Determination of 6PPD-Quinone & Alternative PPD-Quinones in Fish Tissue Using QuEChERS with Serial Push-Thru Cartridge Cleanup

UCT Part Numbers

ECQUUS2-MP
QuEChERS Original Mylar Pouch (4000 mg $MgSO_4$ + 2000 mg NaCl)

ECPURMPSMC
Quick QuEChERS Medium Cartridge (110 mg $MgSO_4$ + 190 mg PSA)

CEC18MC
Clean-Up® C18 Medium Push-Thru Cartridge

LPFLTR01
LipiFiltr® Push Thru Cartridge

SCS27-C18521
SelectraCore® C18 HPLC Column (50 × 2.1 mm, 2.7 µm)

SCS27-C18GDC21
SelectraCore® C18 Guard Column (5 × 2.1 mm, 2.7 µm)

SLGRDHLDR-HPOPT
Guard cartridge holder

Introduction

6PPD-quinone (6PPD-Q) is an ozonation by-product of the tire antioxidant 6PPD, which emerging research shows poses a significant threat to aquatic ecosystems. With this knowledge becoming commonplace, rubber manufacturers are looking into alternative tire antioxidants. Some of these alternatives are also based on the structure of PPD and react similarly to form other PPD-Quinones, with toxicities beginning to be studied [1][2]. As the concern over this class of contaminants grows, developing methods to extract and analyze various matrices is of the utmost importance [3]. Researchers have discovered that the LC50 for a juvenile coho salmon contaminated with 6PPD-Q is as low as 0.094 ng/g [4], requiring high analytical sensitivity.

This application outlines a QuEChERS extraction of a subset of PPD-Qs in salmon, combined with push-thru cartridge clean-up using UCT's Quick QuEChERS®, C18, and LipiFiltr® in series to achieve the best level of sensitivity in the fatty matrix. The extracts are analyzed on UCT's SelectraCore® C18 HPLC column using LC-MS/MS. A matrix-matched calibration, followed by LOD and LOQ studies, determines accuracy and precision. 6PPD-Q, the compound of interest, is calculated using extracted isotope dilution, with the remaining PPD-Qs calculated using an extracted internal standard. Using UCT's SelectraCore® C18 HPLC Column, an optimized analysis method was developed on LC-MS/MS.

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QuEChERS Procedure

1. Sample Pretreatment

a) If using fresh fish, freeze at -40°C overnight to break down cells
b) Thaw and homogenize the sample using a Robot-Coupe or equivalent
c) Weigh 5.5 ± 0.1g thawed sample into a 50mL centrifuge tube
d) Spike extracted internal standard (EIS, 13-C6-6PPD-Q) prepared in acetonitrile into all samples and spike target analytes prepared in acetonitrile into QC. Be cautious, as CPPD-Q will fall out of solution in cold acetonitrile. Prepare solutions of CPPD-Q above 100 µg/mL in DCM and further dilute with acetonitrile for spike solutions. Sonicate the target analyte mixture for 15 minutes before adding it to the sample
e) Vortex to disperse. Let equilibrate for 5 minutes

2. QuEChERS Extraction

a) Add 4mL 1% formic acid in water and a necessary volume of acetonitrile to create a final volume of 14mL, accounting for EIS and spike solution added
b) Shake for 4 minutes on a Spex SamplePrep Geno/Grinder 2010 or equivalent at 1700 rpm
c) Add contents of QuEChERS Original Mylar Pouch (4000 mg $MgSO_4$ + 2000 mg NaCl) (**ECQUUS2-MP**)
d) Immediately shake for 4 minutes on a Spex SamplePrep Geno/Grinder 2010 at 1700 rpm
e) Centrifuge the sample at ≥ 5000 rcf for at least 5 minutes

3. Sample Cleanup

a) Attach the following in series, from top to bottom: Quick QuEChERS Medium Cartridge (110 mg $MgSO_4$ + 190 mg PSA) (**ECPURMPSMC**), Clean-Up® C18 Medium Push-Thru Cartridge (**CEC18MC**), LipiFiltr® Push Thru Cartridge (**LPFLTR01**)
b) Using a 6mL disposable syringe, take 3mL of supernatant, followed by filling the syringe with room air
c) Attach the syringe to the top of the push-thru cartridge series
d) Place a 15mL centrifuge tube in a collection rack
e) Slowly push the supernatant through the cartridge series, followed by the 3mL of void volume in the syringe, into the 15mL centrifuge tube
f) Continue to push air through the cartridge with the syringe until ~2mL of extract is collected
g) Aliquot 500µL of extract into a 2mL polypropylene LC-MS/MS vial
h) Dilute with 500µL 2ng/mL internal standard (D5-6PPD-Q) in acetonitrile
i) Analyze on LC-MS/MS

HPLC/MS Parameters

HPLC Conditions	
HPLC System	SCIEX Exion LC
Delay Column	UCT Selectra® C18, 50 × 4.6 mm, 5 µm (p/n: **SLC-1850ID46-5UM**)
HPLC Column	UCT SelectraCore® C18, 50 × 2.1 mm, 2.7 µm (p/n: **SCS27-C18521**)
Guard Column	UCT SelectraCore® C18, 5 × 2.1 mm, 2.7 µm (p/n: **SCS27-C18GDC21**) Holder: **SLGRDHLDR-HPOPT**
Oven Temperature	45°C
Flow Rate	0.500 µL/min
Injection Volume	10 µL

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Gradient		
Time (min)	**Mobile Phase A (%) 0.2% Formic Acid (aq)**	**Mobile Phase B (%) Acetonitrile**
0	95	5
1.6	50	50
6.8	0	100
8.7	0	100
8.8	95	5
11	95	5
Total Run Time: 11 Minutes		

MS Conditions	
MS/MS System	ABSCIEX Qtrap 6500+
Ionization Mode	ESI +
Curtain Gas (CUR)	40
Collision Gas (CAD)	Medium
IonSpray Voltage (IS)	4500
Temperature (TEM)	600
Ion Source Gas 1 (GS1)	50
Ion Source Gas 2 (GS2)	50

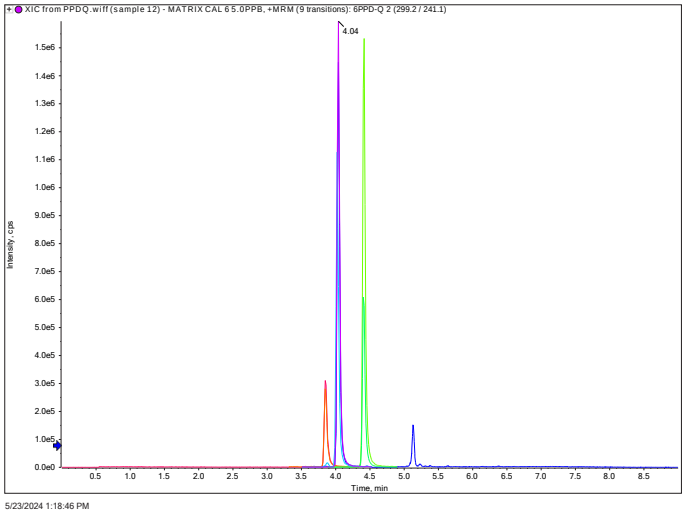

Figure 1. Analytes in Matrix at 18.2 ng per g

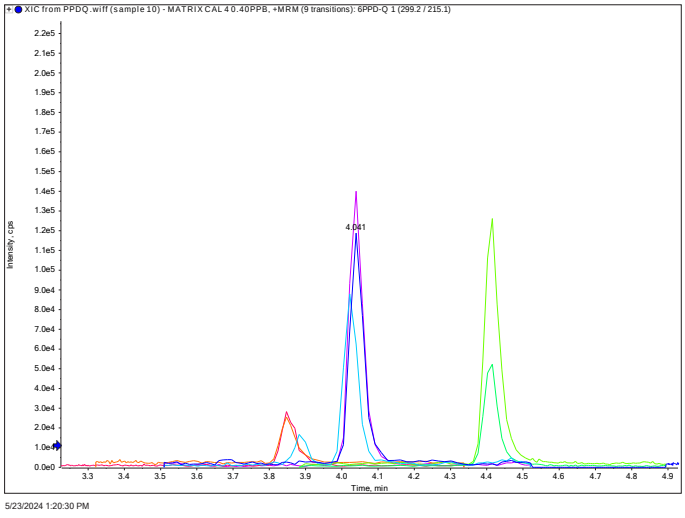

Figure 2. Analytes in Matrix (Zoomed) at 1.5 ng per g

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Calibration Table				
Compound	**RT (Minutes)**	**R^2**	**Calibration Range ng/g**	**Min. S/N**
6PPD-Q	4.0	0.998	0.073 - 73	21
7PPD-Q	4.4	0.999	0.073 - 73	29
CPPD-Q	3.9	0.999	0.073 - 73	13

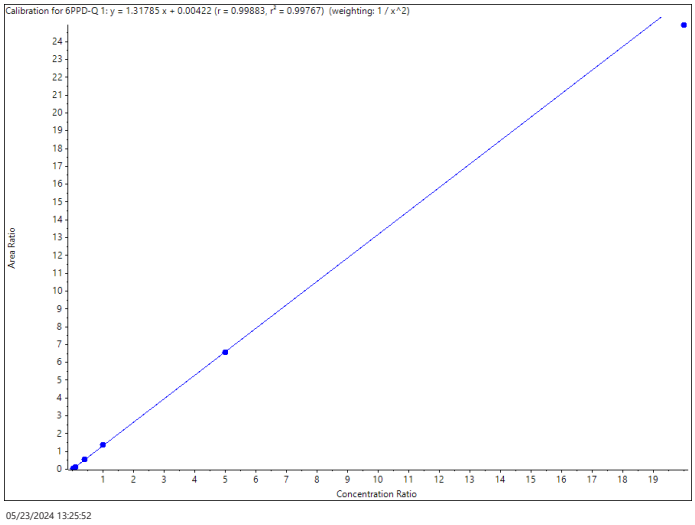


Figure 3. Calibration Curve for 6PPD-Q at 0.073 to 73 ng per g

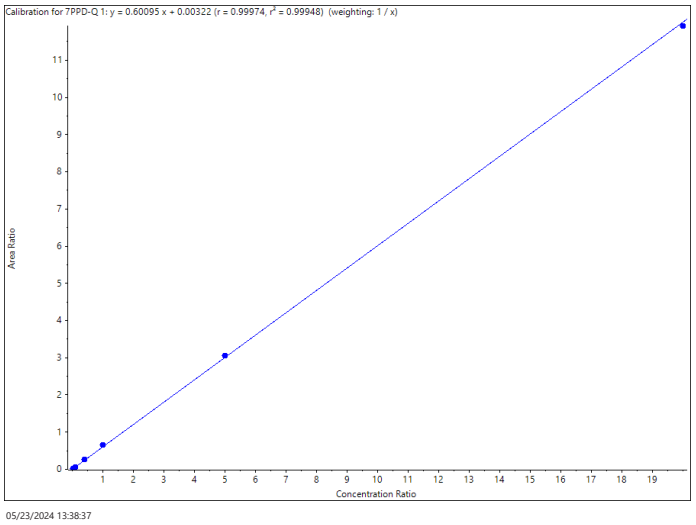


Figure 4. Calibration Curve for 7PPD-Q at 0.073 to 73 ng per g

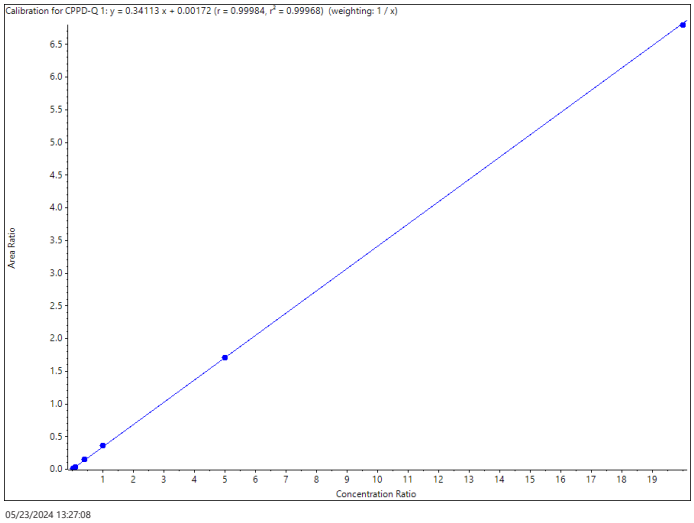


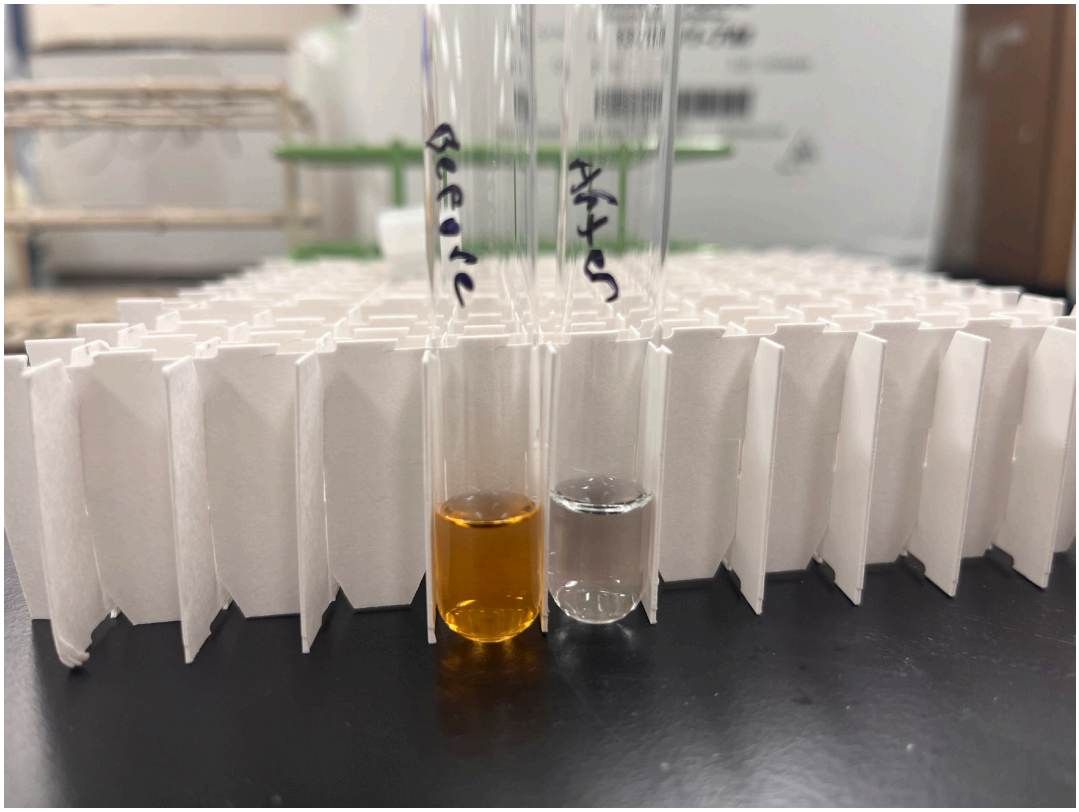
Figure 5. Calibration Curve for CPPD-Q at 0.073 to 73 ng per g

Results

Calculated Results in 5.5g Atlantic Salmon Extract								
Analyte Recovery								
Analyte	**0.073 ng/g (n=7)**				**3.64 ng/g (n=4)**		**18.2 ng/g (n=4)**	
	Matrix Effect Recovery	Recovery %	RSD(%)	LOD (ng/Kg)	Recovery %	RSD(%)	Recovery %	RSD(%)
6PPD-Q	+12	111	9.7	37	91	3.1	91	5.9
7PPD-Q	+7	71	11.4	18	99	12.3	97	13.2
CPPD-Q	+9	94	23.9	73	101	13.8	95	13.1

Extraction/Cleanup Efficiency						
Extracted Internal Standard	**Matrix Effect (%)**	***(%) Recovery** (n=22)**	**RSD (%)**	**Instrument Internal Standard**	**Matrix Effect (%)**	***RSD (%)**
13C6-6PPD-Q	+ 4	86	8.4	D5-6PPD-Q	+5	8.6

**Percent recovery of the extracted internal standard versus the instrumental internal standard
*Calculated by comparing area of 1.0 ng/g matrix matched standards (n=7) to solvent standards (n=3)



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Conclusion

The fatty, complex matrix of fish such as salmonids requires a thorough cleanup to achieve the needed sensitivity for PPD-Qs. With this procedure, PPD-Qs can be detected down to 0.073 ng/g in an extract of 5.5 grams of salmon tissue with acceptable signal-to-noise ratios. UCT's SelectraCore® C18 HPLC Column contains core-shell technology, which reduces backpressure on the LC and band broadening in the chromatography, allowing sharper peak shape and lower detection limits.

Quick QuEChERS® contains PSA to remove sugars, fatty acids, organic acids, and some pigments, while end-capped C18 removes long-chain fatty compounds, sterol, and other non-polar interferences. LipiFiltr® is designed to remove lipids from acetonitrile extracts without retention of analytes. These push-thru cartridges in series allow for a timely cleanup that reduces matrix effects below 12%. The extracted isotope dilution standard allows the most accurate quantitation and calculation of extraction efficiency. Extraction efficiency is 86%, and analyte recoveries meet 70-120% recovery limits with RSDs <15% (<25% at the LOQ).

References

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2. Screening p-Phenylenediamine Antioxidants, Their Transformation Products, and Industrial Chemical Additives in Crumb Rubber and Elastomeric Consumer Products ,Environmental Science & Technology 2023 57 (7), 2779-2791, DOI: 10.1021/acs.est.2c07014

3. Exploration of emerging environmental pollutants 6PPD and 6PPDQ in honey and fish samples,Food Chemistry, Volume 396,2022,133640,ISSN 0308-8146, https://doi.org/10.1016/j.foodchem.2022.133640

4. 6PPD-Quinone: Revised Toxicity Assessment and Quantification with a Commercial Standard, Environmental Science & Technology Letters 2022 9 (2), 140-146, DOI: 10.1021/acs.estlett.1c00910

UCT, LLC • 2731 Bartram Road • Bristol, PA 19007 800.385.3153 • 215.781.9255
www.unitedchem.com Email: methods@unitedchem.com

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