#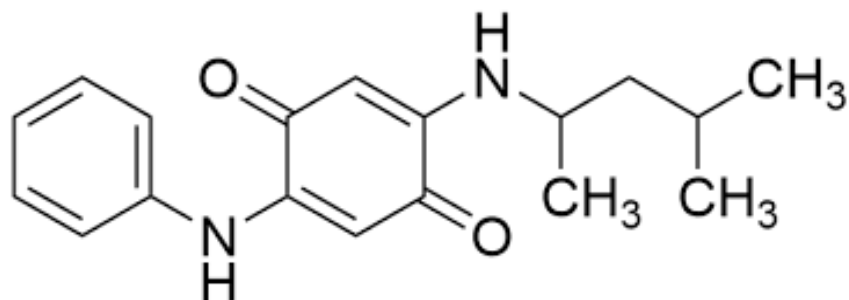 Research Summary for the Single-laboratory Validation Study of a Draft EPA LC-MS-MS Isotope Dilution Method for 6PPD-Quinone

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Acknowledgments

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Research Summary for the Single-laboratory Validation Study of a Draft EPA LC-MS-MS Isotope Dilution Method for 6PPD-Quinone

1.0   Background

A study conducted by the University of Washington and funded by Region 10 EPA and published in *Science* in December 2020 reported that *p*-phenylenediamine (PPD), an antiozonant added to tires, is being converted by ozone to a quinone, 2-anilo-5-[(4-methylpentan-2-yl)amino]cyclohexa-2,5-diene-1,4-dione (6PPD-Q), and that it is found in stormwaters and in Pacific NW rivers subsequent to rainfall events at concentrations toxic to Coho salmon (1).   The paper reported a median lethal concentration (LC50) for Coho salmon of 800 ng/L (ppt).   Subsequent information from the principal scientists indicate that their original calculations were high by as much as an order of magnitude due to an issue with the purity of their standard.   Their revised LC 50 was reported to be 95 ng/L (2).   Commercial standards for the parent compound and an isotopically labeled version became available subsequent to the original paper.

Interest by west coast states to assess the occurrence of this contaminant in watersheds moved quickly, and additional toxicity work is underway.   More recently, a paper was released in February 2022 that indicated 6PPD-Q is also toxic to brook and rainbow trout at similar but higher LC50 concentrations, 590 and 1000 ng/L, respectively (3).   States and NW tribes have emphasized the need for a standard analytical method to analyze this contaminant.   In a letter to the EPA Region 10 Regional Administrator alleging the violation of treaty rights, the Nisqually tribe formally requested that EPA "develop analytical tools and methods to measure and regulate 6PPPD in stormwater" (4).   The high level of interest prompted EPA to initiate work on an EPA method to detect this emerging contaminant in surface and stormwaters.

While the original studies were supported on a high-resolution liquid chromatography time of flight instrument, an isotope dilution-based method that employs a triple quadrupole mass spectrometer (LC-MS-MS) would be optimal, allowing application in more laboratories.   For stormwaters and surface waters containing suspended solids, an extraction-based method is required (5) because 6PPD-Q tends to associate with the total suspended solids.   Incorporating an extraction step also allows the introduction of a wash step to reduce background and improve method sensitivity.   Ideally, this method would have sufficient sensitivity, precision and accuracy to assess surface and stormwater 6PPD-Q concentrations well below the 100 ng/L LC50 for Coho.   Therefore, target method reporting limit for this study was set at 2.0 ng/L.   Surface water and stormwater are more closely associated with the Clean Water Act (CWA) methods program than other EPA method programs (i.e., drinking water, solid waste, or air), and so this method will be considered for publication as a 1600 series EPA CWA method.

Early on in EPA's investigation, technical discussions between key scientists at Region 10 EPA and Eurofins (Sacramento) regarding Eurofins' presentations on occurrence of 6PPDQ in various matrices (surface water, storm water, car wash water) resulted in Eurofins volunteering to partner with EPA on this single-laboratory validation study.   Given the urgent need, EPA pursued this opportunity and developed a QAPP for the single-laboratory study based on the Eurofins' existing standard operation procedure (SOP) and additional EPA technical guidance (QAPP and SOP attached).   Input was sought on

the QAPP from four EPA regional Quality Assurance Managers. In the sections that follow, this document briefly summarizes the early work done by Eurofins and then presents the result of the single-laboratory validation study.

2.0   Initial Work at Eurofins and Method Selection

Upon the initial publication of the landmark 6PPD-quinone (6PPD-Q) paper in *Science* in December 2020, Eurofins Sacramento initiated the development of a quantitative method for 6PPD-Q (CAS 2754428-18-5) with an initial focus on stormwater. The method was LC-MS/MS-based and utilized isotope dilution quantitation with a solid-phase extraction (SPE) to both concentrate the target compound and remove bulk interferences. Since 6PPD-Q had only been very recently reported, an isotopic analogue and a commercially available native compound were not initially available. Eurofins Sacramento maintains multiple high-resolution mass spectrometers (HRMS) which employ exact mass capability and are capable of compound identification without an analytical reference standard. A small volume of water was pulled from the laboratory parking lot storm drain and centrifuged to remove solids prior to infusion into the HRMS (SCIEX x500r). The compound was identified at the published exact mass from the parking lot water and two robust daughter fragments were identified when operating in MS/MS mode. Several directional decisions on chromatography and LC column selection were made using this ad-hoc approach. Later, a small mass of 6PPD-Q was obtained from an academic partner, and although this material proved to be degrading over time, a calibration curve could be formulated and spiked replicates created for SPE evaluation. By Spring 2021, the HPC corporation started producing a 6PPD-Q standard, both in crystalline form and in solution. Not long after, HPC synthesized a deuterated analogue (D_5-6PPD-Q) that was employed as an extracted internal standard (EIS). Eurofins obtained the commercial standards and commenced studies which were documented in an SOP that was first drafted in March 2021. The SOP was shared with EPA as were data regarding the methanol wash used for sample preparation. It was clear from the early Eurofins data that 6PPD-Q had an affinity for suspended material, which ruled out a direct inject method. Data from the methanol wash of the solid-phase cartridges was reviewed by EPA and deemed adequate. These studies were not repeated as part of the single-laboratory validation study. Eurofins reported that their SOP was also applicable for both LC-MS/MS and LC-QToF approaches that use multiple reaction monitoring (MRM). The LC-QToF approach was not evaluated in this study, however, the HRMS aspect of the QToF mitigates potential interferences by the use of high-resolution daughter fragments and should offer even more selectivity for highly complex matrices.

3.0   Study Sample Matrix Selection

Storm and surface waters were collected in the Northwest, the upper Midwest, and the mid-Atlantic portions of the United States. The Northwest stream was the same stream that was the basis for the Science paper (1). The mid-Atlantic surface water, which was collected in Falls Church, VA, was collected from a small pond (~ 1 acre) that is fed by stormwater. At the time of collection, it had a slight oily sheen, which would make it a particularly challenging matrix for the SPE. The bulk matrices and their sample naming conventions area shown below in Table 3-1.

Table 3-1.   Matrices Used in the Single-laboratory Study

Source	Type	Naming Convention
Storm Water Outfall into Puget Sound in Port Orchard (WA)	Storm water	ST1
Storm Water Outfall near Duluth, MN	Storm water	ST2
Blue Ravine and East Natoma, Folsom, CA	Storm water	ST3
Tischer Creek (MN) tributary that flows into Lake Superior near Duluth	Surface water	SU1
Falls Church, VA	Surface water	SU2
Miller Creek, Burien (WA)	Surface water	SU3
American River, Rancho Cordova, CA	Surface water	SU4

4.0   Calibration and Quantitation

Initial Calibration (QAPP Sect. 4.1.1)

Three initial calibration studies were conducted on the instrument used for the study. For each calibration curve, seven calibration standards were injected (20 uL aliquots) that ranged in concentration from 0.025 ng/mL to 10.0 ng/mL, corresponding to sample concentrations of 1.0 ng/L to 400 ng/L. The minimum level of quantitation (ML) for this study has been defined as 2.0 ng/L and is equivalent to the second lowest concentration point in the calibration curve (0.05 ng/mL). Peaks for 6PPD-Q were well resolved allowing valley-to-valley integration. The calibration curves were linear regressions weighted by 1/Conc and/or 1/Conc^2. Table 4-1 summarizes the data collected from the three calibrations performed between March and October 2023. For each calibration curve, the relative standard error (RSE) was calculated using Eq. 2 from the QAPP.

Table 4-1.   Initial Calibration Study Results

Calibration Point	Nominal Value (ng/mL)	% Recovery		
		Cal 1	Cal 2	Cal 3
C1	0.025	100.4	99.7	109.9
C2	0.050	100.4	101.2	100.9
C3	0.10	98.4	99.5	96.9
C4	0.50	95.6	96.9	98.2
C5	1.0	97.9	99.1	94.2
C6	5.0	102.0	100.0	98.7
C7	10.0	103.5	100.4	99.5
RSE (7 Calibration Points)		2.70	1.44	4.96

5.0   Initial Demonstration of Capability (IDC) (QAPP Sect. 4.1.2)

Initial Demonstration of Capability Study

The IDC requirement in the QAPP was unintentionally missed during the project. However, a total of 20 ongoing precision and recovery (OPR) samples (aka laboratory control samples) were prepared and analyzed in conjunction with the study samples. Those OPR samples were run in pairs, with two pairs analyzed with every study sample preparation batch. Those 20 samples represent five sets of four results that each are effectively equivalent to an initial precision and recovery (IPR) study. The mean recoveries and relative standard deviations (RSDs) of those "pseudo" IPR studies are presented in the table and figure below and demonstrate excellent overall precision and recovery for the procedure in a reagent water reference matrix.

Table 5-1.   Pseudo IPR Study Results

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

Figure 5-1.   Mean LCS % Recovery by Batch (n=20)
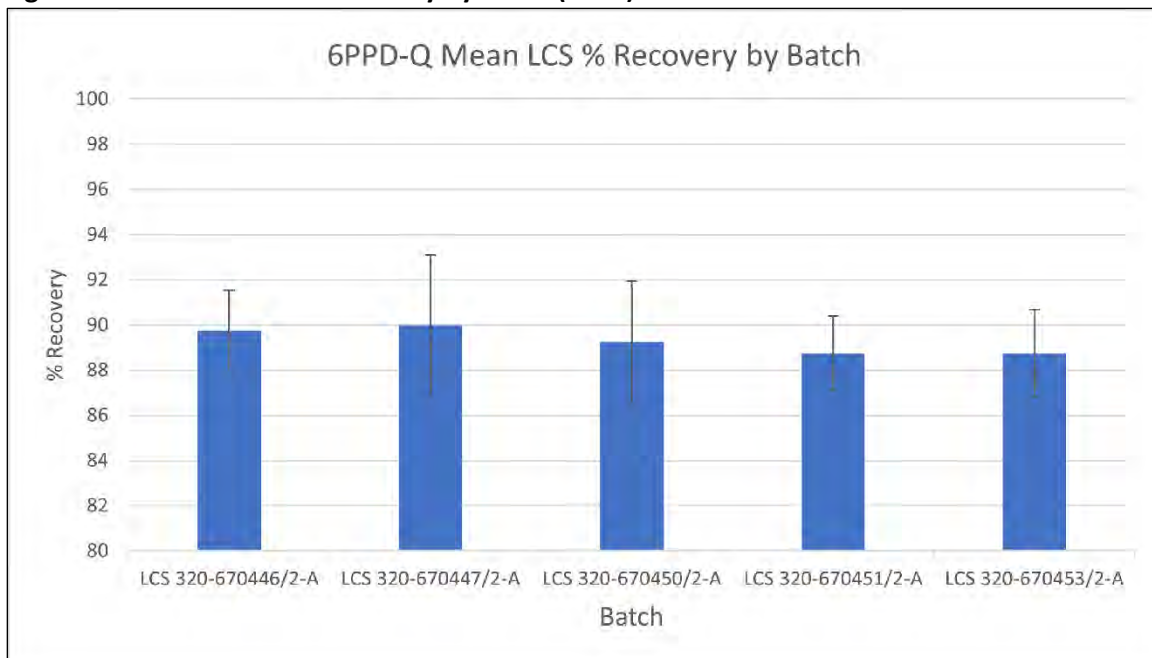
Method Detection Limit Study

An MDL study was performed in triplicate, following the revised procedure found at 40 CFR 136, Appendix B, or at:

https://www.epa.gov/sites/default/files/2016-12/documents/mdl-procedure_rev2_12-13-2016.pdf

Each MDL study consisted of seven replicate reagent water samples spiked with 6PPD-Q and the EIS and carried through the entire analytical process (sample preparation and analysis) and was evaluated in reagent water (only). For this work, reagent water was fortified at a sample concentration equivalent to 2.0 ng/L, which is also the ML for the draft method. Signal-to-noise ratios determined by the Sciex software exceeded 100/1 at these fortification levels, and so the true MDL may be lower. Given the ML reported in the method, these data were deemed sufficient. The EIS was spiked at 50 ng/L, and the reagent water did not contain 6PPD-Q. The MDL studies are summarized in Table 5-2 below. The average MDL for the three determinations was 0.505 ng/L.

Table 5-2.   Results from Three MDL Studies

		Result (ng/L)		
Replicate	Nominal Value (ng/L)	Study 1	Study 2	Study 3
1	2.0	2.07	2.34	2.30
2	2.0	2.00	2.56	2.05
3	2.0	2.03	2.07	1.96
4	2.0	2.34	2.17	2.27
5	2.0	2.10	2.13	2.07
6	2.0	2.32	2.40	2.32
7	2.0	2.23	2.03	2.04
	Average	2.16	2.25	2.16
	Standard Deviation	0.137	0.195	0.150
	MDL (ng/L)	0.430	0.614	0.471

In addition to the spiked reagent water samples, all three MDL studies included the requisite seven method blanks. 6PPD-Q was not detected in any of those method blanks, therefore, the MDL_b calculations did not apply, and the method blanks results need not be summarized here.

6.0   Sample Storage Stability Studies (QAPP Sect. 4.1.5)

The sample storage stability study focused on potential approaches to sample preservation. In addition to cold storage of storm and surface waters at 0-6 °C, two preservative agents were proposed to mitigate oxidative processes and microbial activity until extraction. Both sodium bisulfate (NaHSO$_4$, 1 g/L) and sodium metabisulfite (Na$_2$S$_2$O$_5$, 50 mg/L) were added as solids to the sample bottles. Preservation mechanisms for these agents are described in greater detail in the QAPP. Briefly, the sodium bisulfite will lower the pH of the sample to approximately 3, reducing the potential for microbial activity. Sodium metabisulfite rapidly forms sodium sulfite in water, an ozone scavenger which is employed in water treatment plants to remove residual ozone after drinking water treatment. The rational for the combined preservative actions of these constituents is to halt microbial activity while reducing ozone concentrations in collected samples.

For these studies, 21 sample bottles containing the matrix being studied (reagent water, storm water and surface water, with and without preservatives) were fortified at 200 ng/L and stored headspace free in amber glass bottles at 6 °C with 7 blanks for each matrix (one for each analysis day to determine native concentrations of 6PPD-Q, if any). The EIS was added just prior to extraction, and the samples were extracted and analyzed in triplicate on days 0, 2, 7, 14, 21, 28, and 35. No detections of native 6PPD-Q were observed in any of the associated blanks. These data are summarized in Table 6-1.

The data suggest that the selected chemical preservatives do not aid in preserving spiked 6PPD-Q, but rather appeared to accelerate the degradation/loss of 6PPD-Q. Of course, evaluation of intermediary compounds and end-products is needed to fully characterize the pathway, but based on these results, efforts to employ either sodium bisulfate and/or sodium metabisulfite as preservatives were abandoned. Efforts to preserve 6PPD-Q with ascorbic acid reported by others (7) showed similar loss of 6PPD-Q. It is thought that pH-mediated hydrolysis may play a role. Stability of 6PPD-Q seemed to be acceptable in all unpreserved matrices over at least 14 days. These data are summarized in Table 6-1 and shown in Figures 6-1 and 6-2. Because some of these studies did not have three replicates, the unpreserved storage stability study was repeated. These data are summarized in Table 6.2 and Figure 6-3 below. Both studies warranted a sample holding time of 14 days in the method.

Table 6-1. Sample Storage Stability Studies

Matrix	Day											
	Day 0		Day 2		Day 7		Day 14		Day 21		Day 28	
	Ave	STD	Ave	STD	Ave	STD	Ave	STD	Ave	STD	Ave	STD
Unpreserved Matrices												
Reagent Water	208	2.94	206	0.82	203	4.50	193	10.5	177	10.9	165	13.1
Surface Water	212	2.87	214	3.40	216	4.11	218	4.78	206	2.49	202	2.83
Storm Water	210	OTR	219	OTR	236	17.7	210	1.41	248	OTR	211	OTR
Preserved Matrices												
Reagent Water	208	1.41	22.4	6.67	5.14	NC	0.744	NC	NA	NC	NA	NC
Surface Water	187	11.9	11.2	6.32	ND	NC	ND	NC	NA	NC	NA	NC
Storm Water	156	OTR	10.1	OTR	5.09	2.19	1.64	0.497	NA	NC	NA	NC

NC = could not be calculated because one or more values were below the method reporting limit.

NA = sample not analyzed because prior sample was below method reporting limit.

OTR = only two replicates were available so the STD could not be calculated.

Figure 6-1.   Initial Storage Stability Study in Preserved Waters
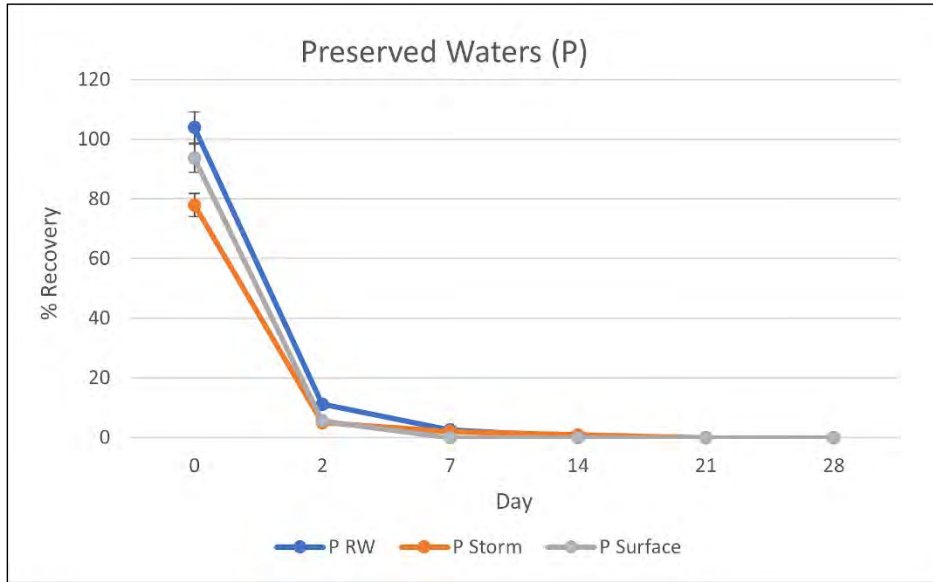
Figure 6-2.   Initial Storage Stability Study in Unpreserved Waters
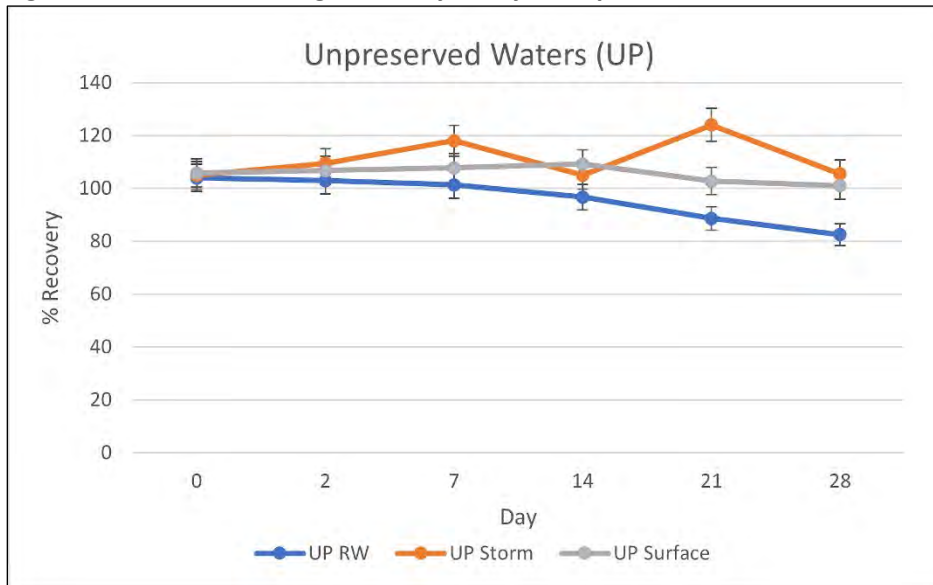
Table 6-2. Repeated Sample Storage Stability Study

	Day						
Reagent Water	**0**	**2**	**7**	**14**	**21**	**28**	**35**
Reagent Water, STD (%)	96.0	100.3	97.1	98.4	100.1	95.4	101.3
Storm Water	0.8	1.0	0.4	0.7	0.5	6.2	1.6
Storm Water, STD (%)	98.8	100.9	98.5	98.7	99.9	98.0	96.1
Surface Water	1.1	0.3	1.9	1.1	0.8	1.7	0.4
Surface Water, STD (%)	96.6	98.7	95.6	89.8	87.6	79.4	74.5
Reagent Water	2.1	1.2	1.4	3.0	0.6	2.9	2.5

Figure 6-3. Repeated Storage Stability Study in Unpreserved Waters
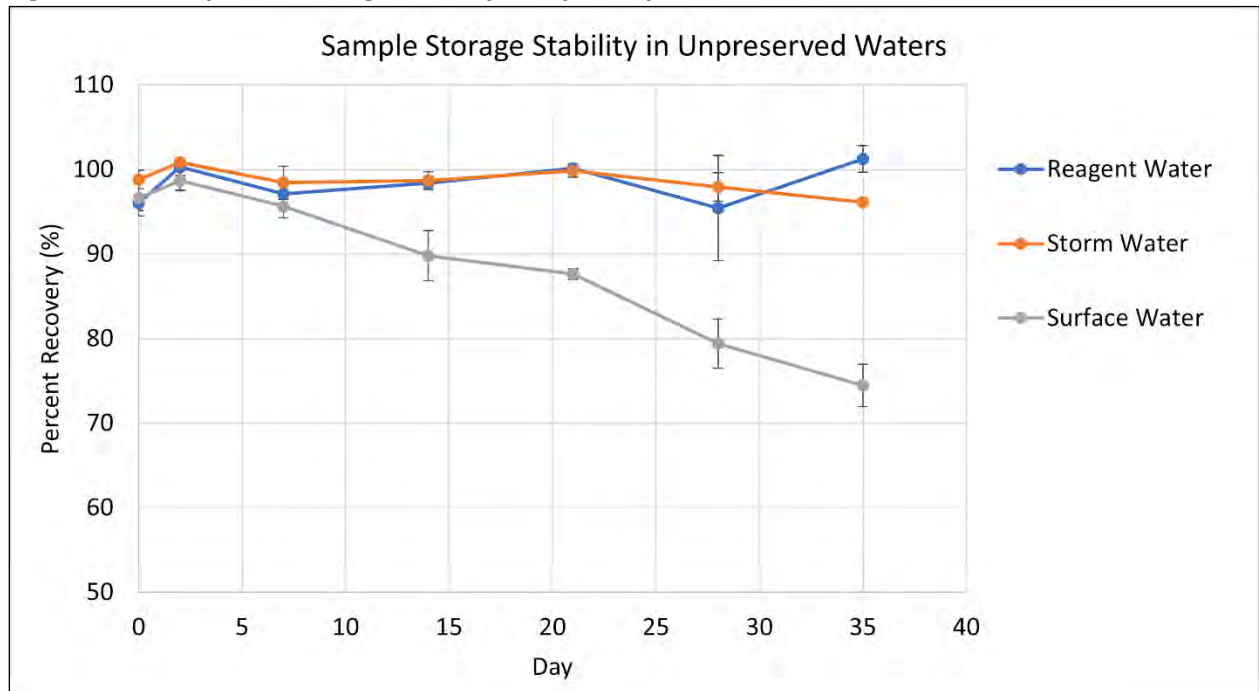
7.0 Determination of Method Precision and Accuracy in Surface Water and Stormwater Matrices (QAPP Sect. 4.1.4)

Three surface and three storm waters collected from different regions of the US. Initial background checked for all waters to check for the presence of 6PPD-Q. No concentrations above the reporting limit were found. Samples aliquoted into 250-mL amber glass bottles from larger LDPE carboys. Samples were then fortified in quadruplicate at low level (20 ppt) and high level (200 ppt) with 6PPD-Q. Blank surface and storm waters were analyzed in duplicate for all iterations to ensure consistently low background concentrations during the analysis. Laboratory Control Samples (LCS) were performed in quadruplicate and no positives observed in any Method Blank (MB). Samples were coded to represent experiment, water type and spike level, i.e., "PAST1-LS-1-A" = precision and accuracy storm 1, low spike bottle, 1 for the initial analysis, and A for the replicate.

Method performance in all three surface and ground water matrices was acceptable at both concentrations. These data are summarized in Table 7.1 and portrayed in Figures 7-1 – 7-3 below. Precision and accuracy at the lower fortification level, which is ten-times the method reporting limit, is similar to the higher level studied. Signal-to-noise at 20 ng/L exceeds 1000/1, which is likely a factor in this observation.

Table 7.1. Method Performance in Surface and Storm Waters Spiked at 2 Concentrations (4 replicates each)

Sample	Matrix	Spike (ng/L)	Average % Recovery	% RSD
PAST	Storm	200	96.5	1.39%
PAST	Storm	20	92.7	2.45%
PASU	Surface	200	94.7	1.76%
All Samples and Matrices (48)			95.2	2.95%

Figure 7.1. Recovery in Stormwaters
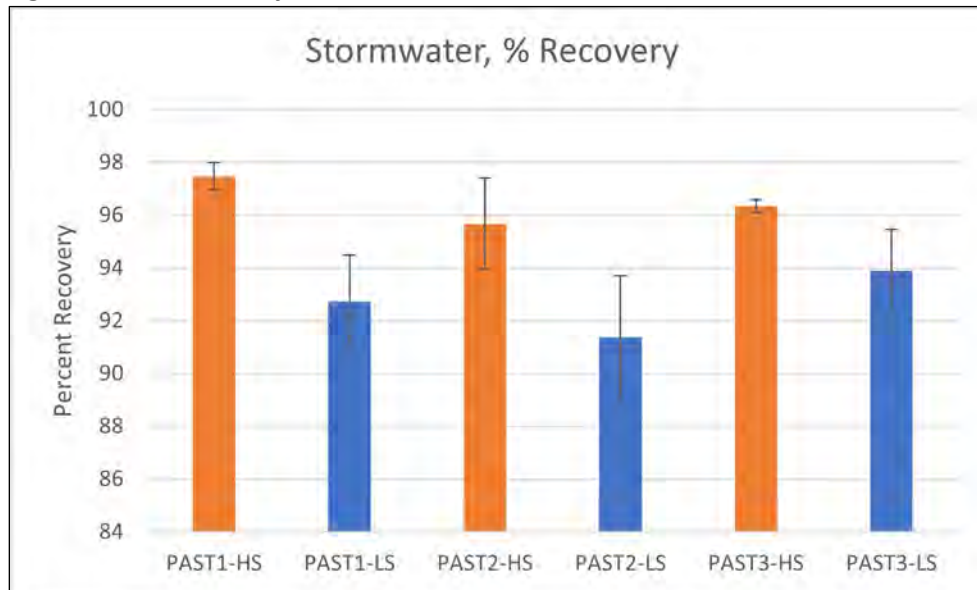
HS=High spike (200 ppt) and LS=Low spike (20 ppt)

Figure 7.2. Recovery in Surface Waters
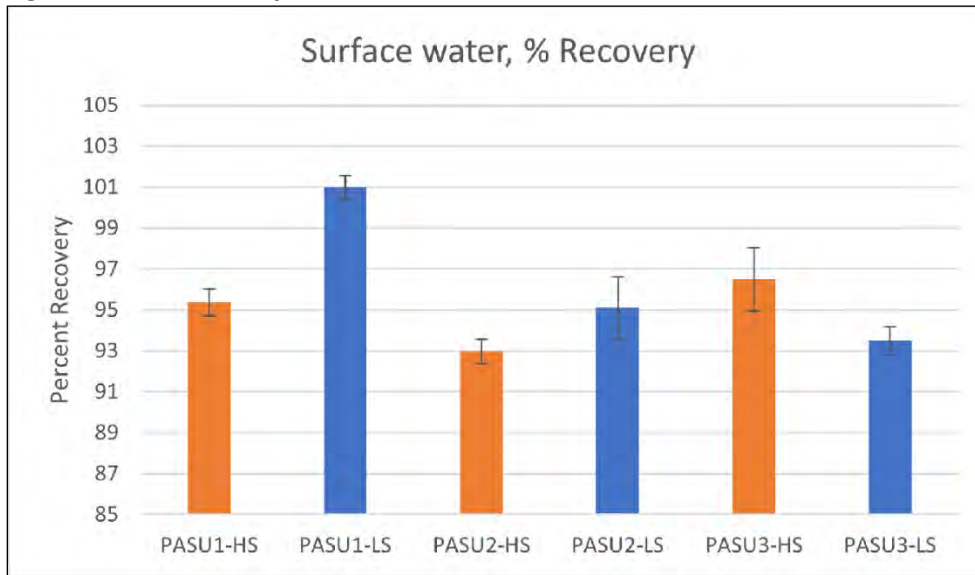
HS=High spike (200 ppt) and LS=Low spike (20 ppt)

Figure 7.3. Composite Data: 3x Storm and 3x Surface Waters, by Spiking Level
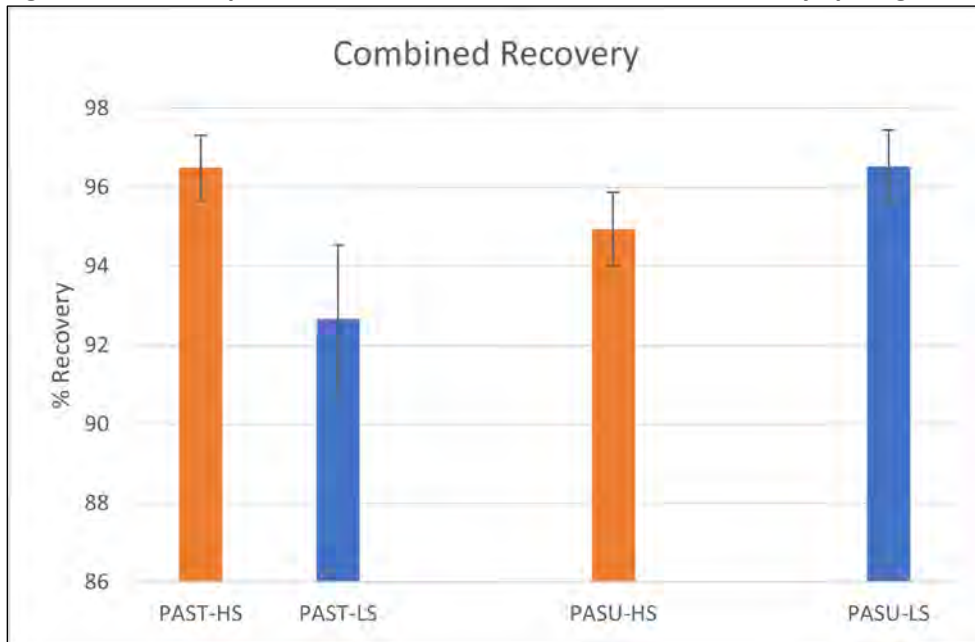
HS=High spike (200 ppt) and LS=Low spike (20 ppt)

8.0.   Extract Storage Stability (QAPP Sect. 4.1.6)

While the QAPP originally only called for storage at freezer conditions of -20 °C, an additional study was conducted at 2 - 6 °C and performed concurrently. These data are plotted in Figure 8-1 below. Recoveries slightly above 100% are thought the be associated with a slight drift in the calibration curve, even though it met all QC criteria. Both storage conditions indicate that extracts may be stored at either temperature for 28 days.

Figure 8-1.   Extract Storage Stability at -20 °C and 2 - 6 °C
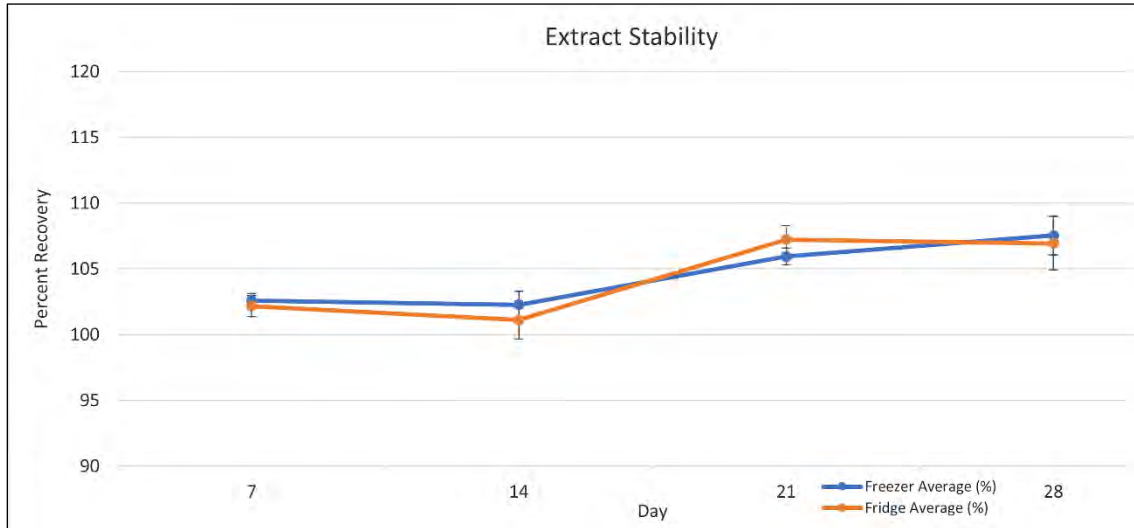
9.0.   Method Robustness

During the course of the study, a total of 20 laboratory control samples (LCSs) were run and analyzed in 5 analytical batches. The individual LCS 6PPD-Q recoveries ranged from 85 to 92% and had a mean recovery of 89.3% with an RSD of 2.7%. These data are plotted by batch in Figure 9-1 below. The dotted line represents the mean recovery from the 20 analyses.

Figure 9-1.   LCS % Recovery by Batch (n=20)
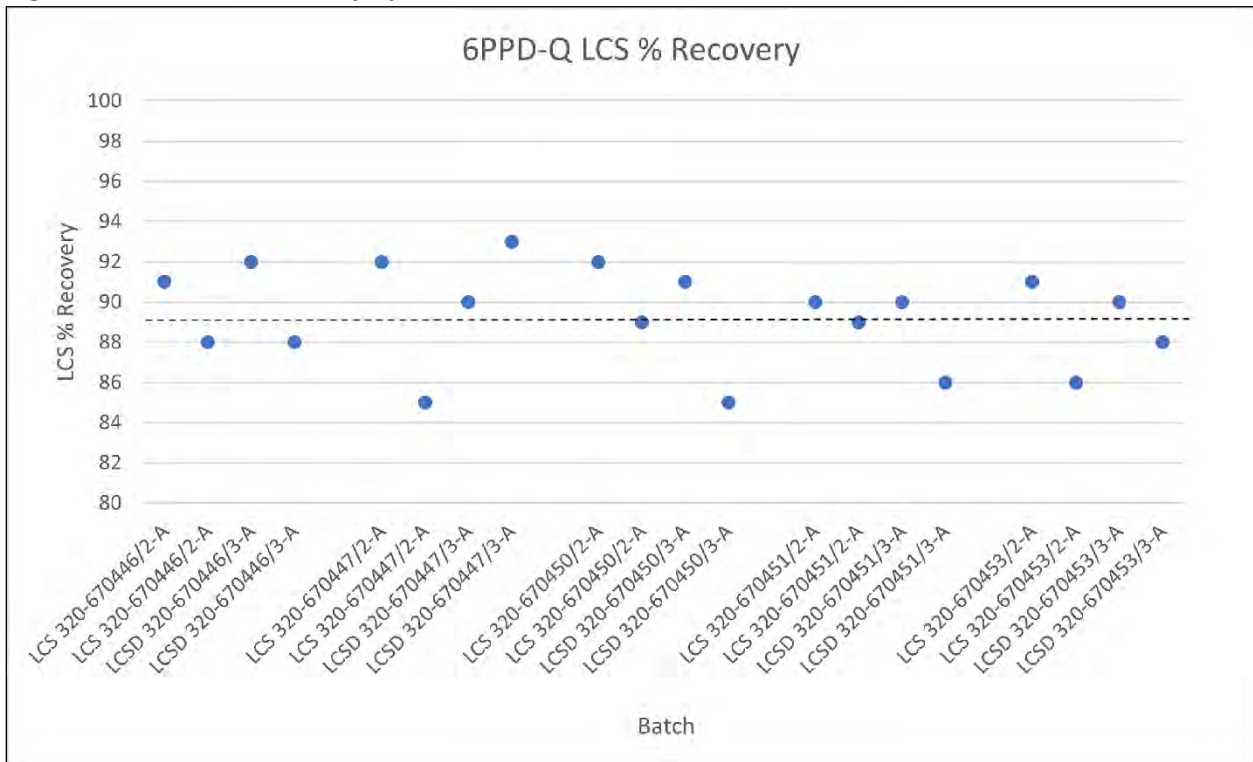
Because this method uses isotope dilution, analyte concentrations are quantitated against the labeled 6PPD-quinone (EIS, $^{13}C_6$-6PPD-Q) and the results for the native 6PPD-Q are recovery corrected. EPA methods that include an EIS also require labs to track the EIS recoveries. During the study, the lab extracted 60 samples, including blanks. The EIS recoveries from those extractions, which were performed by a single analyst, are plotted in Figure 9-2 below. The EIS recoveries averaged 90.5% (dotted green line in the figure) with an RSD of 5.3% in the matrices studied. The EIS recoveries dipped to around 80% for the PASU2 matrix. This was the surface water collected in Falls Church, VA, from a small pond under the influence of a stormwater that had a visible oily sheen on the surface. Overall, EIS recoveries performed quite well in these matrices.

Figure 9-2.   EIS Recoveries for All Matrix Samples
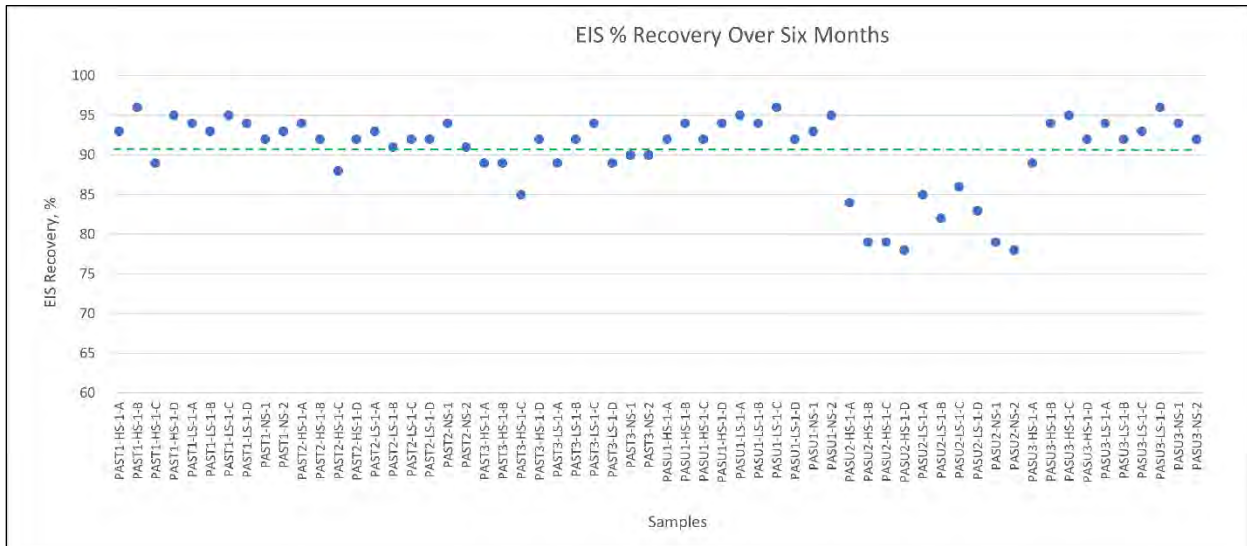
Overall quality control sample performance also was evaluated during the study period. Figure 9-3 represents all 6PPD-Q LCS and LCSD QC replicates over a six-month period. These represent isotope dilution-corrected concentrations. Figure 9-4 presents all $^{13}C_6$-6PPD-quinone (EIS) recoveries over the same time period. These are *not* recovery corrected. The green lines in both figures are at 100% recovery to give the reader perspective on the individual recoveries.

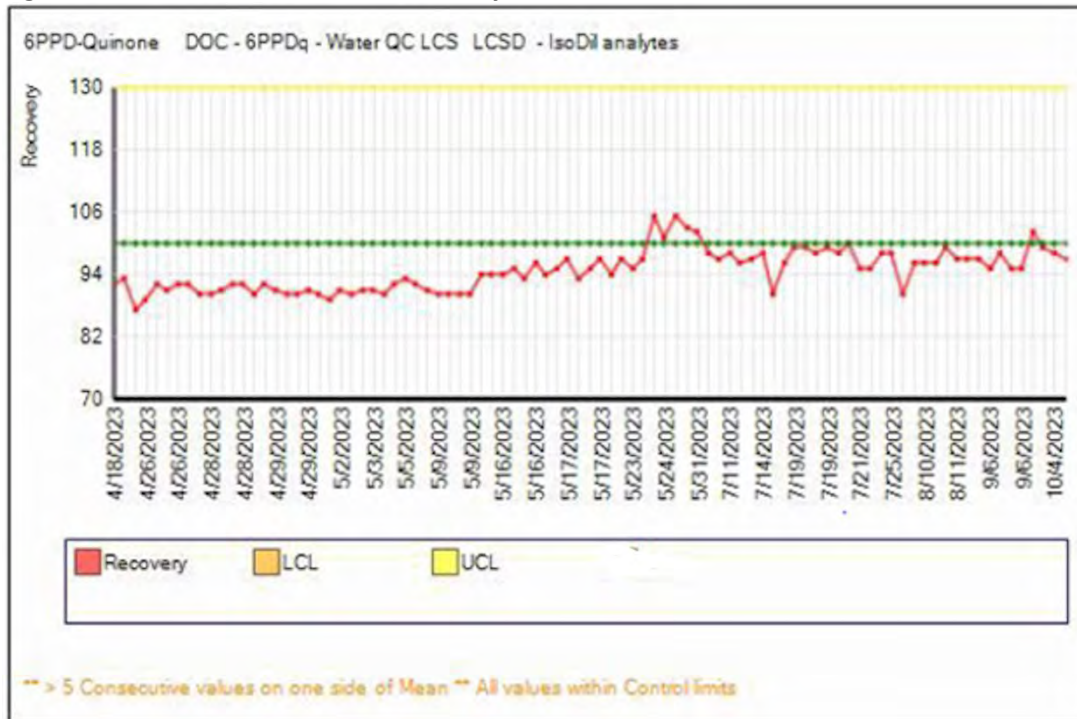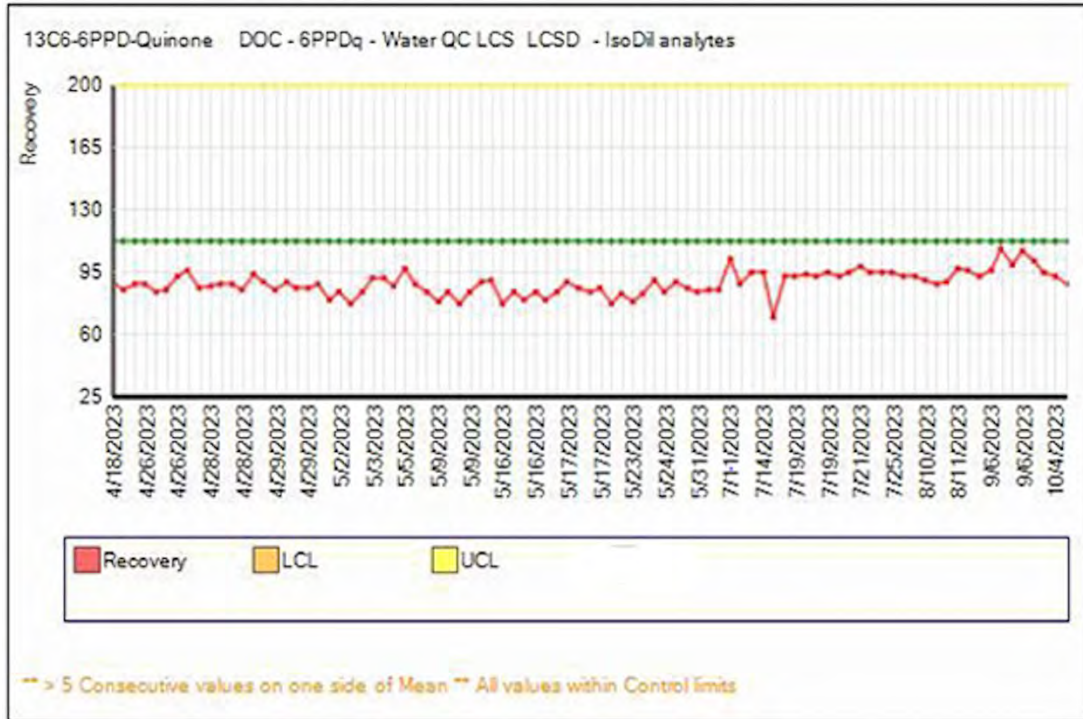**Figure 9-3.   6PPD-Q LCS/LCSD Recovery**

Figure 9-4. $^{13}C_6$-6PPD-Q (EIS) LCS/LCSD Recoveries

10.0   Additional Studies

Early in the study process, multiple isotopes of 6PPD-quinone were sought out. While HPC synthesizes a deuterated analogue, Cambridge Isotope Laboratory (CIL) originally produced a $^{13}C_6$-6PPD-quinone. A second carbon-labeled standard would provide greater stability than a deuterated analogue and was more compatible with the original antimicrobial in the preservation scheme, which reduced pH to around 3. Eurofins reached out to CIL, and they were responsive in synthesizing a $^{13}C_{12}$-6PPD-Q analogue. The thinking was that one ^{13}C standard would be used as an EIS ($^{13}C_{12}$-6PPD-Q) while the other ($^{13}C_6$-6PPD-Q) would be used as a non-extracted internal standard (NIS).

An interesting phenomenon was observed in the $^{13}C_{12}$ ion channel for some real-world stormwater samples outside the scope of the QAPP that were collected mid-winter. This phenomenon was not observed in stormwaters collected during the fall. The chromatographic artifact seen below has nearly the same retention time as the target compound, and what appear to be isomers of the same mass transition are also apparent. This interferant must have the same parent-to-daughter mass transition but could arise from a larger compound that is undergoing some sort of source-induced disassociation. Further investigation into the identity of this artifact on a high-resolution platform indicated that this interference was a different molecule separated by 0.036 amu from $^{13}C_6$-6PPD-Q. No library match was found. The $^{13}C_6$-6PPD-Q and the native analogues do not appear to suffer from this same artifact (see Figure 10-1 below). Based on these data, the spiking scheme was refined to utilize $^{13}C_6$-6PPD-Q as the EIS and the D_5-6PPD-Q as the NIS. The method has a note that cites this potential interference for the $^{13}C_{12}$-6PPD-Q analog.

Figure 10-1.   Interference Seen in Some Stormwaters Collected in Winter 2023

11:0   References

(1) Science, 08 Jan 2021: Vol. 371, Issue 6525, pp. 185-189

(2) LC50 reported by UW researchers that published the Science paper during the CA meeting in July 2021

(3) Brinkmann et al. Environ. Sci. Technol. Lett. 2022 (in press)
https://pubs.acs.org/doi/10.1021/acs.estlett.2c00050

(4) Letter from Willy Frank Jr., Nisqually Tribe chairman to Michell Pirzadeh, EPA Acting Region 10 Regional Administrator, February 16, 2022.

(5) Private discussions with Eurofins researchers who developed the SPE-based extraction method.