is reduced due to competing ions, or ion enhancement, which can lead to false quantification. Such matrix-induced interference can be problematic when quantifying trace amounts of analytes in complex samples, such as biological fluids, environmental matrices, or food products.

During solvent extraction of the sediment samples, a wide range of non-target compounds such as humic acids, lipids, and other organic matter were co-extracted. These matrix components can either interfere with the formation of the ion or alter the charge state and the stability of the analyte's ion. During the ionization step, highly polar matrix components can alter the ionization environment by competing for available ionization energy leading to a lower analyte response. For example, compounds that compete for ionization suppress the ionization of the target analyte, leading to an underestimation of the true concentration. Conversely, some matrix constituents enhance ionization efficiency and falsely elevate the signal response, resulting in overestimation. Both effects introduce substantial error, reducing the reliability of the method.

Typically, in LC-MS/MS analysis, IIS/NIS are used to account for matrix-induced signal variability, as their structural similarity to the analyte allows them to undergo comparable ionization behavior under the same conditions. In our study, the recoveries of both the EIS/SS and the IIS/NIS were influenced by co-extracted matrix components. We observed an inverse relationship, wherein low IIS/NIS recovery correlated with high EIS/SS recovery. Matrix effects are influenced by the nature of the matrix, the extraction procedure, and chromatographic separation (e.g., column and mobile phase composition). Several methodological adjustments were examined to minimize matrix interferences. We employed matrix-matched calibration, modified the solvent polarity, and introduced an acetate salt into the mobile phase to stabilize ionization conditions. Additionally, we evaluated solid-phase extraction (SPE) as a clean-up strategy. While SPE is often used to reduce matrix interference by removing co-extracted impurities, our method detection limit (MDL) study demonstrated that this approach did not increase IIS/NIS recovery. These findings suggest that, for 6PPDQ in complex sediment matrices, optimization of the extraction solvent system was more effective than post-extraction clean-up.

Usually, implementing rigorous sample clean-up, such as solid-phase extraction, minimizes matrix effects; however, our MDL study showed that SPE does not improve IIS/NIS recovery. The performance of the SPE procedure using Agilent HLB Bond Elut cartridges (500 mg, 6 mL) for the extraction of 6PPDQ from both real sediment samples and laboratory-prepared samples was evaluated. The goal of the method was to reduce matrix interferences and improve IIS/NIS recovery. However, following method implementation and testing, the SPE procedure did not yield the desired improvements in extract cleanliness or analytical performance.

The SPE procedure was carried out as follows:

1. Sediment extracts were re-eluted with 10 mL of ammonium acetate extraction solution and vortexed for 15–20 seconds, followed by 2 minutes of sonication. SPE cartridges were prepared by loosely packing clean silanized glass wool to half the height of the cartridge barrel to prevent sorbent loss.

2. The vacuum manifold was fitted with one SPE cartridge, a reservoir, and a reservoir adaptor per sample. Cartridges were pre-conditioned sequentially with 5.0 mL of acetonitrile, followed by 5.0 mL of deionized water. An additional ~5 mL of DI water was added, and the conditioning was repeated if the cartridge ran dry.

3. Samples were loaded at a controlled flow rate (2.5–3 mL/min) to avoid breakthrough or drying of the sorbent. Following sample loading, the original container was rinsed with 5 mL of ammonium acetate extraction solution and applied to the cartridge. Cartridges were then dried under high vacuum for at least 5 minutes.

4. Analytes were eluted into 15-mL polypropylene centrifuge tubes using 5 mL of acetonitrile under low vacuum, allowing for dropwise collection. A second elution step with 4 mL of acetonitrile was performed, yielding a total elution volume of approximately 9–10 mL.

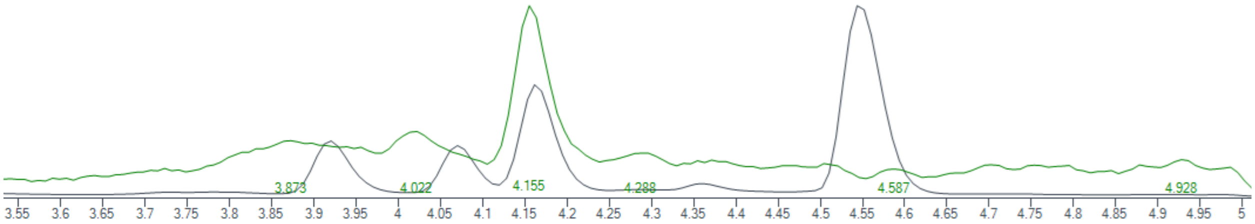


Figure 1. *Chromatogram of Manhole sediment without SPE clean-up. Multiple peaks detected and significant matrix interferences present.*

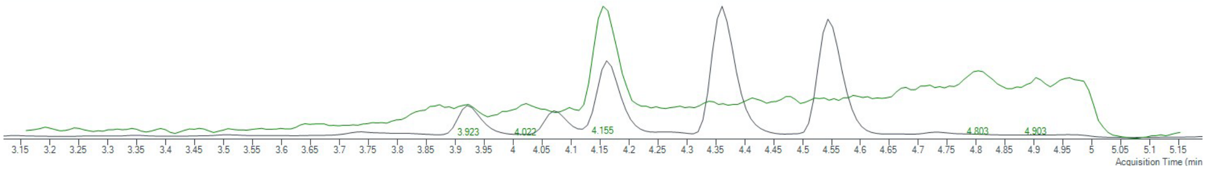


Figure 2. *Chromatogram of Manhole sediment with SPE clean-up. Multiple peaks detected and matrix interferences remain.*

Post-extraction analysis showed that the method did not reduce matrix interferences present in sediment extracts. Instrumental signals showed persistent co-elution of matrix components, and visual inspection of chromatograms indicated no improvement in baseline clarity compared to extracts that were not subjected to SPE (**Figures 1 and 2**). Matrix effects remained substantial, as indicated by variable signal responses and IIS/NIS recoveries. Non-extracted internal standard recovery was not improved following SPE; recoveries were inconsistent and showed no significant difference relative to samples that did not undergo clean-up. This suggests that matrix-derived interferences may have continued to interact with both the target analyte and the internal standard during ionization. Although this SPE method is operationally feasible, it requires further optimization to be analytically useful, as co-eluting interferences were not successfully removed.

HPLC Mobile Phase Optimization

Further optimization strategies were examined as we continued to observe low IIS/NIS recovery caused by matrix effects interfering with ionization efficiency. To improve the analytical performance of our method, we added ammonium acetate into the mobile phase. This modification led to increased recovery of the IIS/NIS leading to more consistent ionization efficiency and improved signal stability at the ESI interface. The inclusion of buffering agents in LC-MS/MS methods is well known to influence various aspects of chromatographic behavior, including retention time, resolution, and signal response. The ionic strength and pH of the buffer can modulate the ionization state of analytes, affecting their interaction with the stationary phase. These buffer characteristics are important when analyzing ionizable compounds. Ammonium acetate was selected due to its ability to control pH, its volatility, and solubility in mixed aqueous-organic mobile phases. This is relevant for phenolic compounds like 6PPDQ, which undergo significant changes in their absorption coefficient. Such compounds contain hydroxyl groups (-OH) attached to an aromatic ring that can gain or lose a proton depending on the pH of the solution, altering its chemical behavior. The acetate buffer used was prepared by dissolving 0.154 g of ammonium acetate (2 mM) in 1 liter of deionized water, and 1 mL of formic acid.

Matrix-matched Calibration

Matrix-matched calibration is an effective approach to minimize matrix effects during quantitative analysis. Conventional calibration was insufficient due to significant signal suppression observed in soil extracts. A matrix-matched calibration curve was constructed using a diluent that reflects the matrix of interest. This strategy was adopted to improve the reliability of quantification as the IIS/NIS did not compensate for variability during analysis. The calibration curve used for our water method was applied and did not yield reliable results. Even with the addition of an IIS/NIS and a buffer, matrix-related variation in results remained, further supporting our decision to use a matrix-matched calibration curve. The diluent was prepared by adding 0.5 g bentonite clay to 10 mL of acetonitrile. This was then used to prepare calibration standards; the calibration curve was created through a linear regression fitting over the range of 0.01 ng/mL to 100 ng/mL.

Method Validation

Limit of Quantitation

The limit of quantitation (LOQ) is defined as the lowest concentration at which the analyte can be reliably quantified with acceptable accuracy and precision. The detection limit corresponds to the smallest amount that results in a signal-to-noise (S/N) ratio of at least 3:1. Based on method performance criteria and laboratory SOPs, the LOQ was set at 5 ng/g. The replicate analyses ($n \geq 7$) of matrix-fortified clay samples spiked at 5 uL/mL over a 3-day period recovered within 70–130% and relative standard deviations below 20%, confirming acceptable method precision and accuracy at the LOQ level. The standard deviation of replicate recoveries was used with the Student’s t-value at the 99% confidence level to calculate the MDLs. A blank-based detection limit (MDLb) was also determined from method blank results. The final verified MDL is the greater of the calculated MDLs or MDLb, which ensures confidence in detection capability in the presence of low-level background variability.

Accuracy

Accuracy was assessed by analyzing matrix-fortified samples at concentrations of 5 ppb and 25 ppb. Percent recovery was calculated by comparing measured concentrations to nominal spike values. Recovery values for both concentrations consistently fell within the acceptable range of 70–130%, demonstrating the method's ability to provide accurate measurements across the specified concentration range.

Precision

Precision was evaluated using replicate analyses ($n = 4$) of LCS samples at a target concentration of 25 ppb over a 3-day period, yielding %RSD values below 15%. These results demonstrate the method’s repeatability and stability over time. Long-term inter-batch precision is monitored through quality control charting of LCS recoveries.

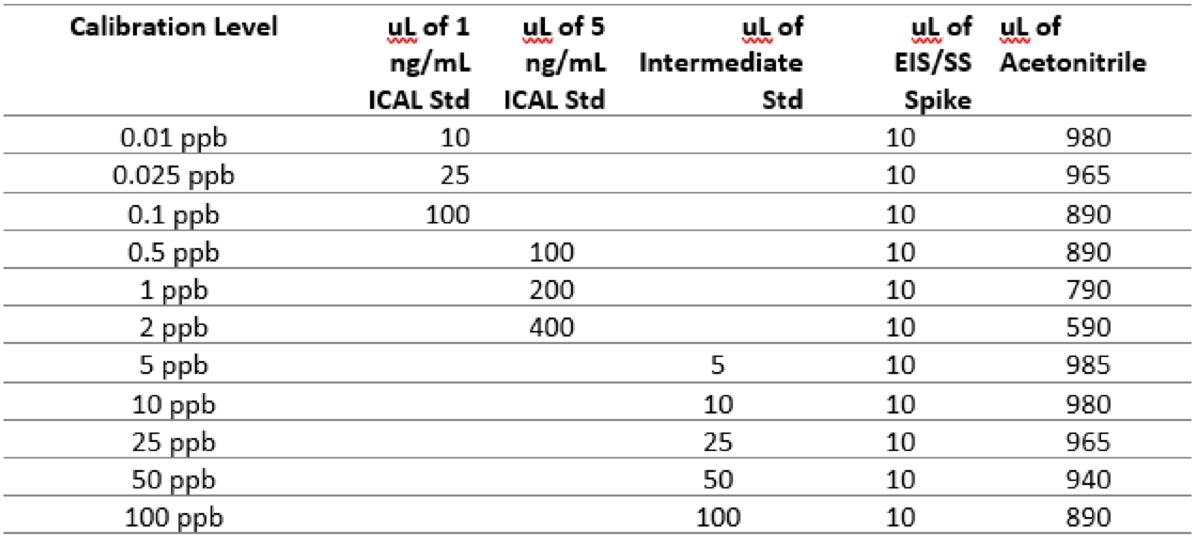
Sensitivity

The sensitivity of the method was evaluated by analyzing blank samples ($n = 7$) over a 3-day period. Additionally, the sensitivity was evaluated based on our ability to detect and quantify the analyte at low concentration levels with acceptable signal-to-noise (S/N) ratios. Analytical sensitivity was demonstrated through successful detection of fortified clay samples at concentrations as low as 5 ppb, with all quantitation ion peaks signal-to-noise (S/N) ratios ≥10:1 and ≥3:1 for confirmation ions at this level, meeting instrumental sensitivity criteria. Sensitivity was further assessed through the verification of the Method Detection Limit (MDL) and Limit of Quantitation (LOQ), both of which were established using replicate fortified samples in a representative matrix. The method demonstrated sufficient responsiveness to enable reliable quantitation at the LOQ, with recoveries and precision (%RSD < 20%) meeting established quality control acceptance criteria. Instrumental sensitivity was confirmed by consistent detection of the lowest calibration standard (0.01 ng/g) and ongoing performance of the low-level calibration verification (LCV) check, which met the required signal-to-noise and ion ratio criteria throughout the validation period.

Linearity

Linearity was assessed using an initial calibration (ICAL) curve with 11 calibration levels ranging from 0.01 to 100 ng/mL (**Table 4**), prepared in matrix-matched solvent. The calibration curve demonstrated a correlation coefficient (R^2) consistently ≥ 0.99, indicating a strong linear relationship between analyte concentration and detector response. Back-calculated concentrations of the standards were within ±20% of their nominal values, confirming the accuracy of the calibration.

Table 4. *Preparation details for each calibration level*



Calibration Level	uL of 1 ng/mL ICAL Std	uL of 5 ng/mL ICAL Std	uL of Intermediate Std	uL of EIS/SS Spike	uL of Acetonitrile
0.01 ppb	10			10	980
0.025 ppb	25			10	965
0.1 ppb	100			10	890
0.5 ppb		100		10	890
1 ppb		200		10	790
2 ppb		400		10	590
5 ppb			5	10	985
10 ppb			10	10	980
25 ppb			25	10	965
50 ppb			50	10	940
100 ppb			100	10	890

The calibration curve was plotted using the ratio of the analyte peak area to the internal standard peak area against the nominal concentration of the calibrators. Linearity was evaluated by determining the equation of line of best fit using least squares linear regression; y= 1.189290x + 0.015043 (**Figure 3)**.

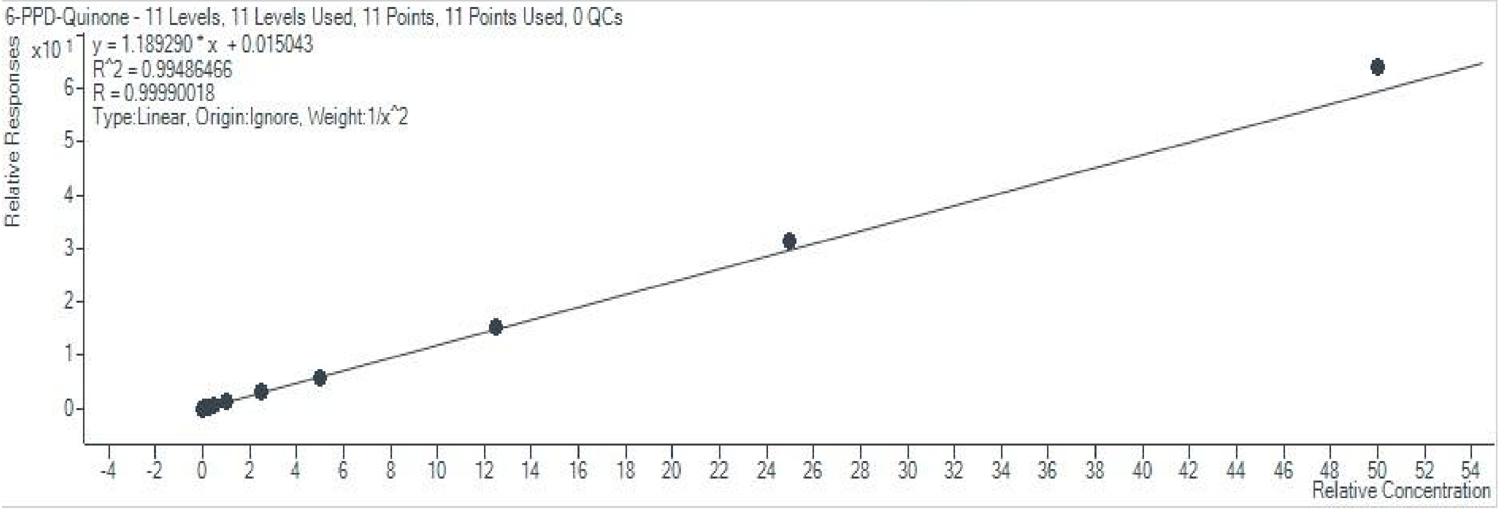


***Figure 3**. Calibration curve for 6PPDQ, showing linear response across 0.01–100 ppb. Linearity was assessed using weighted least squares regression, with $R^2 > 0.99$ indicating strong correlation between concentration and response.*

Stormwater Sediment Sample Preparation

Stormwater sediment samples were removed from storage at -10°C and allowed to thaw at room temperature for 48 hours. Once fully thawed, an appropriate mass of each sample was transferred to a pre-cleaned dish and the wet weight was recorded. Samples were then air-dried under a fume hood overnight to remove residual moisture. The samples were then placed in the oven overnight at 103-105°C to obtain the total percent solid. Following the initial drying step, samples were placed in a desiccator for one hour and then weighed. This process was repeated until a stable dry weight was achieved to ensure consistent moisture removal prior to extraction. Once dried, samples were weighed out for extraction.

Data Analysis

All data processing and statistical analyses were conducted using Python in Jupyter Notebook. Pearson correlation, scatter plots, regression analysis, and density plots were used to evaluate trends and relationships.

Results and Discussion

The developed and optimized method was applied to stormwater sediment samples. All quality control criteria were consistently met, and the method maintained precision even when applied to samples with historically high matrix effects. Stormwater sediment samples collected in Tacoma, WA were prepared and analyzed according to the method. A total of 28 samples were analyzed in two batches (n = 20, n = 8). Each batch included a method blank (BLK), a laboratory control sample (LCS), and a method detection limit check sample (MRL) for quality assurance. The measured concentration of 6PPDQ in the 28 stormwater sediment samples ranged from 11.4 ppb to 691 ppb, with a mean of 160.28 ppb. Though the leaching properties of 6PPDQ from soils to water is not yet fully understood, concentrations as low as 20 ng/L in water are lethal to coho salmon (Tian et al., 2021). The detection of 6PPDQ in stormwater sediment samples at these observed levels highlights its pervasive presence in urban runoff and its ability to bind to sediment matrices for extended periods. The strong adsorption to sediments is likely linked to the high octanol-water partition coefficient (Kow) of 6PPDQ.

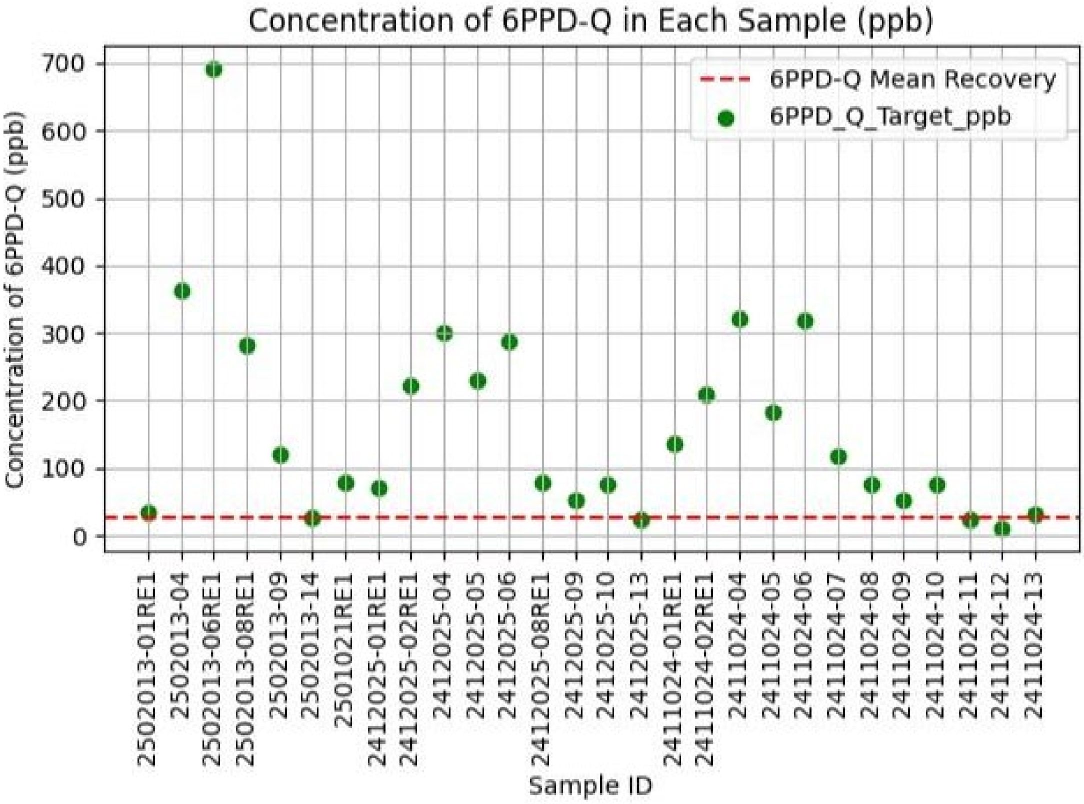


Figure 4. *Scatter plot of measured 6PPDQ concentrations (ppb) in 28 individual stormwater sediment samples.*

The correlation plot between the IIIS/NIS and EIS/SS recoveries shows a moderate negative correlation (r = –0.727, $p < 0.05$). As the recovery of the IIS/NIS increases, the recovery of the EIS/SS decreases, and vice versa. This relationship may reflect competitive matrix interactions where co-extracted components selectively affect the performance of one standard over the other. Such interactions are likely caused by ion suppression during LC-MS/MS analysis. The negative correlation indicates that the non-extracted internal standard response remained sensitive to matrix-induced ion suppression. This suggests that when matrix effects suppress the ionization of the IIS/NIS, the response of the EIS/SS may be concurrently enhanced. Presumably due to ion competition within the electrospray ionization (ESI) source. These findings underscore the intricate complexity involved in working with sediment matrices.

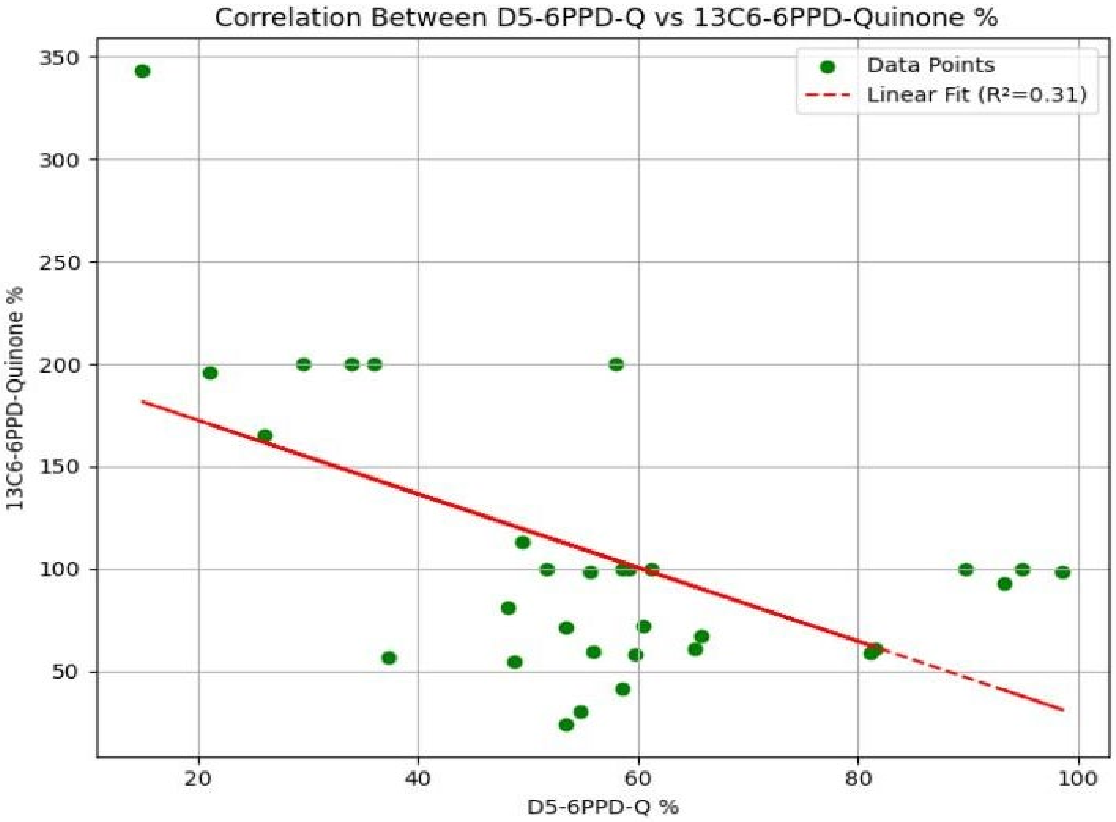


***Figure 5**. Correlation between internal standard and surrogate recoveries (%), with a fitted linear regression ($R^2 = 0.31$).*

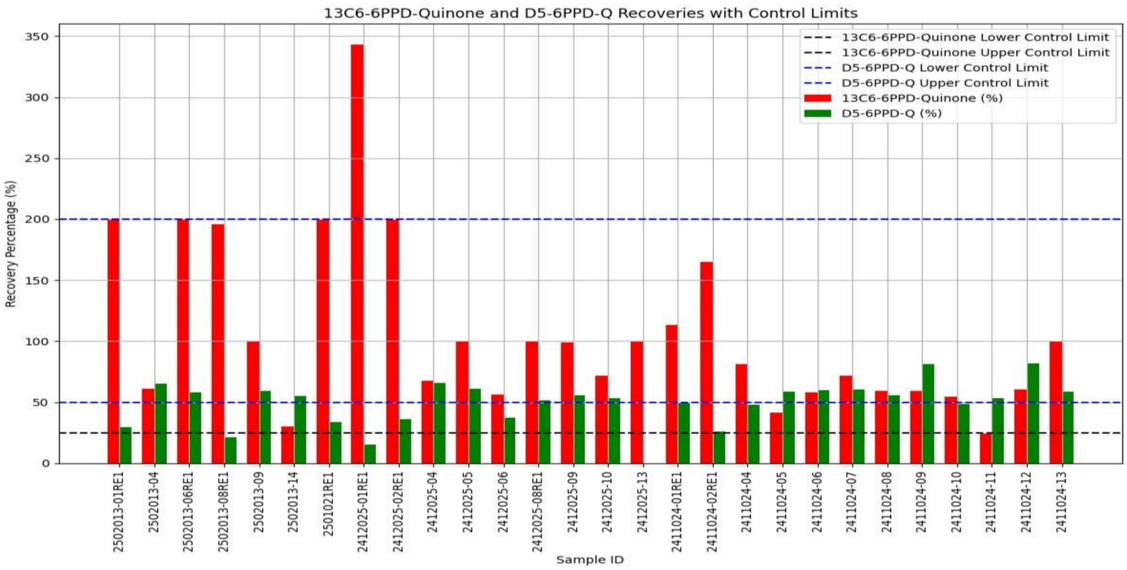


***Figure 6**. Percent recoveries of internal standard (red) and surrogate (green) across stormwater sediment samples, with upper and lower control limits. Notable deviations above or below the control threshold are attributed to matrix effects.*

The density plot illustrates the distribution of 6PPDQ, the EIS/SS, and IIS/NIS across 28 stormwater sediment samples. The IIS/NIS exhibits a sharp peak centered between 50–70%, indicating a narrow and consistent recovery distribution. This suggests that the IIS/NIS behaves predictably and reproducibly under the established analytical conditions. Such performance allows for reliable connection to address matrix effects that influence the accuracy and robustness of quantitation. The high density of values within this narrow range further implies that the extraction method is efficient and well-optimized, with minimal interference even from the complex and heterogeneous sediment matrix. Conversely, the EIS/SS exhibits a broader distribution with a peak around 80–100% and a long right-hand tail extending to values exceeding 300%. This skewed distribution implies substantial inconsistency in its performance that is attributed to interactions with the sediment matrix. We hypothesize that the presence of heavy oils in the samples may have influenced the partitioning behavior of the surrogate standard, resulting in reduced recovery due to sorptive interactions with hydrophobic matrix components. The distribution of the target analyte, 6PPDQ, spans a wide range of values, reflecting the true environmental contamination across the sediment samples. The relatively flat and extended shape of the density curve indicates significant spatial variability in 6PPDQ levels, influenced by differences in runoff composition, sedimentation rates and degradation rates.

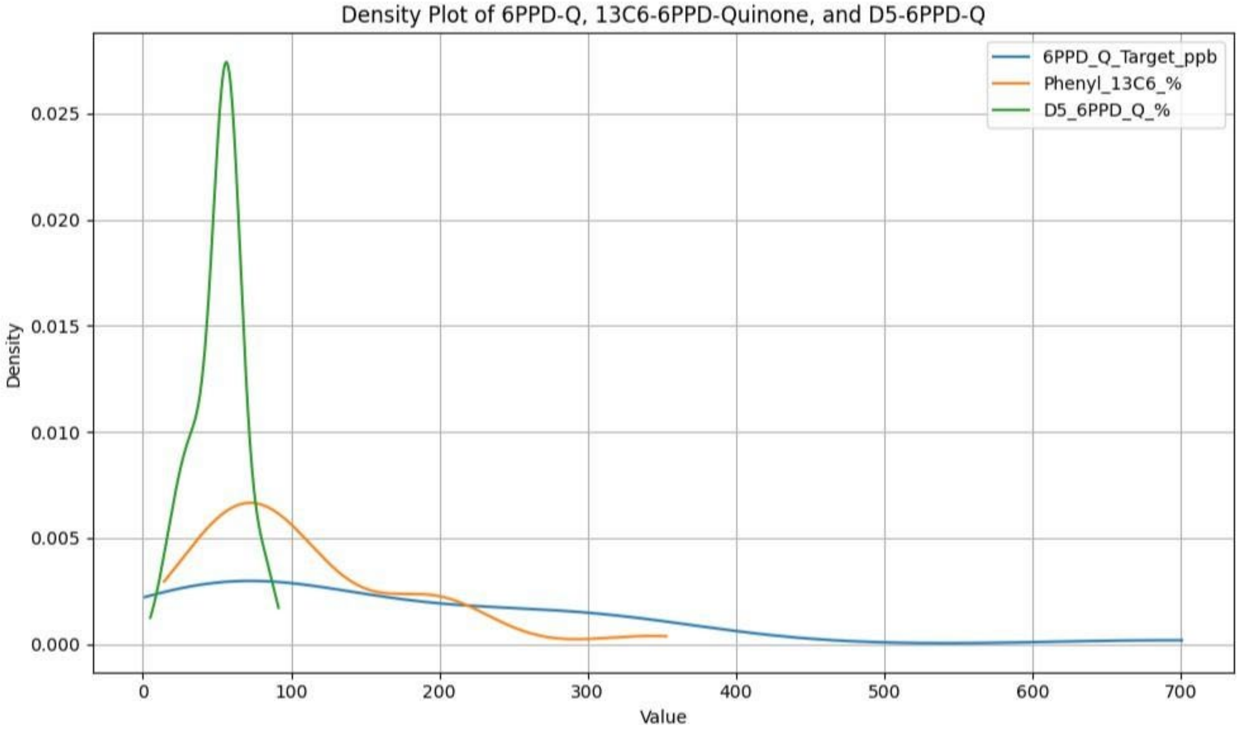


***Figure 7**. Density plot distributions of 6PPDQ target concentrations (ppb) (light blue), internal standard (orange), and surrogate (green) across sediment samples. The variation in distribution shape and spread reflects differences in recovery.*

Chromatograms of the EIS/SS for quality control samples including the BLK, MRL, and LCS, along with two stormwater sediment samples analyzed under the developed LC-MS/MS method are compared below. The method blank shows no discernible background signal confirming the absence of contamination during sample preparation and analysis. The sediment samples chromatograms reveal target peaks that are comparable in retention time, shape, and resolution to those observed in the fortified QC samples. This alignment suggests that matrix effects such as ion suppression were minimal under the optimized method. The clean baselines and absence of significant co-eluting interferences confirms the selectivity of the method. The consistency across all chromatograms substantiates the method's robustness and applicability for trace-level quantification of 6PPDQ in sediment samples.

***Figure 8**. Chromatogram of Method Blank (BLK)*

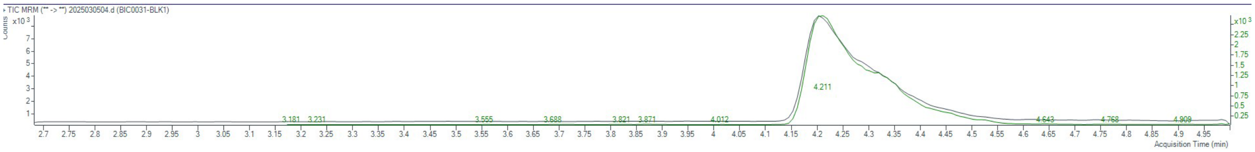


***Figure 9**. Chromatogram of Method Reporting Limit (MRL)*

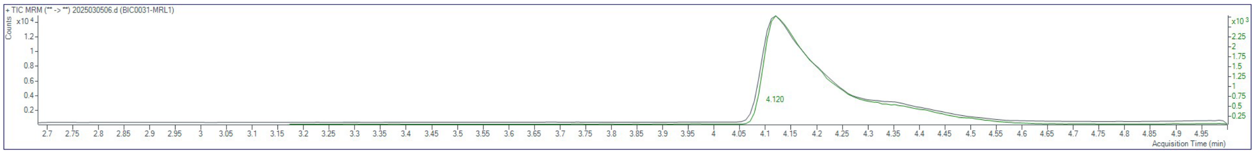


***Figure 10**. Chromatogram of Laboratory Control Sample (LCS)*

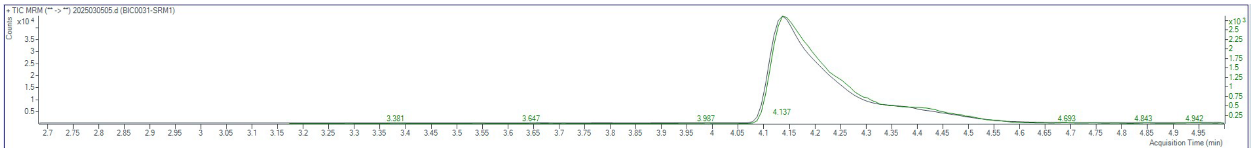


***Figure 11**. Chromatogram of a Stormwater Sediment Sample*

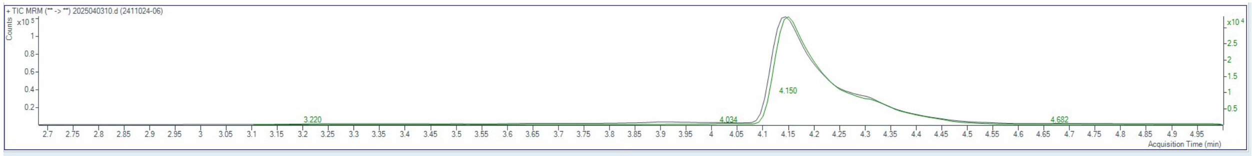
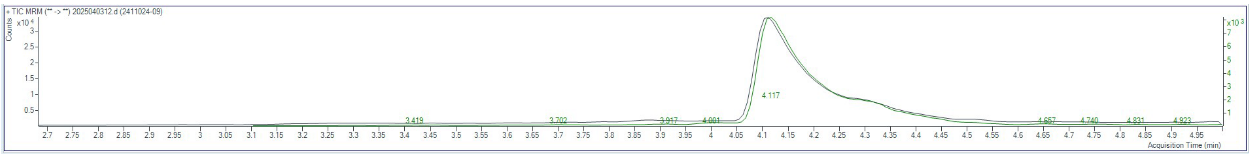


***Figure 12**. Chromatogram of a Stormwater Sediment Sample*



Conclusion

This study presents the first comprehensive and rigorously validated analytical method for the quantification of 6PPDQ in sediment matrices. The final method, employing ultrasonic-assisted extraction with a Hexane: Ethyl acetate solvent system and LC-MS/MS quantitation with matrix-matched calibration, demonstrated high sensitivity, selectivity, and reproducibility. Matrix effects were reduced through optimized extraction parameters rather than post-extraction clean-up. The inverse correlation observed between the extracted internal standard and the non-extracted internal standard recoveries needs further understanding as matrix dynamics is the root cause. Application of the method to field-collected stormwater sediments revealed widespread levels of 6PPDQ contamination. These findings not only validate the robustness and environmental relevance of the developed method, but also reinforce the urgent need for further leaching characteristic and land-use studies, regulatory action and remediation efforts to address tire-derived contaminants in urban runoff. Elucidating the leaching behavior of 6PPDQ from stormwater-impacted sediments across varying salinities and geochemical conditions is critical for informing fate modeling and for developing effective management strategies. This research establishes a new analytical benchmark for sediment-based monitoring of 6PPDQ and offers a foundational tool for advancing both scientific understanding and policy development. We hope the methodological innovations and environmental data generated herein will serve as a model for future research.

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Appendix

Table 1. Media types tested: Ottawa sand, diatomaceous earth, ratios of Ottawa sand & diatomaceous earth, and Bentonite clay

Media types	Ratio	% Avg recovery for 50 ppb 6PPD-Q	% Avg recovery for 5 ppb 6PPD-Q
Ottawa sand	100	0%	0%
Diatomaceous earth	100	73%	53%
Ottawa sand: Diatomaceous earth	75:25	82%	65%
Ottawa sand: Diatomaceous earth	50:50	71%	46%
Diatomaceous earth: Ottawa sand	25:75	64%	38%
Bentonite clay	100	84%	73%

Table 2. Gradient elution profile (time, flow rate, composition) and the pressure

Time (min)	A [%]	B [%]	Flow [mL/min]	Max. Pressure Limit [bar]
0.00	30.00	70.00	0.400	1000.00
0.30	30.00	70.00	0.400	1000.00
3.00	80.00	20.00	0.400	1000.00
4.00	95.00	5.00	0.400	1000.00
5.00	100.00	0.00	0.400	1000.00

Table 3. Retention time, transition ions, and fragmentor for 6PPD-Q, D5-6PPD-quinone, and 13C6-6PPD-quinone

Analyte	RT [min]	Precursor ion	Product ion	Fragmentor
6PPD-Quinone	4.2	299.1	241.1	

			215.1 187.1	105
D5-6PPD-quinone	4.2	304.1	246.1 220.1 192.1	110
13C6-6PPD-quinone	4.2	305.1	247.1 221.1	110

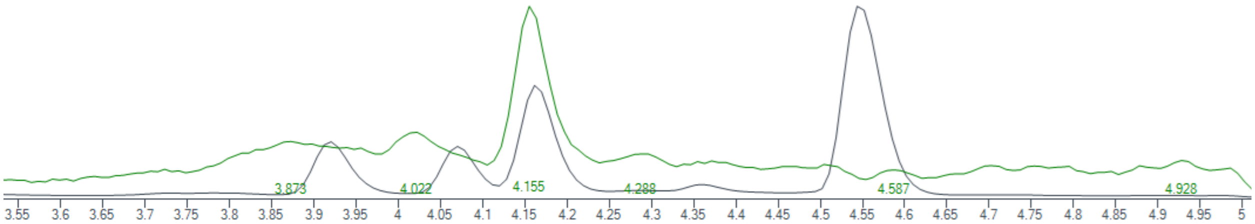

Figure 1. Chromatogram of Manhole sediment sample without SPE clean-up. Multiple peaks detected and significant matrix interferences present.

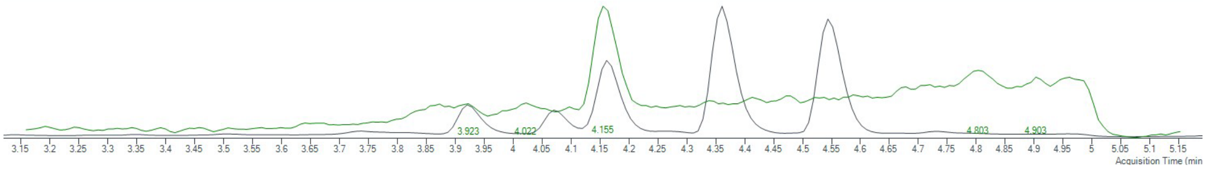

Figure 2. Chromatogram of Manhole sediment sample with SPE clean-up. Multiple peaks detected and matrix interferences remain.

Table 4. Preparation details for each calibration level

Calibration Level	**uL of 1 ng/mL ICAL Std**	**uL of 5 ng/mL ICAL Std**	**uL of Intermediate Std**	**uL of EIS/SS Spike**	**uL of Acetonitrile**
0.01 ppb	10			10	980
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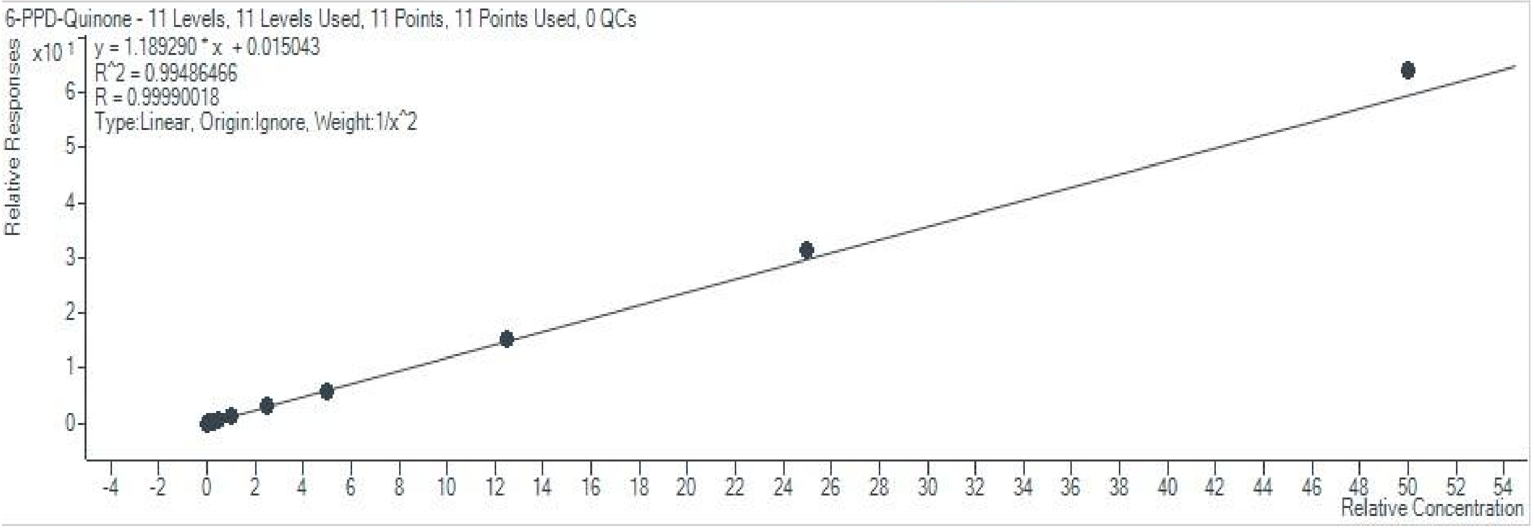


Figure 3. Calibration curve for 6PPD-Q, showing linear response across 0.01–100 ppb. Linearity was assessed using weighted least squares regression, with $R^2 > 0.99$ indicating strong correlation between concentration and response.

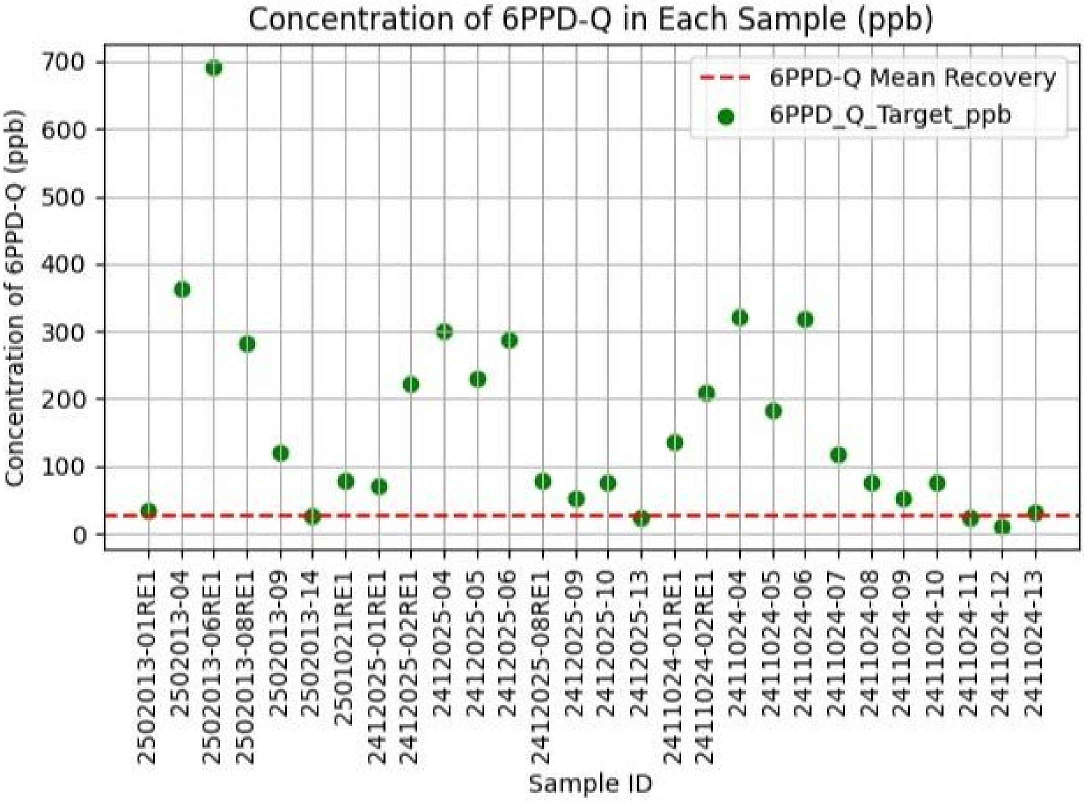
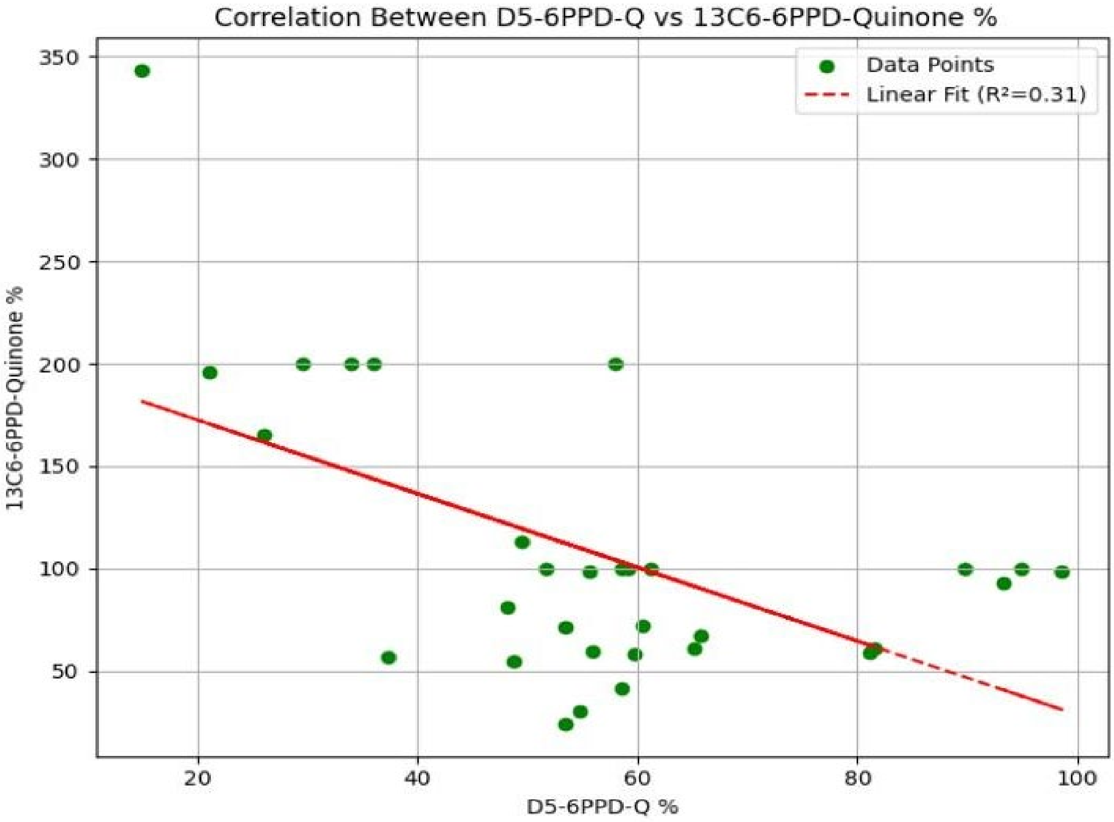


Figure 4. Scatter plot of measured 6PPD-Q concentrations (ppb) in 28 individual stormwater sediment samples.



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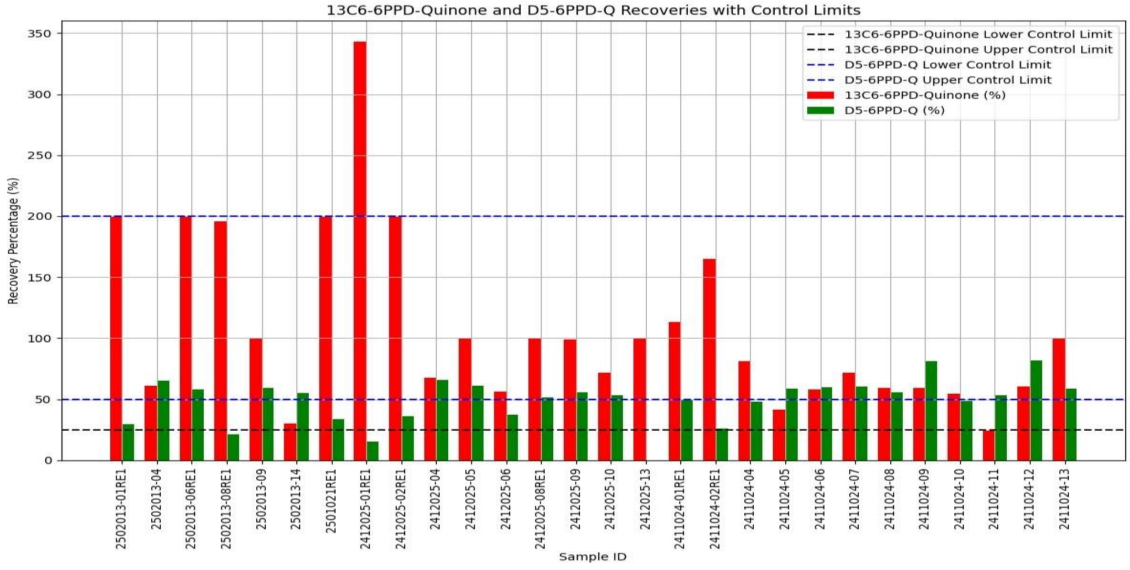

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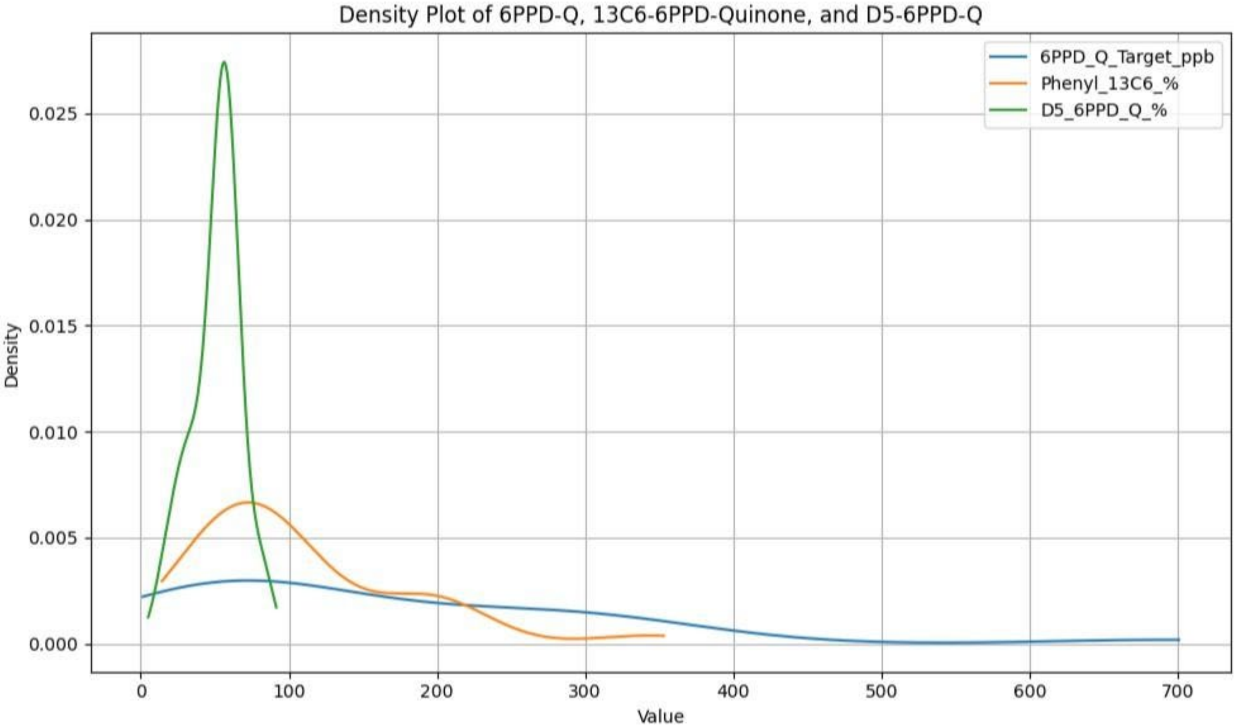


Figure 7. Density plot distributions of 6PPD-Q target concentrations (ppb) (light blue), internal standard (orange), and surrogate (green) across sediment samples. The variation in distribution shape and spread reflects differences in recovery.

Figure 8. Chromatogram of Method Blank (BLK)

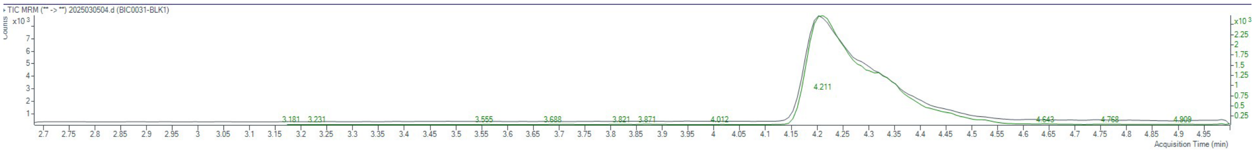
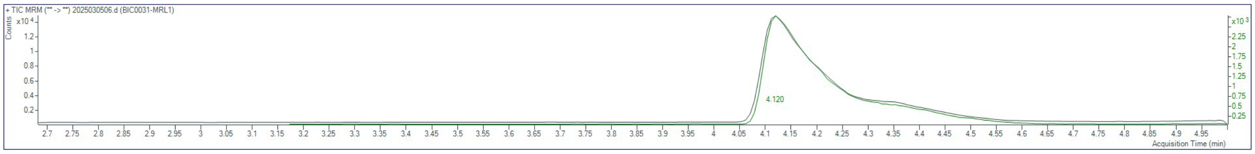


Figure 9. Chromatogram of Method Reporting Limit (MRL)



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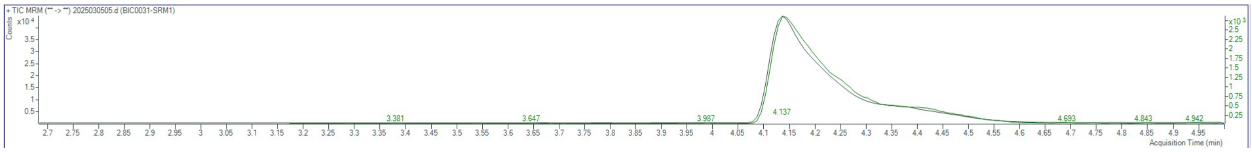

Figure 11. Chromatogram of a Stormwater Sediment Sample

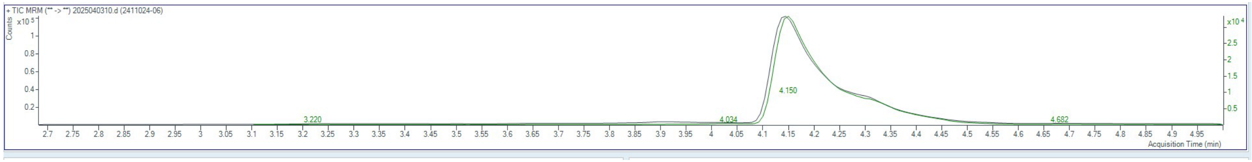

Figure 12. Chromatogram of a Stormwater Sediment Sample