

Quantification of Caffeine as an Anthropogenic Marker in Western Lake Superior

A Thesis

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ABSTRACT

Caffeine (1,3,7-trimethylxanthine) is a widely used anthropogenic marker to trace untreated wastewater in natural water samples. Understanding possible locations of wastewater inputs and the extent to which contaminants in untreated wastewater can spread is significant in maintaining and preserving Lake Superior's ecosystems and water quality. For this study, an existing method based on solid phase extraction using hydrophilic-lipophilic-balanced (HLB) resin and electrospray mass spectrometry was revised and applied to quantify the caffeine content in western Lake Superior. Method development addressed sample preparation techniques, determinations of the limits of detection and quantification of caffeine, and quantitative analysis for analyte ion fragmentation. Once the method was optimized for analyte quantification, western Lake Superior surface water samples were collected at 5 stations, including off and near shore sites, to gain insight into possible contaminant distributions. Caffeine measurements ranged from 5-26 ng/L with higher concentrations near shore and depleted concentrations offshore.

1. INTRODUCTION

Caffeine as a Wastewater Tracer

Anthropogenic tracer molecules are used in marine and freshwater ecosystems to analyze the impacts of various routes of contamination such as urban runoff, atmospheric inputs from transport or industrial processes, direct and indirect inputs from agricultural activities, and human waste contamination. Potential wastewater inputs into aquatic systems have a direct negative impact that could lead to serious environmental issues due to their delivery of nutrients, bacteria, and harmful contaminants. Traditionally, wastewater inputs into aquatic ecosystems are analyzed by general water quality parameters, such as implementing bacterial indicators (e.g. coliform counts). However, other studies have utilized chemical tracer molecules to analyze possible contamination due to the better source specificity and reliability of the tracers, shorter analysis times, and to address concerns about direct chemical inputs or the persistence of biologically active molecules during wastewater treatment (Ogunseitan, 1996; Glassmeyer et al., 2005; Peeler et al., 2006). Common anthropogenic tracer molecules for wastewater emissions include compounds from household chemicals, pharmaceuticals, personal care products, and biogenic hormones (Kolpin et al., 2002, Weigel et al., 2004; Wu et al., 2009; Zhou et al., 2010; Hedgespeth et al., 2012; Blair et al., 2013; Ferrey et al., 2015; and others).

The Great Lakes make up 84% of North America's fresh water and about 21% of the world's supply of fresh surface water (USEPA, 2012). Only a few studies have

analyzed wastewater contaminants in the Great Lakes watersheds, and these studies have focused on rivers, near shore, and tributary sites (Metcalf et al., 2003; We et al., 2009; Li et al., 2010; Csiszar et al., 2011). None of these studies have collected and analyzed wastewater tracer molecule data for open-water Lake Superior. Lake Superior is the world's largest lake by surface area, exhibiting a complex hydrodynamic system and high dilution of riverine inputs. The St. Louis estuary and the region encompassing the western arm of Lake Superior is the most highly populated area in the Lake Superior watershed, and thus the far-western lake is a key location for investigating potential wastewater emissions and contaminant distributions within Lake Superior's surface waters. Previous studies have reported areas within the Lake Superior watershed to have considerable caffeine concentrations, indicating the importance of extending caffeine measurements to Lake Superior itself (Ferrey, 2011; Christensen et al., 2012).

The identification of pharmaceutical and personal care product residues in a water system indicates a serious ecological concern as wastewater can deliver pathogens, unknown transformation byproducts, and endocrine disrupting chemicals (EDC) (Uslu et al., 2011; Jongh et al., 2012; Bolong et al., 2009; and others). The detection of antibiotic resistant bacteria in surface water biofilms also suggests a serious threat as such bacteria provide a source of transferable resistant traits for emerging pathogens derived from untreated wastewater (Ash et al., 2002; Schwartz et al., 2003). Untreated wastewater containing contaminant residues and biofilms can cause aquatic organisms to become susceptible to stunted growth, obtain reproductive problems, and developmental mutations, while humans are vulnerable to serious health risks upon exposure (Cevasco et

al., 2008; Hogan et al., 2008; Owen et al., 2007; Schwartz et al., 2003). This is an expanding area of research as there is marginal data for the toxicity and bioaccumulation of PPCP and prescription drug residues present in trace quantities. Caffeine itself is considered a low-risk toxicity contaminant in surface waters; however, utilizing it as an anthropogenic marker for wastewater emissions can help us further understand the spatial distribution of such emissions before they seriously impact the aquatic and human population (Wu et al., 2014). Caffeine was chosen as the tracer molecule of interest for this study due to its common consumption by humans, measurable quantities in untreated wastewater, and its physical and chemical stability. Therefore by quantifying caffeine, the potential extent and impact of more harmful contaminants that enter Lake Superior through sewage overflow, septic system and sewer line leakages, etc. can be determined.

Caffeine is one of the most commonly proposed indicators of human-derived waste, and has been utilized as a traceable biomarker for the spread of untreated wastewater contaminants in rivers, lakes, groundwater, and marine environments (Hillebrand et al., 2012; Loos et al., 2013; Glassmeyer et al., 2005, Weigel et al., 2004; Zhou et al., 2010; and others). Caffeine has been reported to be a stable compound under variable conditions in natural aquatic systems. It has been measured in remote locations with minimal human impacts, suggesting compound stability within aquatic ecosystems (Weigel et al., 2004; Kurissery et al., 2012). Caffeine has a high water solubility of >20 g/L at 25 °C (Yalkowsky et al., 2016) and a low octanol-water partition coefficient ($\log k_{ow} < 0$), showing negligible sorption to solids and thus negligible sedimentation (Gaspari & Bonati, 1987; Standley et al., 2000). Caffeine also has negligible volatility

due to a low Henry's law constant (1.1×10^{-11} atm m³/mol at 25 °C) (EPA, 2012).

Globally, caffeine is ranked as one of the top most commonly consumed ingredients, as it is a component in many beverages, foods, and pharmaceuticals (Gardinali et al., 2002; Heckman et al., 2010). Over 60 plant species naturally produce caffeine, however, most are found in tropical ecosystems and therefore caffeine excreted by plants is negligible in this study of caffeine in surface water collected from temperate Lake Superior (Bradley et al., 2006). The average daily consumption of caffeine by Americans is 168 mg per day, with <5% of it excreted unchanged in the urine (Heckman et al., 2010; Magkos and Kavouras, 2005). The caffeine compound is metabolized into more than 20 metabolites, primarily dimethylxanthines (e.g. paraxanthine), which are not found in plants or food (Buerge et al., 2003; Heckman et al., 2010; Stavric, 1988). Most of the caffeine in wastewater treatment plant (WWTP) influents enters the sewer system by the disposal of coffee, tea, soda, etc. down the drain, which accounts for most of the caffeine found in WWTP effluents all over the world. Caffeine is efficiently removed (>99%) in well-functioning wastewater treatment plants; concentrations of caffeine in untreated wastewater are usually orders of magnitude higher than in treated wastewater (Ternes et al., 2001; Loos et al., 2013; Buerge et al., 2006; Blair et al., 2015). Thus, measurable caffeine concentrations in Lake Superior would most likely indicate the presence of untreated wastewater contamination in lake water.

Wastewater Tracer Analysis

Extraction Techniques

Assessing caffeine in natural water samples, as compared to source beverages or plants, becomes a competence challenge for analytic analysis. Caffeine is generally present in trace amounts in the water column (e.g parts per trillion), therefore samples must be concentrated in order to quantify above the instrumental detection limit. Solid phase extraction (SPE) is a separation technique for concentrating analytes from natural matrixes for further quantitative or qualitative analysis. SPE provides environmental and safety benefits over traditional liquid-liquid extraction techniques by limiting hazardous organic solvent waste. Various SPE cartridges have been used for molecule tracer studies where multiple chemical compounds are usually analyzed. C₁₈ cartridges were tested for extracting caffeine; however, Oasis hydrophilic-lipophilic-balanced (HLB) cartridges are currently considered the primary sorbent for pharmaceuticals and have been utilized in many studies that included caffeine analysis (Buchberger, 2011; Loos et al., 2013; Verenitch & Mazumder, 2008; Zhou et al., 2010; Kurissery et al., 2012; and others). The HLB cartridge resin consists of a mixture of hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene (Waters, 2014). Inconsistent flow rate and repeated cartridge dryness are reported not to hinder extraction efficiency, therefore they were used for this study (Waters, 2014). Table 1.1 provides an overview of cartridge efficiencies where an isotopically labeled caffeine compound was added to the matrix prior to extraction, and quantified using optimized instrumentation.

Table 1.1: Previous cartridge efficiencies for caffeine. Error is associated with duplicate or *n* sample analyses.

| Cartridge | Matrix | pH Adjustment | % Spike Recovery | Instrumentation | Reference |
|--------------------------|---------------------|---------------|----------------------|-----------------|----------------------------|
| Oasis HLB | | | | | |
| 200 mg, 6cc | Ultrapure Water | Non | 72 | UHPLC-QTRAP/MS | Loos et al., 2013 |
| | | 7.5 | 80-103 | IT-GC/MS/MS | Verenitch & Mazumder, 2008 |
| | | < 4 | 92 (± 13 , n=3) | UPLC-MS/MS | Zhou et al., 2010 |
| | Rivers | < 4 | 84 (± 8 , n=3) | UPLC-MS/MS | Zhou et al., 2010 |
| | | 7.5 | 66-98 | IT-GC/MS/MS | Verenitch & Mazumder, 2008 |
| | | Lakes | 7.5 | 61-116 | IT-GC/MS/MS |
| | WWTP Effluent | 7.5 | 65-120 | IT-GC/MS/MS | Kurissery et al., 2012 |
| | | < 4 | 89 (± 14 , n=3) | UPLC-MS/MS | Zhou et al., 2010 |
| 500 mg, 6cc | | Rivers | Non | 97 | ESI-LC/MS/MS |
| | Lakes | 8 | 71-118 | ESI-LC/MS/MS | Li et al., 2010 |
| C ₁₈ | | | | | |
| 500 mg | MQW | Non | 85 (± 3 , n=5) | HPLC/DAD | Chen et al., 2002 |
| | WWTP Effluent | 7-7.5 | 41 (± 1 , n=3) | ESI-LC/MS/MS | Ternes et al., 2001 |
| | Rhine Surface water | 7-7.5 | 48 | ESI-LC/MS/MS | Ternes et al., 2001 |
| <i>r</i> C ₁₈ | Groundwater | 6-8 | >75 | GC/MS | Nakada et al., 2008 |

Solvent Techniques

The primary eluent for releasing caffeine from the HLB cartridges was methanol. Caffeine is soluble in many solvents including methanol with a solubility of 1.36 g/L at 25 °C (Shalmashi & Golmohammad, 2010). The volume of methanol employed varied between studies depending on resin amount and particle size (see Table 1.2); a modified EPA 1694 method from Ferrer et al., 2010 was applied in this work. A wash step prior to caffeine elution was also utilized for some HLB cartridges shown in Table 1.2. For this study, a series of washes (from a method by Waters, 2014) were used to remove additional interferents and enhance analyte recovery (see Table 2.1).

Table 1.2: Elution and wash steps from previous studies in relation to resin amount and particle size for analyte recovery.

| HLB Cartridge (mg) | Particle Size (μm) | Wash Steps | Elution Solvent | Reference |
|-------------------------------|---|----------------------------|---|---|
| 1000 | 60 | 10 mL Reagent Water | 12 mL Methanol | Englert, 2007 |
| 60-540 | 30/60 | Optional | 2-4 mL Methanol | Waters, 2014 |
| 200 | 30 | 2 mL 25% Methanol/Water | 1 mL Methanol; 6 mL Methanol/methyl tert-butyl ether (1:9) | Verenitch & Mazumder, 2008; Kurissery et al., 2012 |
| ∞ 200 | 30 | 20 mL Milli-Q | 6 mL Methanol | Loos et al., 2013 |
| 200 | 30 | Non | 6 mL Methanol | Zhou et al., 2010 |
| 500 | 60 | Non | 8 mL Methanol | Ferrer et al., 2010 |
| 200 | 30 | 2 mL Methanol | 3 x 3 mL 2% Formic acid in Methanol | Li et al., 2010 |

ESI-MS/MS Techniques

Anthropogenic tracer molecules are primarily quantified through mass spectrometry analysis. Gas chromatography mass spectrometry (GC-MS) analyses have been previously utilized for caffeine quantification (Buerge et al., 2006; Bradley et al., 2007; Kurissery et al., 2012 and others); however liquid chromatography mass spectrometry (LC-MS) offers an additional benefit over GC-MS through simplified sample preparation techniques developed for complex matrixes. Previous studies have utilized ESI-LC/MS/MS for analyte quantification (Ternes et al., 2001; Englert, 2007; Ferrer et al, 2010; Wu et al., 2014; Lindholm et al., 2016) where a solvent gradient mobile phase was applied for LC column separation upon ionization. This is beneficial for reducing spectral interferences upon quantifying a series of analytes. However, in this caffeine-focused study, the use of an LC separation step and a solvent gradient mobile phase was unnecessary. Sample carryover issues were observed in preliminary data utilizing an LC column without a solvent gradient mobile phase. This was minimized using a solvent gradient, however, the sample analysis time dramatically increased which seemed unnecessary for analyzing one analyte. The elimination of the LC column and use of direct sample injection into the electrospray ionization source eliminated detectable carryover and reduced analysis time. It can also provide enhanced sensitivity due to lower sample dilution caused by an increased mobile phase volume for LC column separation. Since chromatographic separation was eliminated, multiple reaction monitoring (MRM) transitions were used to minimize interference from the sample matrix in the analyses for

natural caffeine. This detection technique simplified the analytical protocol and provided sufficient caffeine identification.

Project Objectives

While caffeine concentrations in the St. Louis River and estuary, a key tributary system in western Lake Superior, have been studied (Ferrey, 2011; Christensen et al., 2012), the presence of untreated wastewater containing harmful contaminants has not been much addressed in western Lake Superior itself. Therefore, the objective of this study was to modify previous anthropogenic tracer methods to efficiently extract low concentrations of caffeine from natural water samples and to quantify the caffeine concentrations in western Lake Superior as an unambiguous tracer of anthropogenic inputs most likely from untreated wastewater. Personal care products, pharmaceuticals, artificial sweeteners, and other substances have also been used to trace wastewater inputs. However, because caffeine has been previously measured in significant quantities in untreated wastewater and exhibits low reactivity within surface waters, caffeine was considered the best choice for this study.

2. METHOD

Analytical Method Development

Standard Preparation

Natural caffeine and ^{13}C -labeled caffeine working standards (see Appendix A for calibration standards and correlating concentrations) were prepared in glass autosampler vials on the day of MS analysis. Aliquots of stock solutions of ^{12}C -caffeine or $^{13}\text{C}_3$ -caffeine were diluted to a final volume of 1.5 mL in a solution of 90% HPLC grade water, 0.1% ACS certified formic acid, and 10% LC-MS grade methanol. The ^{12}C -caffeine stock solution was prepared by weighing powdered caffeine (Sigma, Ultra) on a microbalance into a tin capsule that was directly added to a volumetric flask, and then diluted with 90% water, 0.1% formic acid, and 10% methanol. The $^{13}\text{C}_3$ -caffeine stock solution was prepared by making a 90% water, 0.1% formic acid, and 10% methanol solution using a pre-prepared diluted stock from the received 1 mg/mL caffeine- $^{13}\text{C}_3$ -solution in methanol as delivered by Sigma-Aldrich (product # 603295). The more dilute standards were prepared directly from the 90:10 water methanol stock; the more concentrated standards were made after concentrating aliquots of the diluted methanol stock of $^{13}\text{C}_3$ -caffeine by drying at 50°C under an N_2 gas stream (using a Flexivap apparatus) and re-dissolving in 90% water, 0.1% formic acid, and 10% methanol. Linear regression relationships between concentration and precursor to product ion response were determined for both ^{12}C -caffeine and $^{13}\text{C}_3$ -caffeine.

Solid Phase Extraction

A Waters Oasis HLB cartridge is designed for use with a wide range of polar compounds, including acidic, neutral, and basic compounds across a pH range of 0-14 (Water, 2014). pH comparisons of sample water prior to extraction show that there was little variation in recovery as a function of pH; the HLB resin performed similarly under neutral, acidic, and alkaline conditions (Ferrer et al., 2010). Therefore in this method, collected water samples were extracted using 3cc/540 mg (60 μ m) Waters Oasis HLB cartridges without prior pH adjustment. To optimize recoveries and remove additional interferences, a series of washes after sample loading were employed in this study. The order of cartridge washes is listed in Table 2.1. Each wash was collected and analyzed separately by MS to investigate possible caffeine losses.

Table 2.1: Wash series for column efficiency.

| | |
|---------|---|
| Wash #1 | 2mL of 95% water 5% methanol |
| Wash #2 | 2mL of 2% formic acid in 95% water 5% methanol |
| Wash #3 | 2mL of 5% ammonium hydroxide in 95% water 5% methanol |

All glassware used in the extraction process was rinsed with deionized water and a series of solvents (methanol, acetone, and then hexane) between samples. Samples and method blanks were loaded onto SPE cartridges on the same day. Two glass separatory funnels and one Teflon separatory funnel, all three with Teflon stopcocks, were used to hold the initial sample (or method blank) and to control the flow of sample by gravity

onto the extraction cartridges. Extraction apparatus was pressurized using a hand pump with a pressure gage for controlling and for monitoring flow rate. After extraction, all cartridges were labeled and refrigerated in individual ziplock bags until caffeine elution the following day. Before concentrating samples, Flexivap needles were rinsed in the same manner as extraction glassware, and any additional glassware used was methanol rinsed. Sample eluents were concentrated under a N₂ gas stream using a Flexivap apparatus set at 50°C and re-dissolved in 90% water, 0.1% formic acid, and 10% methanol. For all samples and method blanks, the initial volume of sample loaded onto a cartridge was 1000 mL; final volume after elution and concentration of the eluate was 500 µL leading to a concentration factor of 2,000-fold. This was estimated to place the natural samples within the detectable range of the MS protocol (see Appendix B).

Instrument Analysis and Quantification

Caffeine concentrations in the extracts were analyzed by direct injection using an Agilent 6460 triple-quadrupole tandem LC-MS system with an Agilent 1260 infinity injector. The injection volume was 15 µL with a mobile phase flow rate of 0.2 mL/min; the caffeine retention time was 0.2 minutes and each analysis was performed over 3 minutes to minimize carryover between samples. A mobile phase of LC-MS Chromasolv grade 90% water with 0.1% formic acid and 10% HPLC grade methanol was used as it led to increased sensitivity relative to 100% methanol. Samples were diluted in the same solution as the mobile phase to avoid additional matrix effects. After LC injection, the samples were directly introduced into the ESI source before entering the mass

spectrometer. Since no supplementary analytes were quantified and MRM appeared to provide sufficient resolution for caffeine quantification, no LC column was used. Direct injection also eliminated caffeine carryover between samples, as was seen in preliminary data using a reverse phase C₁₈ LC column. The source parameters for the mass spectrometer are shown in Table 2.2 and follow existing SOP protocols (Ferrer et al., 2010).

The triple quadrupole LC-MS system was run using positive ion electrospray in MRM mode for the chromatograph segments of caffeine and labeled ¹³C₃-caffeine. The fragmentor voltage, collision energies and MRM transitions for caffeine and labeled caffeine previously optimized for the Agilent 6460 triple quadrupole LC-MS system (Ferrer et al., 2010) were used for caffeine analyses within this study. The MRM chromatograph segment details are shown below in Table 2.3. The fragmented product ions were analyzed using MassHunter Qualitative Analysis Software (Agilent) using manual peak integration. For natural caffeine, the first ion segment transition (195→138 m/z) is used as the quantifier ion given its higher abundance, and the second transition (138→110 m/z) is used as a qualifier for caffeine confirmation. Since samples underwent direct injection without prior LC column separation, MRM transitions provide more accurate ¹²C caffeine identification, removing additional compound interferences. Labeled caffeine was analyzed by a single ion transition, and a qualifier ion transition did not seem necessary. The abundance ratio between the qualifier and quantifier ion is fixed from a method-determined value of 0.2 (or 20%), with a tolerance for ratio acceptance of ±20%. Product ion identifications (Zumwalt et al., 2007) are predicted based on known

fragmentation patterns (Figure 2.1) and the elemental composition of the fragments.

Labeled caffeine is assumed to undergo similar ionization and fragmentation for product ion quantification.

Table 2.2: ESI-MS/MS Source Parameters.

| | |
|--------------------------|------|
| Gas Temp (°C) | 250 |
| Gas Flow (L/min) | 10 |
| Nebulizer Pressure (psi) | 45 |
| Sheath Gas Temp (°C) | 375 |
| Sheath Gas Flow (L/min) | 11 |
| Capillary Voltage (V) | 4000 |
| Nozzle Voltage (V) | 0 |

Table 2.3: MRM Segment Details.

| Compound | Precursor Ion Mass (m/z) | Product Ion Mass (m/z) | Dwell (ms) | Fragmentor Voltage (V) | Collision Energy (eV) | Cell Acceleration (V) | Polarity |
|--|-------------------------------------|-----------------------------------|-----------------------|-----------------------------------|----------------------------------|----------------------------------|-----------------|
| Caffeine | 195 | 138 | 200 | 110 | 15 | 7 | Positive |
| Caffeine | 195 | 110 | 200 | 110 | 25 | 7 | Positive |
| ¹³ C ₃ -Caffeine | 198 | 140 | 200 | 110 | 15 | 7 | Positive |

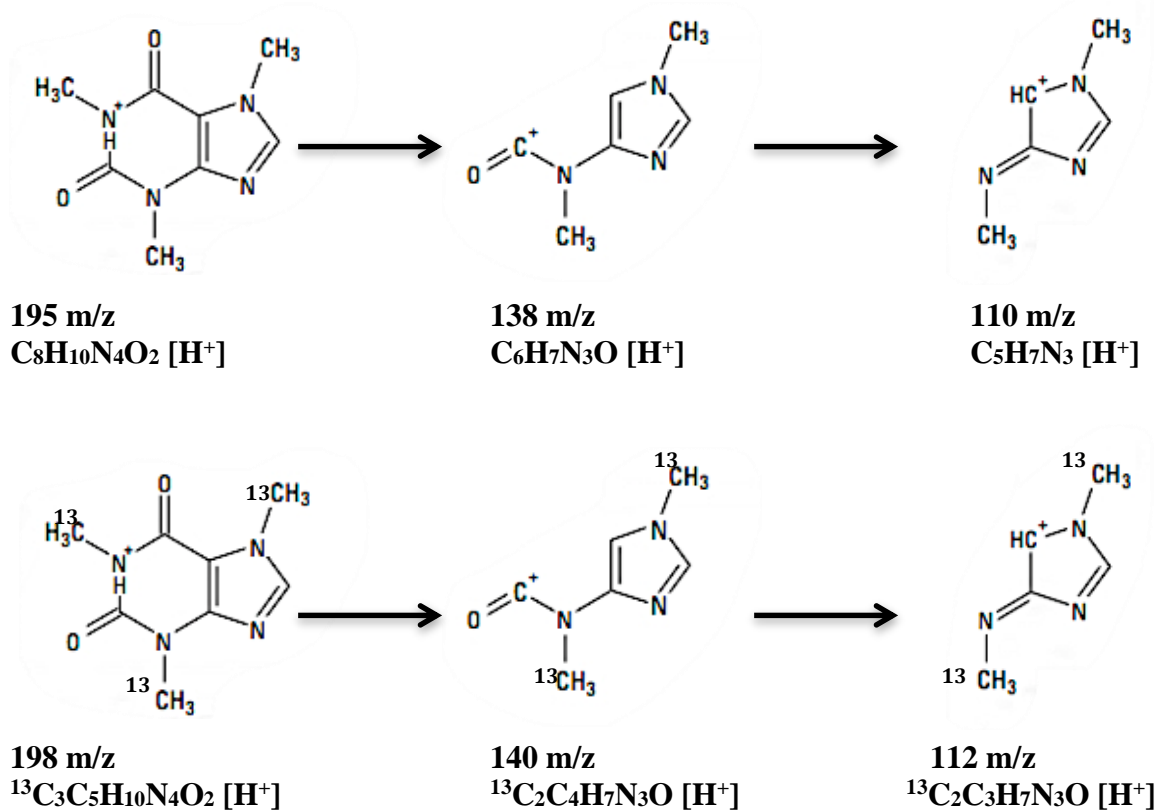


Figure 2.1: Predicted ionized caffeine molecule and fragmented ion structures.

Quantifying Internal Standard Recoveries

$^{13}C_3$ -caffeine is used as an internal standard to monitor solid phase extraction efficiency and to compensate for any natural caffeine loss (Kurissery et al., 2012; Buerge et al., 2003; Loos et al., 2013; and others). For this study, the internal standard was also used to correct for any caffeine loss during sample storage (e.g sorption and/or degradation). The internal standard is assumed to have the same physical and chemical behavior as ^{12}C -caffeine, therefore, all surface water samples were spiked with the internal standard on the day of collection to monitor this effect. The spike for each 1 L surface water and method blank samples was 1 mL of a 500,000 ng/L $^{13}C_3$ -caffeine stock

solution (in methanol). Samples were concentrated to a final volume of 0.5 mL in 90% water, 0.1% formic acid, and 10% methanol solution for a final concentration of 1,000 µg/L for $^{13}\text{C}_3$ -caffeine. Three injections of the wash filtrates and methanol eluent samples were analyzed by MS, if above the minimum detection signal (MSD) and calculated lower limit of detection (LLOD) concentration. These wash concentrations were combined mathematically for sufficient internal standard recovery and caffeine quantification. Based upon the EPA 1694 method, the acceptable internal standard recovery range is 31-200% (Englert, 2007). All reported error in this study is based upon instrument reproducibility among replicate injections, and propagation of error (following Harris, 2010) is utilized for calculations (see Appendix C for details).

Quality Control

A method blank (consisting of 1000 mL of HPLC-grade water) and 2 spiked method blanks (where $^{13}\text{C}_3$ -caffeine was added to address extraction efficiency) were processed in the same manner as samples in order to correct for contamination during sample processing. The 2 spiked method blanks used $^{13}\text{C}_3$ -caffeine added at the same concentration as in the collected surface water samples. The recoveries of the internal standard were calculated using the ion segment 198→140 m/z for $^{13}\text{C}_3$ -caffeine to determine the concentration using the linear regression equation from the $^{13}\text{C}_3$ -caffeine calibration plot. The recovery of this isotopically labeled internal standard (approximately 42-130% for spiked samples and method blanks) was used as a conversion factor to adjust the caffeine recovery data to initial caffeine concentrations in

the lake water samples. The natural or ^{12}C -caffeine concentration was calculated using the ion segments $195 \rightarrow 138$ m/z from instrument response, and calculated using the linear regression equation from the ^{12}C -caffeine calibration plot measured at the beginning of the MS run. This concentration was then multiplied by the spike-determined correction factor to calculate the true concentration of ^{12}C -caffeine present in the method blanks and surface water samples (see Appendix C and I for an example calculation and data details respectively). The 2 method blanks were averaged together and used to blank correct the calculated natural caffeine concentrations in the Lake Superior surface water samples to eliminate possible caffeine contamination during sample preparation.

All samples and method blanks were measured in triplicate; instrument blanks consisted of multiple injections dispersed through the analyses. A few instrument blanks had area responses above the minimum detection limit. Therefore to correct for any caffeine carryover, all samples, standards, and method blank area response replicates were instrument blank-corrected using the average of the multiple instrument blank peak areas for correlating ion transitions. This was done prior to all quantitation calculations.

Potential ion suppression or enhancement was monitored using periodic check standards for natural and labeled caffeine compounds. The calibration curve was also run twice (at the beginning and near the end of the suite of sample analyses) to further correct for instrument drift. The caffeine concentrations for sample stations were calculated using the beginning calibration curve and the wash eluent samples, analyzed near the end of the sample suite, were calculated using the calibration curve that was rerun towards the end based on correlating ion segments. Total concentrations are acquired from concentrated

samples, and the actual caffeine concentration in each sample is calculated by dividing the total caffeine by the 2,000-fold concentration factor (from SPE processing).

LLOD and LLOQ

The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) for ^{12}C and $^{13}\text{C}_3$ -caffeine were determined for the Agilent 6460 triple-quadrupole tandem LC-MS using methods defined in Harris (2010). The same series of standards used for calculating the concentration of extracted caffeine samples were used for determining the detection and quantification limit. A new instrument blank of 90% water, 0.1% formic acid, and 10% methanol was prepared. The instrument blank was analyzed 7 times, along with the lowest calibration standard to determine the instrument's minimum detection signal, defined as 3 times the standard deviation of the low concentration standard plus the average of the blank response. The rest of the calibration standards were analyzed to create a linear regression for calculating the detection and quantification limit in terms of analyte concentration. Assuming the linear regression y-intercept is equal to the average of the instrument blank response, the LLOD is defined as the 3 times the standard deviation of the low concentration standard divided by the linear regression slope. The LLOQ is defined as 10 times the standard deviation of the low concentration standard divided by the linear regression slope. See Table 2.4 for MSD, LLOD, and LLOQ for the correlating caffeine compounds. Additional information is given in Appendix D.

Table 2.4: Results from instrumental caffeine limitations.

| | Minimum Detection Signal | LLOD (ng/L) | LLOQ (ng/L) |
|---|---------------------------------|--------------------|--------------------|
| ¹² C-Caffeine: | 124 | 558 | 1859 |
| ¹³ C ₃ -Caffeine: | 117 | 336 | 1220 |

Statistics

Linear regression, t-tests, and ANOVA were performed using Excel 2013.

Field Application

Sampling Stations

Surface-water samples for caffeine were collected at 5 stations (A-E, Figure 2.2) in western Lake Superior on May 13th, 2016. Note that E represents the near shore (close to St. Louis Estuary) sample and D is the most offshore sample. See Appendix E for further sampling site information.



Figure 2.2: Western Lake Superior sample collection stations.

Surface Water Collection

Water from 5 m water depth was collected in western Lake Superior aboard the R/V Blue Heron using a CTD rosette with attached Niskin bottles for water collection. The surface water was positive-pressure filtered through a $\sim 0.7 \mu\text{m}$ (GF/F) filter using nitrogen gas and stainless steel pressure canisters. For caffeine samples, the filtrate was collected into combusted 1-liter glass bottles and refrigerated (<12 hours) until return to the Large Lakes Observatory. Samples were then spiked with $^{13}\text{C}_3$ -caffeine and refrigerated until SPE.

Analytic Techniques

Prior to extraction, samples were brought to room temperature. Extraction was performed using 3cc/540 mg (60 μ m) Waters Oasis HLB cartridges. Each cartridge was pre-treated with 2 mL of LC-MS grade methanol followed by 2 mL HPLC grade water prior to sample extraction. 1-liter water samples were loaded onto the column at a flow rate <15 mL/min. After extraction, the outside of the cartridge was rinsed with methanol to remove any possible contamination. The column was then dried for approximately 5 minutes with an N₂-stream to remove any water residue on the resin. A series of 3 washes was eluted through the column using N₂ pressure and collected directly into a LC-MS vial. 8 mL of methanol was then used to elute caffeine from the column into a glass test tube; the sample was then blown down to a volume of approximately 1 mL with an N₂ gas stream on a Flexivap at 50°C. This was then transferred into a LC-MS vial using Pasteur pipets followed by 3 rinses of methanol. The 3 wash samples and caffeine sample were concentrated via N₂ gas stream to near dryness and reconstituted to a quantitative volume of 0.5 mL in 90% HPLC grade water, 0.1% ACS certified formic acid, and 10% LC-MS grade methanol. All sample were sonicated and vortex mixed before ESI-MS/MS analysis.

3. RESULTS

Caffeine Standard Calibration Curves

^{12}C -caffeine and $^{13}\text{C}_3$ -caffeine standard calibration series were analyzed at the beginning and end of the sample suite (see Figures 3.1a & 3.1b). Instrument sensitivity significantly decreased through the course of the run; it is hypothesized that this is due in part to a decrease in source cleanliness over the course of the ~21 hours it took to analyze our standards, samples, and blanks. The shift in sample solvent composition for the wash 1 and wash 2 samples analyzed near the end of the analyses may also have played a role. Therefore, the caffeine concentrations within samples, blanks, and washes were quantified using the linear regressions for the calibration standards analyzed closest to the time of sample analysis; i.e., the methanol extracts (analyzed first) were quantified using the beginning calibration curves (Figure 3.1a) and the washes (analyzed near the end of the run) were quantified using the later calibration curves (Figure 3.1b).

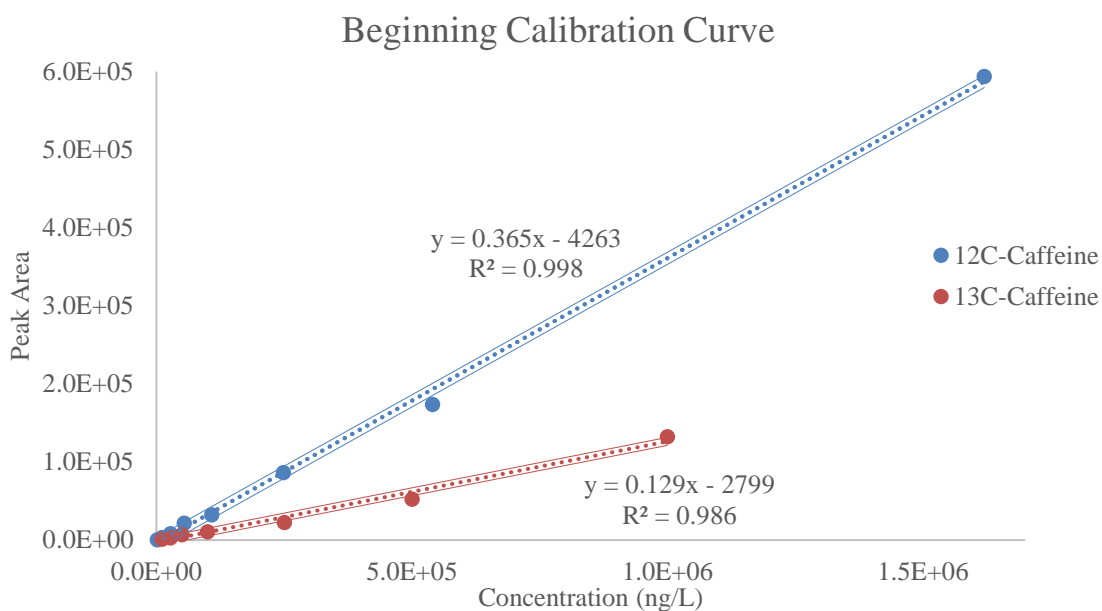


Figure 3.1a: Beginning calibration curve for natural and labeled caffeine. Solid lines represent upper and lower 95% confidence intervals, dashed line represents the linear regression fit.

Table 3.1a: Regression statistics for beginning calibration curves for natural and label caffeine standards.

| | Parameter | Coefficients | Standard Error | 95% CI |
|--|-----------|--------------|----------------|----------------|
| ¹² C-Caffeine | Intercept | -4263 | 3811 | [-13589, 5062] |
| | Slope | 0.365 | 0.006 | |
| ¹³ C ₃ -Caffeine | Intercept | -2799 | 2534 | [-9000, 3401] |
| | Slope | 0.129 | 0.006 | |

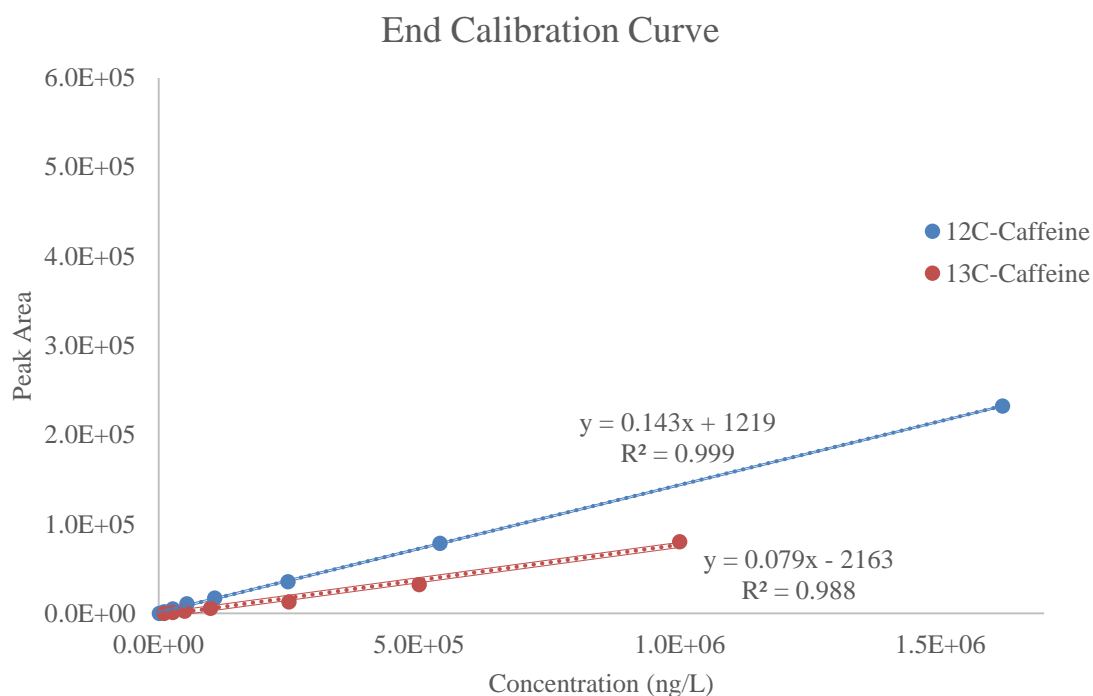


Figure 3.1b: End calibration curve for natural and labeled caffeine. Solid lines represent upper and lower 95% confidence intervals, dashed line represents the linear regression fit.

Table 3.1b: Regression statistics for end calibration curves for natural and label caffeine standards.

| | Parameter | Coefficients | Standard Error | 95% CI |
|--|-----------|--------------|----------------|---------------|
| ¹² C-Caffeine | Intercept | 1219 | 451 | [116, 2322] |
| | Slope | 0.143 | 0.0007 | |
| ¹³ C ₃ -Caffeine | Intercept | -2163 | 1453 | [-5719, 1393] |
| | Slope | 0.079 | 0.004 | |

Natural caffeine and labeled caffeine surprisingly yielded significantly different calibration curves (e.g. Figure 3.1a) (statistical regression data is shown in Tables 3.1a & 3.1b). Ionization and fragmentation patterns within MS analyses are assumed to behave similarly for isotopologues. However, because each slope is significantly different to its isotopologue ($p < 0.05$, $t > 2.179$), further investigation was implemented to determine whether this difference is primarily from ionization or fragmentation. The calibration standards were reintegrated from the precursor ion only (see Figure 3.2) (statistical regression data is shown in Table 3.2), where it was observed that the slopes were indeed significantly different ($p < 0.05$, $t > 2.179$) suggesting that calibration curve variance between these isotopologues takes place prior to fragmentation.

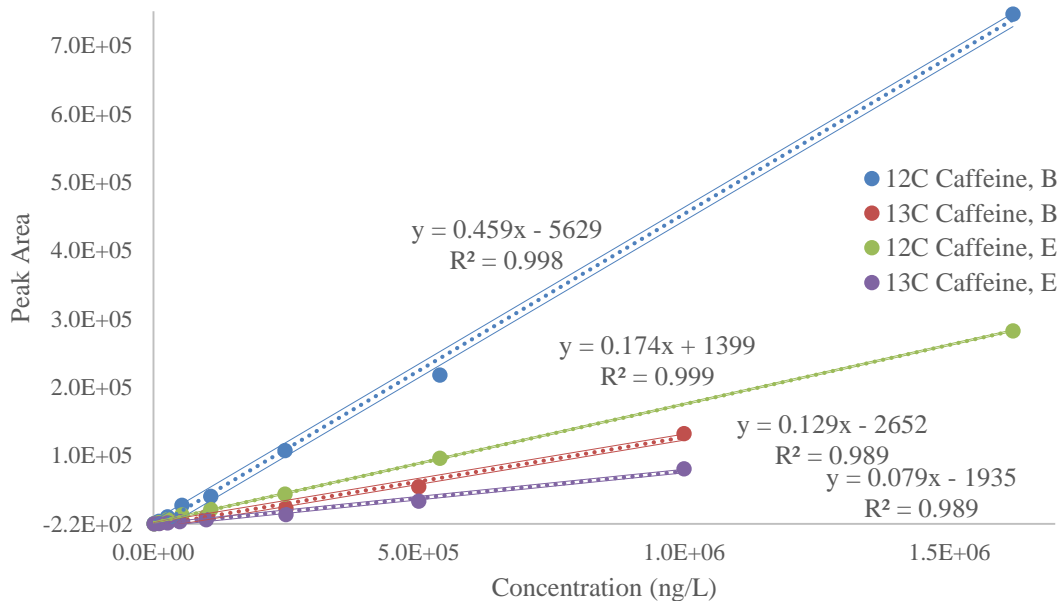


Figure 3.2: Beginning (B) and end (E) calibration curves from precursor ion integration for natural and labeled caffeine. Solid lines represent upper and lower 95% confidence intervals, dashed line represents the linear regression fit.

Table 3.2: Regression statistics for beginning and end calibration curves from precursor ion integration for natural and label caffeine.

| | Parameter | Coefficients | Standard Error | 95% CI |
|---|-----------|--------------|----------------|----------------|
| ¹² C-Caffeine, B | Intercept | -5629 | 4824 | [-17433, 6175] |
| | Slope | 0.459 | 0.008 | |
| ¹³ C ₃ -Caffeine, B | Intercept | -2652 | 2318 | [-8325, 3021] |
| | Slope | 0.129 | 0.006 | |
| ¹² C-Caffeine, E | Intercept | 1399 | 582 | [-24, 2823] |
| | Slope | 0.174 | 0.001 | |
| ¹³ C ₃ -Caffeine, E | Intercept | -1935 | 1375 | [-5299, 1429] |
| | Slope | 0.079 | 0.003 | |

Due to this phenomenon, additional exploration of the effects of this difference on the calculations of caffeine concentrations in this study seemed appropriate. Calculations for labeled caffeine concentrations utilizing only the natural caffeine calibration curves were performed on the extracts and washes (the calculated concentrations for the wash samples were generally negative, and therefore considered not detectable). Using the ¹²C-caffeine calibration curves for isotopically labeled caffeine led to significantly lower calculated concentrations for the internal standard, resulting in decreased spike recoveries (15-25%). A one-way ANOVA test was applied between the different calibration curve approaches (¹²C-caffeine calibration curves vs. ¹³C₃-caffeine curves) using the 3 replicate injections for ¹³C₃-caffeine concentrations from each station's extract (see Table 3.3). The labeled caffeine concentrations for all extract samples were significantly different (p<0.05).

Table 3.3: Statistical difference for labeled caffeine concentrations in the methanol extracts utilizing different quantification approaches.

| | w/ ¹³ C Calibration Curve Caffeine Conc (µg/L) | w/o ¹³ C Calibration Curve Caffeine Conc (µg/L) | p-Value |
|---|--|---|---------|
| A | 656 | 236 | 2.81E-7 |
| | 647 | 233 | |
| | 636 | 229 | |
| B | 579 | 209 | 1.93E-7 |
| | 589 | 212 | |
| | 596 | 215 | |
| C | 691 | 248 | 1.10E-6 |
| | 697 | 250 | |
| | 669 | 240 | |
| D | 559 | 202 | 4.51E-6 |
| | 578 | 208 | |
| | 594 | 214 | |
| E | 419 | 152 | 5.10E-8 |
| | 428 | 155 | |
| | 422 | 153 | |

Using the two different quantification approaches for the internal standard based recoveries greatly affects the measurements of caffeine concentrations in Lake Superior surface water (as shown in Figure 3.2). Due to the low spike recoveries obtained without utilizing a ¹³C₃-caffeine calibration curve, the extraction efficiency correction factor increases, leading to an overestimate of natural caffeine concentrations in the water samples. Therefore, for the rest of this study, the quantification method using both ¹³C₃-

caffeine and ^{12}C -caffeine calibration curves were used to calculate labeled and natural caffeine concentrations respectively. Previous studies have utilized internal standardization methods (e.g isotope dilution) by using peak area ratios relative to a labeled internal standard to correct for ionization discrepancies (Buerge et al., 2003; Cahill et al., 2004; Kurissery et al., 2012; Niesser et al., 2013; and others). This is very useful for multiple analyte analyses, however, since caffeine was the only analyte of interest, the approach used within this study does produce appropriate quantitation. Thus far, this is the only study that we know of to compensate for calibration curve variance utilizing a two calibration approach between a compound and the compound specific isotopically labeled internal standard for tandem MS analysis. For future studies, it should be cautioned that caffeine isotopologues may vary in response factor. Therefore, it is necessary to implement an internal standardization approach for calibration or produce a separate calibration series for labeled caffeine. It is suggested that producing separate calibration series (as seen in this method) may produce a better quantitation due to eliminating the possible unknown interactions between caffeine isotopologues during ESI-MS/MS detection.

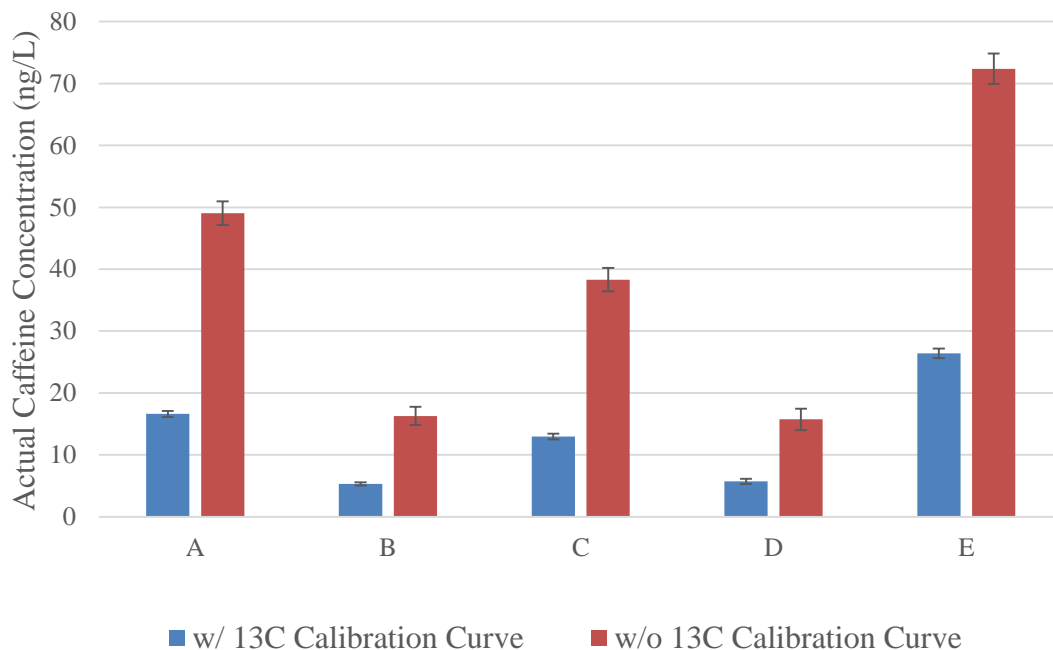


Figure 3.3: Comparison of actual caffeine concentrations with (w/) and without (w/o) utilizing the labeled calibration curves for quantifying natural caffeine in western Lake Superior surface water. Error bars were propagated from standard deviations obtained by 3 ESI/MS/MS sample injections.

Solid Phase Extraction

Extraction efficiency was greatly enhanced by using a series of washes to improve analyte recovery; these included an acid and base wash prior to methanol elution. Without the wash steps, preliminary data (see Appendix F) repeatedly produced poor analyte recovery (e.g. 10%). Therefore, the wash series were prudent for recovery, and wash filtrates were analyzed to monitor possible caffeine loss during elution. The analysis of the wash samples considerably shifted instrument sensitivity (e.g. wash 3)

possibly due to differing solvent composition. However, these samples were analyzed at the end of the sample suite to avoid sensitivity variation upon caffeine sample analysis.

Method blanks containing internal standard demonstrated high extraction efficiency, however extraction efficiencies were overall lower in lake water samples (see Table 3.3 & 3.4). It is hypothesized here, as before (Ferrer et al., 2010; Buchberger, 2011), that the more complex sample matrix in natural waters affected caffeine's interactions with the resin.

Table 3.4: Method blank recoveries containing internal standard. Error was propagated from standard deviations obtained by 3 ESI/MS/MS sample injections.

| | % Recovery |
|--|-------------------|
| Method Blank + $^{13}\text{C}_3$ -Caffeine-1 | 130 \pm 1 |
| Method Blank + $^{13}\text{C}_3$ -Caffeine-2 | 97 \pm 3 |

Table 3.5: Station sample recoveries containing internal standard. Error was propagated from standard deviations obtained by 3 ESI/MS/MS sample injections.

| Station | % Recovery |
|----------------|-------------------|
| A | 68 ±1 |
| B | 62 ±1 |
| C | 72 ±2 |
| D (offshore) | 58 ±3 |
| E (near-shore) | 42 ±1 |

Quality Control

Method Blanks

A method blank consisting of HPLC grade water and 2 method blanks consisting of HPLC grade water spiked with ¹³C₃-caffeine were extracted and analyzed with samples to correct for caffeine contamination and to estimate extraction efficiency. The method blank consisting of HPLC grade water had no detectable natural caffeine contamination, however there were measurable amounts of ¹³C₃-caffeine above the LLOQ in the wash 3 sample, indicating contamination from the internal standard stock solution during wash sample preparation (see Table 3.6). The 2 method blanks containing internal standard had high extraction efficiency along with measurable ¹²C-caffeine reported below in Table 3.7. The natural caffeine contamination is above the LLOQ, however this does not hinder caffeine quantification for surface water samples after method blank corrections (utilizing the mean ¹²C-caffeine ng/L) due to the larger peak

areas (approximately 2 times larger) for natural caffeine in surface water samples. The source of natural caffeine contamination is unknown based on preliminary contamination tests. Detailed information for method blanks is given in Appendix I.

Table 3.6: Method blank results without internal standard from sample series.

Error was propagated from standard deviations obtained by 3 ESI/MS/MS sample injections.

| | Total ¹²C-Caffeine (ng/L) | Total ¹³C-Caffeine (µg/L) | Actual ¹³C-Caffeine (ng/L) |
|--------------|---|---|--|
| Method Blank | n/d | 30 ±1 | 14.9 ±0.5 |

n/d: not detectable

Table 3.7: Method blank results containing internal standard from sample series and the reported natural caffeine mean used for method blank corrections. Error was propagated from standard deviations obtained by 3 ESI/MS/MS sample injections.

| | Method Blank + ¹³C Spike-1 | Method Blank + ¹³C Spike-2 |
|---|--|--|
| % Recovery | 130 ±1 | 97 ±3 |
| Total ¹²C-Caffeine (µg/L) | 9.7 ±0.1 | 13.1 ±0.4 |
| Actual Conc. (ng/L) | 4.85 ±0.06 | 6.6 ±0.2 |
| <hr/> | | |
| Mean ¹²C-Caffeine (µg/L) | 11.8 ±0.4 | |
| Mean Actual Conc. (ng/L) | 5.9 ±0.2 | |

Check Standards

A check standard from each calibration series (¹²C-caffeine and ¹³C₃-caffeine) was injected multiple times throughout the sample series to monitor instrumental sensitivity. Detailed data is shown in Appendix J. The check standard concentrations for ¹²C-caffeine and ¹³C₃-caffeine are calculated using the initial (beginning of run) calibration curves (Figure 3.1a) for corresponding ion transitions (see Table 2.3). The check standard concentrations are also calculated utilizing the later calibration curve to distinguish where instrument sensitivity decreases throughout sample analysis (see Table 3.8). The end calibration curve was run after wash 1 and wash 2 samples and before wash

3, where possible suppressed ionization efficiencies were observed (analysis time of ~13h). However, this is not the case for check standards run during wash 3 samples (analysis time of ~20h). Enhanced ionization efficiencies were first observed during preliminary sample runs that included wash 3 during sample preparation. Wash 3 consists of 5% ammonium hydroxide in 95:5 water:methanol (pH ~12) which greatly increased instrument response and thus caffeine concentrations, compared to wash 1 and wash 2 samples. Therefore, wash 3 samples were analyzed at the very end of the sample analysis to avoid such a significant ionization enhancement. However, it is suggested that ionization efficiencies are potentially affected from all wash samples that contain possible trace amounts of varying solvents that are reaching the ionization source. Based upon the results, little caffeine is lost in the wash steps and it is suggested to either eliminate caffeine quantification in these or analyze each set of washes in a separate sample batch with its own instrument optimization and calibration curve.

Table 3.8: Natural and labeled caffeine check standards with expected concentrations and calculated concentrations utilizing the beginning and end calibration curves. Error was propagated from standard deviations obtained by 3 ESI/MS/MS sample injections.

| | Analysis Time (~h) | ¹² C-Caffeine; ¹³ C ₃ -Caffeine Expected Conc (µg/L) | ¹² C-Caffeine; ¹³ C ₃ -Caffeine Beginning Calc Conc (µg/L) | ¹² C-Caffeine; ¹³ C ₃ -Caffeine End Calc Conc (µg/L) |
|---------------------------------|--------------------|--|---|---|
| | 7 | 54; 50 | 86 ±26; 82 ±3 | 182 ±66; 125 ±4 |
| | 10 | 54; 50 | 49.2 ±0.5; 57 ±4 | 87 ±1; 86 ±6 |
| | 13 | 54; 50 | 49 ±1; 57.9 ±0.9 | 86 ±2; 86 ±1 |
| | 20 | 54; 50 | 148 ±12; 146 ±5 | 340 ±30; 230 ±8 |
| Total Analysis Time: | 21 | | | |
| Sample Range (µg/L): | | n/d; 1000 | 11-53; 423-715 | |

Caffeine Quantification

Caffeine samples were stored for ~38 days before SPE, undergoing possible sorption or degradation. Once extracted and concentrated, a preliminary MS analysis for all caffeine samples and wash eluents was implemented to determine approximate concentration ranges. After this initial survey, all samples were refrigerated in the dark (for ~21 days) until final quantitative MS analysis. Recoveries of the internal standard in surface water samples are calculated to account for possible caffeine loss during sample storage and to monitor extraction efficiency. Table 3.9 below shows quantified results for sample stations. Reported error is associated with instrumental deviation from 3 sample injections. It is assumed that natural caffeine and labeled caffeine react similarly, e.g., in terms of sorption, bioavailability, and extraction efficiency; therefore loss terms and extraction efficiencies determined from the labeled compound are applied to correct the natural caffeine measurements performed here. All recoveries of the internal standard were comparable, except for the sample from station E. This could be due to the dissolved organic matter (DOM) matrix present in near shore samples, where trace amounts of caffeine may be preferably bound, hindering extraction efficiency, as hypothesized by Ferrer et al., 2010 and Buchberger, 2011. It could also be due to an enhanced microbial presence in the near-shore sample leading to greater caffeine degradation, although all samples were filtered (<0.7 μm) to minimize particles including bacteria. Station E was determined to give the lowest internal standard recovery and highest predicted caffeine concentration, thus providing possible evidence that near-shore

samples may contain higher amounts of caffeine, but will hinder extraction efficiency due to matrix complexity.

Table 3.9: Western Lake Superior caffeine results. Error was propagated from standard deviations obtained by 3 ESI/MS/MS sample injections.

| Stations | A | B | C | D | E |
|--|--------------|--------------|--------------|--------------|--------------|
| Internal Std % Rec | 68 ±1 | 62 ±1 | 72 ±2 | 58 ±3 | 42 ±1 |
| Correction Factor | 1.48 | 1.33 | 1.40 | 1.73 | 2.36 |
| Total Caffeine (µg/L) | 30.5 ±0.4 | 13.9 ±0.1 | 27.0 ±0.2 | 13.4 ±0.2 | 27.3 ±0.6 |
| Corrected Caffeine Conc. (ng/L) | 45.0 ±0.9 | 22.4 ±0.3 | 37.7 ±0.9 | 23.2 ±0.8 | 65 ±2 |
| Blank Corrected Conc. (ng/L) | 33 ±1 | 10.7 ±0.5 | 26.0 ±0.9 | 11.5 ±0.9 | 53 ±2 |
| Actual Conc. (ng/L) | 16.6 ±0.5 | 5.3 ±0.3 | 13.0 ±0.5 | 5.7 ±0.4 | 26.4 ±0.8 |



Figure 3.4: Previously reported caffeine concentrations within the Lake Superior watershed and caffeine concentrations in ng/L for western Lake Superior (Ferrey, 2011; Christensen et al., 2012). Error was propagated from standard deviations obtained by 3 ESI/MS/MS sample injections.

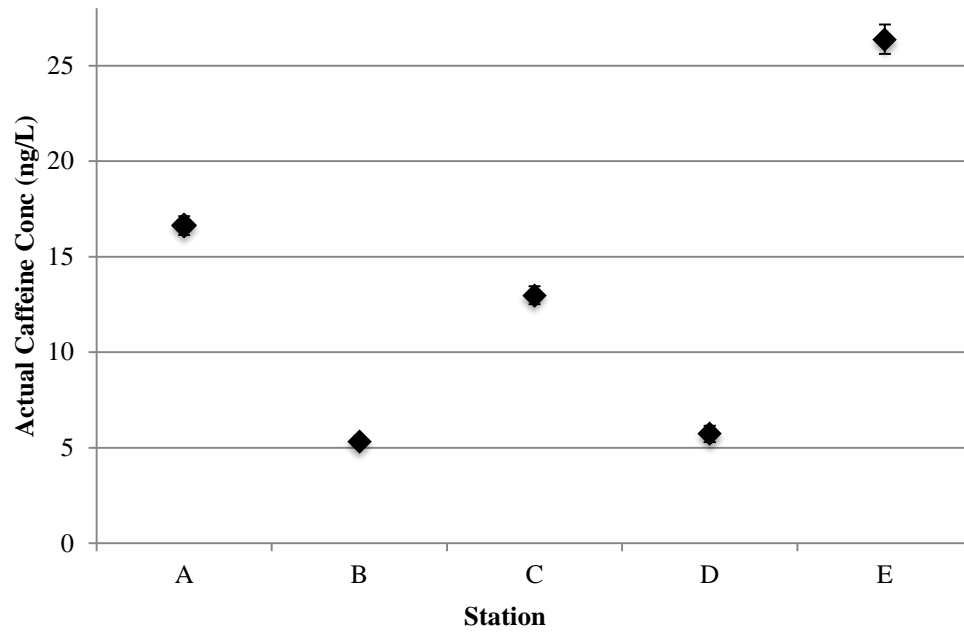


Figure 3.5: Reported caffeine concentrations for western Lake Superior. Error bars are derived from 3 injections on each sample. Error bars were propagated from standard deviations obtained by 3 ESI/MS/MS sample injections.

4. DISCUSSION

Caffeine Results

Caffeine has been analyzed and quantified in various aquatic systems worldwide, and has been primarily used as a tracer molecule to assess significant wastewater contamination within surface waters. Previously measured caffeine quantities have primarily depended on the surrounding environment and the wastewater treatment process, thus producing a wide range of caffeine concentrations attributed to an anthropogenic source in natural systems (see Table 4.1). The wastewater contamination for western Lake Superior is supported through traceable caffeine concentrations, where reported concentrations (see Table 4.1) are comparable to previously quantified caffeine concentrations within lakes.

Table 4.1: Typical caffeine concentrations in various systems attributed to an anthropogenic source.

| System | Location | Caffeine Conc. (ng/L) | Reference |
|-----------------------|-----------------|------------------------------|---|
| <i>WWTP/Septic</i> | China | <500 | Zhou et al., 2010 |
| <i>Tank Effluents</i> | | | |
| | Canada | 16,000-292,000 | Rogers et al., 1986 |
| | Switzerland | 28-355 | Buerge et al., 2006 |
| | Norway | <126,000 | Weigel et al., 2004 |
| | USA | >100,000 | Umari et al., 1995; Godfrey et al., 2007 |

| | | | |
|-----------------------|-----------|----------------------------|---|
| | | 16-8,000 | Glassmeyer et al., 2005 |
| | | <9,000 (Duluth, Minnesota) | Ferrey, 2011 |
| <i>Rivers/Streams</i> | China | 900-7,000 | Zhou et al, 2010 |
| | Canada | 2-15 | Verenitch & Mazumder, 2008 |
| | USA | 10-100 (Mississippi) | Barber et al., 1995 |
| | | 14-6,000 | Kolpin et al., 2002 |
| | | 70-250 | Standley et al., 2000 |
| | | 24-32 | Gardinali et al., 2002 |
| | | <3,000 | Glassmeyer et al., 2005 |
| | | <1,080 (Minnesota) | Ferrey, 2011 |
| | | <769 (Duluth, Minnesota) | Ferrey, 2011; Christensen et al., 2012 |
| <i>Lakes/Ponds</i> | USA | <43 (Minnesota) | Ferrey et al., 2015 |
| | | 5-26 (W. Lake Superior) | This Study |
| | Canada | 5-77 | Kurissery et al., 2012 |
| | | 2-22 | Verenitch & Mazumder, 2008 |
| | | 46 (Lake Erie) | Metcalf et al., 2003 |
| | | <33 (Lake Ontario) | Li et al., 2010 |
| | Australia | <700 | Chen et al., 2002 |
| <i>Groundwater</i> | USA | 100-290 | Fram et al., 2011 |
| | | <206 | Godfrey et al., 2007 |
| | Germany | 4-27 | Hillebrand et al., 2012 |
| | Australia | <500 | Chen et al., 2002 |
| <i>Seawater</i> | USA | <12 | Gardinali et al., 2002 |
| | | <166 | Peeler et al., 2006 |
| | Canada | 5-149 | Verenitch & Mazumder, 2008 |

| | | |
|--------|--------|---------------------|
| Norway | 7-87 | Weigel et al., 2004 |
| Italy | 82-367 | Loos et al., 2013 |

Caffeine concentrations in western Lake Superior were in the range 5-26 ng/L where station E had the highest concentration and stations B and D had the lowest concentrations. There were higher caffeine concentrations near-shore and depleted concentrations offshore, as would be expected from sewage overflow and wastewater point sources. In order to determine the quantity of caffeine needed to support Lake Superior's surface water concentrations, the annual input of caffeine was calculated based on lake volume and caffeine removal through degradation following a similar model to that reported by Buerge et al., 2006. The photodegradation half-life for caffeine was estimated to be 10 years, showing negligible removal compared to biodegradation (Buerge et al., 2006). With biodegradation as the primary removal of caffeine, with an average rate constant of 0.005 d^{-1} (Buerge et al., 2006), approximately $1.1 \times 10^8 \text{ g/yr}$, or 1.2×10^9 cups of coffee would need to enter Lake Superior annually in order to reach a minimum steady-state concentration of 5 ng. With a population of 600,000 for Lake Superior's watershed, this would be equivalent to 0.5 g/per person/ day or 5 cups of coffee/per person/day. This model may present unrealistic assumptions for Lake Superior primarily due to the physical differences between Lake Superior and Lake Greifensee. It is probable to assume that on average Lake Superior may be 10-12 °C colder than summer temperatures in Lake Greifensee, resulting in decreased biodegradation reaction rates (e.g. approximately half). Therefore, the estimate could be refined to daily input of

caffeine for Lake Superior of 0.2 to 0.3 g/per person. This is still a high estimate (2 to 3 cups of coffee per person per day), most likely due to the use of the entire volume of Lake Superior in the calculation. The measurements of detectable caffeine concentrations in this thesis are for western Lake Superior. It is likely that there is slow mixing (e.g. 1-2 years, J. Austin, personal communication) between the western and eastern side of Lake Superior. Thus the volume of the lake to be included in this simple box model should be adjusted or a more complicated model addressing lake physics should be applied.

However, further investigation for Lake Superior biodegradation rates and additional sample collections would be necessary for a more accurate model. When comparing caffeine concentrations from western Lake Superior to other surface water systems throughout the world, Lake Superior presents minimal wastewater contamination.

However, Lake Superior caffeine concentrations are comparable to measurements seen in other great lake systems, such as in Lake Erie and Lake Ontario. This suggests that western Lake Superior may experience more contamination per person compared to other great lakes, due to the decreased human population, the increased dilution that caffeine inputs would undergo, and the assumption that degradation rates would be slower in Lake Superior (due to colder average surface water temperatures).

The traceable caffeine concentrations in Lake Superior suggest there was a source(s) of contamination during the time of (or before) sample collection. In previous chemical monitoring studies, caffeine concentrations within Lake Superior's watershed (see Figure 3.4) were reported to be 59 ng/L in the St. Louis Bay, 769 ng/L in a downstream sample from the St. Louis Bay, and 87 ng/L in the Superior Bay (Ferrey,

2011; Christensen et al., 2012). This suggests that the Western Lake Superior Sanitary District (WLSSD) may be the source of contamination between the St. Louis Bay and downstream from the St. Louis Bay. In 2009-2011, the MPCA measured a maximum caffeine concentration of 9000 ng/L in the WLSSD effluent, thus providing evidence of traceable caffeine from this considerable potential contaminant source (Ferrey, 2011). Additionally, the St. Louis River had a maximum caffeine concentration of 200 ng/L that was reported below Fond du Lac, as well as an undetectable concentration above this region (Ferrey, 2011; Christensen et al., 2012). This indicates the river reach near Fond du Lac as a potential location for some untreated wastewater contamination into the St. Louis River. Untreated wastewater contamination may also be attributed to other riverine inputs aside from the St. Louis River. It is predicted that rivers along the north shore may report similar or possibly increased concentrations (e.g 10-80 ng/L) to the St. Louis River. Additional factors such as increased river flow, biodegradation, adsorption, and/or minimized human influence must be considered. However, if it were assumed that 10 or more rivers along the north shore contained a caffeine maximum of 80 ng/L, the annual input of caffeine could increase by an order of magnitude (with increased river flow) than the annual input delivered by the St. Louis River (assuming a maximum input of 200 ng/L). This suggests that the quantity of rivers along the north shore with traceable caffeine content can rapidly become a primary contributor to Lake Superior surface water contamination. The estimations based upon previous studies (see Table 4.1), and the previous caffeine concentrations reported within Lake Superior's watershed provide foundational insight for determining possible point sources of untreated wastewater

contamination that may be extending into Lake Superior's open waters. Overall, there do not appear to be significant wastewater processing inefficiencies or source leakages/overflows around the region encompassing the western arm of Lake Superior. However, this study is based upon only 5 surface water samples collected during the month of May, and further investigation across seasons and storm events, and including more sampling stations would be prudent.

Analytic Method

Method development for caffeine quantitation was refined from previous chemical monitoring studies where caffeine was included. Instrumental drift and/or matrix effects during ionization caused shifts in instrumental performance during the course of the analyses. This was seen through implementing periodic check standards throughout sample analyses in order to monitor instrument sensitivity. Caffeine standard calibration series were re-analyzed towards the end of the sample suite (after wash 1 and wash 2, and before wash 3) in order to compensate for decreased sensitivity upon quantitation. This shift is seen comparing beginning and end calibration curves for natural and labeled caffeine standards. The instrumental performance (ionization efficiency) between natural and labeled caffeine shows a significant difference as can be seen in their respective calibration-curve slopes. This significant variance was surprising as these isotopologues are assumed to experience similar ionization and fragmentation patterns. Additional investigation was done to determine if this significant calibration curve variance developed primarily from differences in ionization or from fragmentation.

This was done by integrating the precursor ions 195 m/z and 198 m/z for natural and labeled caffeine respectively. It was determined that both slopes for beginning and end calibration curves were indeed significantly different ($p < 0.05$; $t > 2.179$) prior to fragmentation concluding that similar ionization for these two isotopologues should not be assumed. Therefore, utilizing a two calibration approach (as was done in this study) or internal standardization methods (e.g. isotope dilution) utilizing ratio calculations would be necessary for successful caffeine quantification using an isotopically labeled internal standard.

Surface water sampling stations closer to the St. Louis estuary may contain higher concentrations of DOM that directly affect spike recovery. This may be due to the competition of interferents for specific sites in the sorbent producing a lower recovery for the labeled caffeine compound. Station E demonstrates such a possibility, where the highest natural caffeine concentration and lowest recovery were seen. Other studies have reported a similar theory, where high recoveries were obtained for reagent water containing internal standard compared to low recoveries reported from more complicated matrices (e.g. increased amounts of suspended solids) (Ferrer et al., 2010; Buchberger, 2011). Additional preliminary data in this project supports this hypothesis. A near-shore (post-storm) grab sample was collected, spiked, and analyzed on the same day along with a spiked method blank to determine if an increase in DOM affected analyte recovery (see Appendix G for details). The spiked method blank had a higher SPE recovery (18%) and the grab sample reported a lower recovery (4%). Unfortunately, the results were quantified utilizing only a ^{12}C -caffeine calibration curve due to the initial assumption that

isotopologue standards would produce similar regressions curves. This explains the low recovery for the method blank in comparison to the reported spiked method blanks within this study (e.g. 97-130%) where a $^{13}\text{C}_3$ -caffeine calibration curve was used for quantitation. Therefore, the preliminary results suggest that the varying SPE efficiencies may support the theory proposed by Ferrer et al., 2010 and Buchberger, 2011, but further analyses are necessary to more fully test the hypothesis.

The reported caffeine concentrations were corrected for extraction inefficiencies and other losses using the correction factor acquired from internal standard recovery in each sample. Sorption or degradation may be a participant in recovery loss during sample storage. Preliminary data suggested caffeine to experience rapid sorption in MQW during storage. It was observed that caffeine underwent sorption within 1 day in glass 1 L bottles, glass LC-MS vials, and high density polyethylene (HDPE) bottles (see Appendix H for details). However, the extent of sorption was lower in glass bottles, therefore 1 L glass bottles were best suited for sample collection. However, it has been hypothesized that the combustion of glassware may form possible active sites on the interior surface causing an increase in caffeine sorption (Englert, 2007). All glass 1 L bottles for sample collection were previously combusted, therefore it becomes beneficial to add the internal standard to the surface water samples the day of collection (as done here) to correct for possible sorption. Caffeine samples may also experience degradation by microbes during sample storage, however, further investigation is needed for validation. It is predicted that natural caffeine may experience sorption, degradation, and/or recovery loss during SPE;

unfortunately the extent of natural caffeine loss from each mechanism remains unclear, as it is difficult to untangle these effects from those of variable extraction efficiency.

Caffeine Limitations as an Anthropogenic Marker

Caffeine quantification is a useful proxy for wastewater inputs within natural systems, however caffeine has been estimated to degrade in the water column biologically, chemically, and photochemically (Buerge et al., 2003; Snyder et al., 2004; Buerge et al., 2006; Bradley et al., 2006; Loos et al., 2013). Caffeine degradation rates are difficult to quantify in natural conditions within lakes because of their dependence on several parameters such as microbial activity, trophic conditions, temperature, water depth, physical distribution, etc.

For future caffeine tracer studies, it is important to understand how the seasonal fluctuations in caffeine concentrations are dependent on changes in degradation rates and contaminant input. It has been estimated that caffeine concentrations increase during the summer months due to an increase in contaminant influx. For example, in Ontario Canada, an increase in human activity on Lake Simcoe correlated with an increase in caffeine concentrations (Kurissery et al., 2012). It has also been reported that an increase in precipitation correlated with sewer and septic overflows, thus increasing traceable caffeine concentrations (Buerge et al. in 2006). In contrast, in other locations, caffeine and other pharmaceuticals have been measured at higher concentrations during the winter months, however this is believed to be due to changes in degradation rates. Such a seasonal trend was detected in a few lakes in central Finland, as well as in the Charleston

Harbor in South Carolina where it was predicted that higher caffeine concentrations were obtained by WWTP inefficiency caused by decreased biodegradation from lowered temperatures, as well as reduced photodegradation within lake surface waters (Hedgspeth et al., 2012; Lindholm-Lehto et al., 2016). Due to the aquatic complexity among varying systems and trace amounts of caffeine present in surface waters, it becomes difficult to quantify degradation rates, address changes in contaminant influx, and determine how caffeine concentrations are affected. Caffeine samples for this study were collected and analyzed only during the month of May. Therefore, additional surface water samples would need to be collected seasonally and over several years, to estimate changes in wastewater contamination and degradation for caffeine. At the present time, caffeine degradation processes in Lake Superior surface waters remain little known and further investigation into degradation rates would be a logical next step in this area of research.

5. CONCLUSION AND RECOMMENDATIONS

This study successfully quantified caffeine, an anthropogenic marker, in western Lake Superior surface water samples. A revised SPE method was implemented to improve caffeine recovery and to decrease the limit of quantitation by 2,000-fold before ESI-MS/MS analysis. Instrumental MDS, LLOD, and LLOQ were reported for natural and labeled caffeine compounds prior to quantification. The quantitation approach was based on the variance observed between ^{12}C -caffeine and $^{13}\text{C}_3$ -caffeine calibration curves. As a result, both the natural and labeled caffeine linear regressions obtained from the quantifier ion transition were utilized to account for the ionization and possible fragmentation discrepancies. Observed instrumental drift over the course of these analyses, and possible matrix effects from wash samples were accounted for by utilizing two sets of calibration curves, one near the beginning and one near the end of the analyses. A total of five western Lake Superior sampling locations were analyzed for caffeine content. Station D and E represent the most offshore and nearest shore water samples respectively. The calculated caffeine concentrations correlate well with the hypothesis that caffeine concentrations will be greater near-shore with lower concentrations away from human population centers.

Our data show that there are trace amounts of wastewater contamination in western Lake Superior surface waters. However, due to the dilution factor of riverine inputs and other wastewater contaminant transporters within this large lake, it is possible there is a significant volume of contamination entering the lake. Further investigation

would be needed to determine point sources of contamination. Over time, the physical and chemical attributes of Lake Superior surface water could be greatly influenced with an inflow of contaminants producing potential health risks for the aquatic ecosystem and human populations. This study constructs a basis for future work investigating Lake Superior surface water contamination from anthropogenic compounds in wastewater, supports evidence of contaminant distribution, and provides an efficient SPE method for caffeine extraction and ESI-MS/MS analysis within surface waters.

Recommendations

- Further evaluation is recommended with additional samples. While this study provides evidence of wastewater contamination in Western Lake Superior, it is only based on 5 sampling sites visited during the month of May. It would be useful to study how caffeine concentrations change seasonally and as a function of storm events. An annual report of caffeine concentrations would be useful for recognizing the primary sources of contamination.
- Collecting samples at more sampling stations would be useful to further analyze contaminant distributions and how these may be affected annually by the physical lake characteristics.
- Stream water samples could also be collected on the north shore of Lake Superior for determining comparable surface water caffeine concentrations. Areas of higher caffeine concentrations could pinpoint other anthropogenic sources to further prevent contamination.

- Further investigation of the ESI-MS/MS variability between ^{12}C -caffeine and $^{13}\text{C}_3$ -caffeine would be beneficial to strengthen instrumental calibrations.
- Other chemical indicators such as caffeine metabolites could also be analyzed with variations of this method. Metabolite information would reinforce confirmation of wastewater inputs and help to constrain caffeine degradation and possible sorption in the lake environment.
- Implementing lab studies would be useful for approximating caffeine degradation in Lake Superior surface waters and applying to mass balance equations for predicting caffeine concentrations.

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7. APPENDICIES

APPENDIX A

Standard Calibration Series

¹²C-Caffeine, Beginning

Stock Preparation Details:

Caffeine, Sigma Ultra (Sigma C8960; CAs58-08-2; batch 023k0054)

Mass = 0.162 mg diluted in 100 mL 90:10 water:methanol

Stock Concentration = 1,620,000 ng/L

| Cal | Concentration (ng/L) | Peak Area 195→138 | Peak Area 195→110 | Ratio |
|-----|----------------------|----------------------|----------------------|-------|
| 1 | 1361 | 371 | 95 | 0.26 |
| 2 | 10800 | 2984 | 750 | 0.25 |
| 3 | 27000 | 8276 | 2121 | 0.26 |
| 4 | 54000 | 21568 | 5562 | 0.26 |
| 5 | 108000 | 32089 | 8107 | 0.25 |
| 6 | 248400 | 86236 | 21510 | 0.25 |
| 7 | 540000 | 173526 | 41410 | 0.24 |
| 8 | 1620000 | 593664 | 139649 | 0.24 |

¹²C-Caffeine, End

| Cal | Concentration (ng/L) | Peak Area 195→138 | Peak Area 195→110 | Ratio |
|-----|----------------------|----------------------|----------------------|-------|
| 1 | 1361 | 326 | 73 | 0.22 |
| 2 | 10800 | 1875 | 453 | 0.24 |
| 3 | 27000 | 5092 | 1181 | 0.23 |
| 4 | 54000 | 10840 | 2448 | 0.23 |
| 5 | 108000 | 17332 | 3829 | 0.22 |
| 6 | 248400 | 35811 | 7809 | 0.22 |
| 7 | 540000 | 78663 | 16909 | 0.21 |
| 8 | 1620000 | 232520 | 49228 | 0.21 |

¹³C₃-Caffeine, Beginning

Stock Preparation Details:

Sigma-Aldrich (603295-1 mL Caffeine-¹³C₃; 99 atom % ¹³C; lot # Ls-68-304)

¹³C₃-Caffeine = 1 mg/mL; 0.5 mL diluted in 1000 mL methanol= 500,000 ng/L

90:10 water methanol stock prepared from 500,000 ng/L in methanol for more dilute standards

Concentrated standards pulled from 500,000 ng/L stock in methanol, dried, and reconstituted in 90:10 water methanol

| Cal | Concentration (ng/L) | Peak Area 198→140 |
|-----|----------------------|-------------------|
| 1 | 1366 | -113 |
| 2 | 10833 | 972 |
| 3 | 27000 | 2700 |
| 4 | 50000 | 6699 |
| 5 | 100000 | 10361 |
| 6 | 250000 | 22433 |
| 7 | 500000 | 52470 |
| 8 | 1000000 | 132457 |

¹³C₃-Caffeine, End

| Cal | Concentration (ng/L) | Peak Area 198→140 |
|-----|----------------------|-------------------|
| 1 | 1366 | -153 |
| 2 | 10833 | 318 |
| 3 | 27000 | 1191 |
| 4 | 50000 | 2696 |
| 5 | 100000 | 6035 |
| 6 | 250000 | 13238 |
| 7 | 500000 | 32352 |
| 8 | 1000000 | 80445 |

APPENDIX B

Sample Concentration Equation

| Concentration of ¹³ C ₃ -caffeine stock | Volume of spike | Volume spiked sample | Concentration of ¹³ C ₃ -caffeine in spiked mix | | | |
|---|-----------------|----------------------|---|---------|---|----------|
| 500,000 ng/L | x | 1.00 mL | = | 1000 mL | x | 500 ng/L |

| Sample Volume loaded | Concentration of ¹³ C ₃ -caffeine in spiked mix | Volume sample eluted | Concentration in eluted sample | | | |
|----------------------|---|----------------------|--------------------------------|------|---|-------------|
| 1000 mL | x | 500 ng/L | = | 8 mL | x | 62,500 ng/L |

| Volume sample eluted | Concentration in eluted sample | Final volume of extract | Concentration of extract | | | |
|----------------------|--------------------------------|-------------------------|--------------------------|--------|---|----------------|
| 8 mL | x | 62,500 ng/L | = | 500 uL | x | 1,000,000 ng/L |

$$\frac{\text{Final Concentration}}{\text{Initial Concentration}} = \frac{1 \times 10^6 \frac{\text{ng}}{\text{L}}}{500 \frac{\text{ng}}{\text{L}}} = 2,000 \text{ fold}$$

APPENDIX C

Quantitation & Propagation of Error Example Calculations

Station A

Wash Samples:

| ¹² C-Caffeine (ng/L) | Wash #1 | Wash #2 | Wash #3 |
|---------------------------------|---------|---------|---------|
| Average = | n/d | 15625 | 666 |
| Std Dev = | | 124 | 386 |

Methanol Extract Sample:

| ¹² C-Caffeine (ng/L) | Extract |
|---------------------------------|---------|
| Average = | 14185 |
| Std Dev = | 107 |

Total ¹²C-Caffeine (ng/L) = 15625 + 666 + 14185 = 30476

$$\text{Absolute Uncertainty} = \sqrt{(124)^2 + (386)^2 + (107)^2} = 419$$

$$\% \text{ Relative Uncertainty} = \left(\frac{419}{30476} \right) * 100 = 1.38$$

Wash Samples:

| ¹³ C ₃ -Caffeine (ng/L) | Wash #1 | Wash #2 | Wash #3 |
|---|---------|---------|---------|
| Average = | n/d | n/d | 30599 |
| Std Dev = | | | 641 |

Methanol Extract Sample:

| ¹³ C ₃ -Caffeine (ng/L) | Extract |
|---|---------|
| Average = | 646074 |
| Std Dev = | 9941 |

Total ¹³C₃-Caffeine (ng/L) = 30599 + 646074 = 676674

$$\text{Absolute Uncertainty} = \sqrt{(641)^2 + (9941)^2} = 9962$$

$$\% \text{ Recovery} = \left(\frac{676674}{1000000} \right) * 100 = 68$$

$$\% \text{ Relative Uncertainty} = \left(\frac{9962}{676674} \right) * 100 = 1.47$$

$$\text{Spike Correction} = \frac{100}{68} = 1.48$$

$$\text{Corrected }^{12}\text{C-Caffeine Total (ng/L)} = 30476 * 1.48 = 45038$$

$$\% \text{ Relative Uncertainty} = \frac{\sqrt{(1.38)^2 + (1.47)^2}}{100} = 0.02$$

$$\text{Absolute Uncertainty} = 0.02 * 45038 = 908$$

Spiked Method Blanks

$$\text{Average }^{12}\text{C-Caffeine (ng/L)} = 11782$$

$$\text{Absolute Uncertainty} = 383$$

$$\text{Method Blank Corrected }^{12}\text{C-Caffeine Total (ng/L)} = 45038 - 11782 = 33256$$

$$\text{Absolute Uncertainty} = \sqrt{(908)^2 + (383)^2} = 985$$

$$\text{Actual Natural Caffeine Concentration } \left(\frac{\text{ng}}{\text{L}}\right) = \frac{33256}{2000} = 16.6$$

$$\text{Absolute Uncertainty} = \frac{985}{2000} = 0.5$$

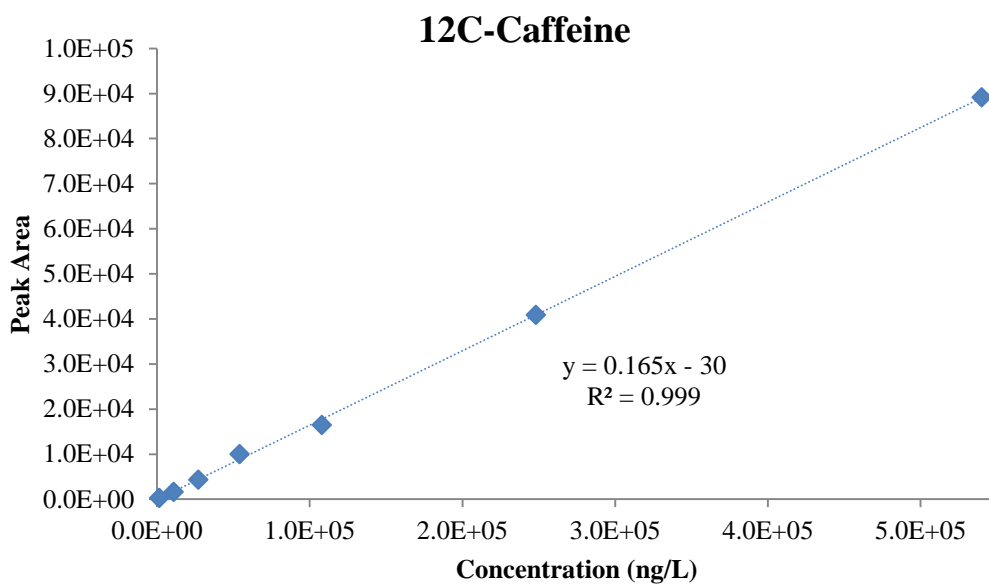
APPENDIX D

LLOD and LLOQ

¹²C-Caffeine

| Instrument Blank | Peak Area 195→138 | Peak Area 195→110 | |
|---------------------|----------------------|----------------------|---------------|
| 0 | 37 | 14 | |
| 0 | 31 | 9 | |
| 0 | 33 | 13 | |
| 0 | 30 | 15 | |
| 0 | 29 | 8 | |
| 0 | 23 | 11 | |
| 0 | 41 | 8 | Average = 32 |
| Cal 1 (ng/L) | | | Std Dev = 6 |
| 1361 | 374 | 76 | |
| 1361 | 350 | 102 | |
| 1361 | 370 | 86 | |
| 1361 | 329 | 69 | |
| 1361 | 346 | 92 | |
| 1361 | 290 | 86 | |
| 1361 | 311 | 86 | Average = 339 |
| | | | Std Dev = 31 |

| Cal | Concentration (ng/L) | Peak Area 195→138 | Peak Area 195→110 | Ratio |
|-----|-------------------------|----------------------|----------------------|-------|
| 1 | 1361 | 339 | 85 | 0.25 |
| 2 | 10800 | 1630 | 392 | 0.24 |
| 3 | 27000 | 4388 | 1058 | 0.24 |
| 4 | 54000 | 10028 | 2310 | 0.23 |
| 5 | 108000 | 16461 | 3832 | 0.23 |
| 6 | 248000 | 40929 | 9388 | 0.23 |
| 7 | 540000 | 89163 | 19916 | 0.22 |



s = cal 1 standard deviation
b = y-intercept
m = slope

$$\text{Minimum Detection Signal} = (3 \times s) + \text{mean}_{\text{blank}}$$

$$LLOD = \frac{3 \times s}{m}$$

$$LLOQ = \frac{10 \times s}{m}$$

¹³C₃-Caffeine

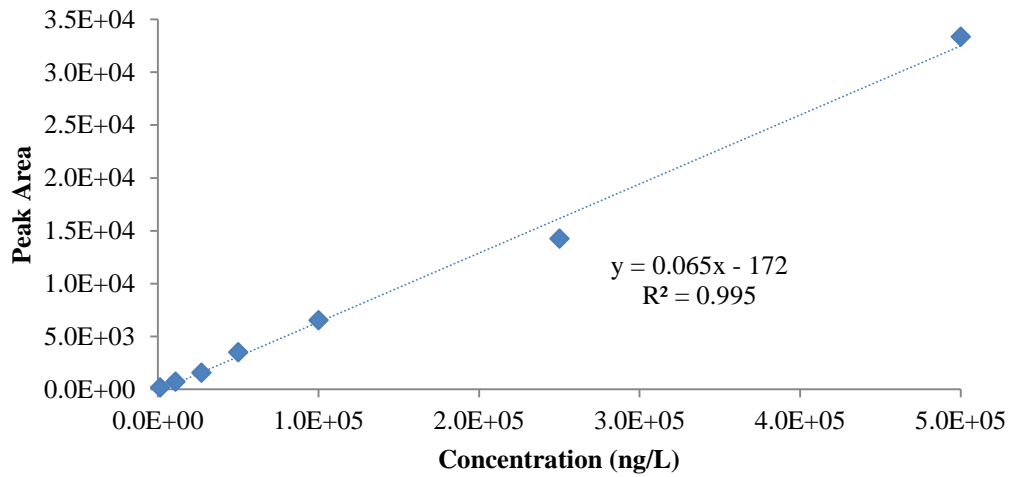
| Instrument Blanks & Cal 1 (ng/L) | Peak Area 195→138 | |
|----------------------------------|-------------------|--------------|
| 0 | 96 | |
| 0 | 96 | |
| 0 | 93 | |
| 0 | 102 | |
| 0 | 82 | |
| 0 | 91 | |
| 0 | 91 | |
| | | Average = 93 |
| | | Std Dev = 6 |

| | | |
|------|-----|--|
| 1366 | 181 | |
| 1366 | 175 | |
| 1366 | 168 | |
| 1366 | 186 | |
| 1366 | 183 | |
| 1366 | 193 | |
| 1366 | 179 | |

| | |
|-----------|-----|
| Average = | 181 |
| Std Dev = | 8 |

| Cal | Concentration (ng/L) | Peak Area 195→138 |
|-----|----------------------|-------------------|
| 1 | 1366 | 181 |
| 2 | 10833 | 724 |
| 3 | 27000 | 1579 |
| 4 | 50000 | 3517 |
| 5 | 100000 | 6536 |
| 6 | 250000 | 14253 |
| 7 | 500000 | 33371 |

¹³C₃-Caffeine



APPENDIX E

Sampling Station Details

| Station | Latitude | Longitude | Depth (m) | Total Water Depth (m) | (mm/dd/yr) | Time Filtered | Time Spiked | TOC (mg/L) |
|----------------|-----------------|------------------|------------------|----------------------------------|-------------------|--------------------------|------------------------|-----------------------|
| A | 46°99'74" N | 91°37'53" W | 5 | 156 | 05/13/16 | 733 | 1810 | 1.44 |
| B | 46°93'93" N | 91°33'84" W | 5 | 124 | 05/13/16 | 824 | 1810 | 1.46 |
| C | 46°86'56" N | 91°28'18" W | 5 | 49 | 05/13/16 | 930 | 1810 | 1.48 |
| D | 47°31'62" N | 90°53'88" W | 5 | 79 | 05/13/16 | 236 | 1810 | 1.32 |
| E | 46°83'24" N | 91°74'89" W | 5 | 67 | 05/13/16 | 1444 | 1810 | 1.42 |

APPENDIX F

Preliminary Recoveries With and Without Wash Series

Grab samples were collected, spiked, and analyzed within the same day. Quantitation for both samples were calculated utilizing precursor ion responses.

| | Location (Duluth, MN) | Vol IS (mL) | % Recovery |
|---|-----------------------------------|--------------------|-------------------|
| Near-shore Grab Sample w/o wash series | Lake Superior Bay (post-storm) | 0.5 | 10 |
| Near-shore Grab Sample w/ wash series | Brighton Beach | 1 | 59 |

IS: Internal standard

Further investigation would be needed to determine if % recovery is a function of DOM quantity and/or spike concentration.

APPENDIX G

Preliminary SPE Matrix Effects

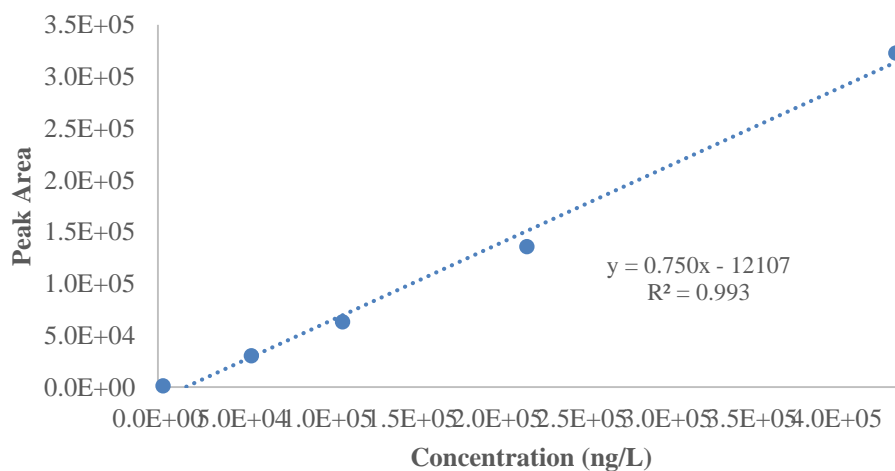
Near-shore grab sample and method blank data

Samples spiked with 1 mL of 500,000 ng/L (in methanol) ¹³C₃-Caffeine stock

Extracted 500 mL; Final volume= 0.5 mL

| Cal | Conc (ng/L) | Peak Area 195-->138 |
|-----|-------------|----------------------------------|
| 1 | 2900 | 1467 |
| 2 | 55100 | 30314 |
| 3 | 108750 | 63171 |
| 4 | 217500 | 135712 |
| 5 | 435000 | 322944 |

12C-Caffeine Calibration Curve



| Water Method Blank + ¹³C Spike | | Peak Area |
|--|--|-------------------------------------|
| | | ¹³C: 198-->140 |
| Wash #1 | | n/d |
| Wash #2 | | -47 |
| Wash #3 | | -19 |

| <u>MeOH Elution</u> | Peak Area | Conc (ng/L) |
|----------------------------|------------------|-------------------------------------|
| | | ¹³C: 198-->140 |
| | 263429 | 367558 |

| Results: | | | |
|-------------------------------------|---------------------|--------------------------|--------------|
| ¹³C: 198-->140 | | | |
| | Total (ng/L) | Spike Conc (ng/L) | % Rec |
| | 367558 | 2000000 | 18 |

| Grab Sample + ¹³C Spike | | Peak Area | Conc (ng/L) |
|---|--|-------------------------------------|--------------------|
| | | ¹³C: 198-->140 | |
| Wash #1 | | n/a | |
| Wash #2 | | 1252 | 17820 |
| Wash #3 | | 3234 | 20464 |

| <u>MeOH Elution</u> | Peak Area | Conc (ng/L) |
|----------------------------|------------------|-------------------------------------|
| | | ¹³C: 198-->140 |
| | 15642 | 37016 |

| Results: | | | |
|-------------------------------------|---------------------|--------------------------|--------------|
| ¹³C: 198-->140 | | | |
| | Total (ng/L) | Spike Conc (ng/L) | % Rec |
| | 75301 | 2000000 | 4 |

APPENDIX H

Sorption Preliminary Data

Expected Concentration = 100%

| Day | Container | % Concentration |
|------------|------------------|------------------------|
| 1 | Glass 1-L | 78 |
| | LC-MS Vial 2 mL | 48 |
| | HDPE 500 mL | 67 |
| | LC-MS Vial 2 mL | 52 |
| 2 | Glass 1-L | 65 |
| | HDPE 500 mL | 61 |

Glass and HDPE samples were stored for 1 and 2 days. Each LC-MS vial originally came from the corresponding glass or HDPE container. The expected concentration was the same for all samples.

APPENDIX I

Blank Data

Instrument Blank

| TIC Area | Peak Area 198-->140 | Peak Area 195-->138 | Peak Area 195-->110 |
|-----------------|-----------------------------------|-----------------------------------|-----------------------------------|
| 92 | 22 | 36 | 5 |
| 63 | 38 | 18 | 7 |
| 40 | 33 | 14 | 2 |
| 57 | 30 | 18 | 9 |
| 26 | 21 | 20 | 5 |
| 44 | 32 | 17 | 3 |
| 33 | 26 | 14 | 3 |
| 35 | 25 | 15 | 2 |
| 130 | 31 | 78 | 34 |
| 68 | 34 | 41 | 18 |
| 52 | 23 | 0 | 0 |
| 62 | 51 | 0 | 0 |
| 54 | 29 | 30 | 13 |
| 78 | 74 | 0 | 0 |
| 0 | 0 | 0 | 0 |
| 44 | 48 | 0 | 0 |
| 46 | 36 | 0 | 0 |
| 30 | 20 | 0 | 0 |
| 45 | 30 | 0 | 0 |
| 38 | 27 | 0 | 0 |
| 35 | 19 | 0 | 0 |
| 47 | 32 | 0 | 0 |
| 29 | 28 | 0 | 0 |
| 42 | 43 | 0 | 0 |
| 127 | 111 | 0 | 0 |
| 96 | 75 | 0 | 0 |
| 90 | 69 | 0 | 0 |
| 93 | 82 | 20 | 0 |
| 58 | 52 | 0 | 0 |
| 87 | 59 | 18 | 0 |
| 2066 | 1547 | 65 | 22 |
| 81 | 67 | 11 | 5 |
| 57 | 38 | 0 | 0 |
| 75 | 67 | 0 | 0 |
| 53 | 47 | 0 | 0 |
| 52 | 44 | 0 | 0 |
| 9005 | 8141 | 348 | 202 |
| 1765 | 1369 | 93 | 42 |
| 83 | 82 | 0 | 0 |
| 2100 | 1883 | 47 | 48 |
| 1030 | 837 | 41 | 30 |

| | | | |
|------|------|-----|-----|
| 213 | 151 | 20 | 8 |
| 1977 | 1412 | 334 | 124 |
| 89 | 77 | 16 | 6 |
| 1345 | 953 | 235 | 85 |
| 2738 | 2124 | 165 | 77 |
| 1305 | 1153 | 94 | 45 |
| 791 | 657 | 66 | 22 |
| 326 | 245 | 43 | 22 |
| 212 | 161 | 32 | 17 |
| 160 | 90 | 35 | 12 |
| 173 | 78 | 47 | 15 |
| 152 | 70 | 42 | 14 |
| 101 | 72 | 52 | 21 |
| 99 | 56 | 54 | 28 |
| 152 | 99 | 53 | 24 |
| 127 | 52 | 50 | 35 |
| 150 | 63 | 57 | 19 |
| 146 | 59 | 49 | 24 |
| 152 | 52 | 46 | 21 |
| 92 | 40 | 34 | 18 |
| 138 | 46 | 78 | 37 |
| 211 | 89 | 93 | 41 |
| 178 | 83 | 72 | 27 |
| 154 | 69 | 52 | 25 |
| 132 | 61 | 59 | 14 |
| 137 | 63 | 60 | 14 |
| 145 | 88 | 63 | 15 |
| 125 | 74 | 47 | 19 |
| 142 | 56 | 47 | 21 |
| 142 | 80 | 54 | 20 |
| 94 | 62 | 49 | 12 |
| 89 | 61 | 40 | 16 |
| 139 | 53 | 102 | 23 |
| 192 | 47 | 81 | 21 |
| 155 | 51 | 67 | 25 |
| 114 | 36 | 57 | 16 |
| 138 | 57 | 56 | 22 |
| 479 | 42 | 365 | 111 |
| 523 | 37 | 381 | 128 |
| 386 | 41 | 368 | 131 |
| 282 | 48 | 196 | 91 |
| 340 | 40 | 221 | 117 |
| 351 | 210 | 90 | 75 |
| 317 | 131 | 90 | 63 |
| 342 | 194 | 93 | 53 |
| 306 | 143 | 84 | 54 |
| 307 | 157 | 70 | 56 |
| 596 | 391 | 137 | 94 |
| 648 | 404 | 145 | 78 |
| 433 | 236 | 139 | 61 |

| | | | |
|-----|-----|-----|----|
| 415 | 195 | 131 | 56 |
| 342 | 206 | 83 | 66 |
| 376 | 197 | 136 | 48 |
| 326 | 160 | 125 | 61 |
| 358 | 150 | 118 | 55 |
| 279 | 118 | 135 | 56 |
| 333 | 119 | 169 | 38 |
| 268 | 103 | 126 | 48 |
| 277 | 93 | 135 | 65 |
| 328 | 97 | 193 | 69 |

| | | | | |
|-----------------|-----|-----|----|----|
| Average= | 392 | 273 | 72 | 31 |
|-----------------|-----|-----|----|----|

Method Blank Data

Negative peak areas are considered n/d

Wash Samples:

| | | ¹³ C: Peak Area 198→140 | | | Calc Conc (ng/L) |
|-------------------|---|---------------------------------------|---------|---------|-----------------------|
| <i>Injection:</i> | | Wash #1 | Wash #2 | Wash #3 | Wash #3 |
| | 1 | -116 | -231 | 280 | 30878 |
| | 2 | -152 | -209 | 171 | 29500 |
| | 3 | -123 | -215 | 130 | 28982 |
| | | | | | Average= 29786 |
| | | | | | Std Dev= 980 |

78

| | | Peak Area 195→138 | | | Peak Area 195→110 | | | Ratio |
|-------------------|---|----------------------|---------|---------|----------------------|---------|---------|---------|
| <i>Injection:</i> | | Wash #1 | Wash #2 | Wash #3 | Wash #1 | Wash #2 | Wash #3 | Wash #3 |
| | 1 | -37 | -41 | 144 | -4 | 116 | 251 | 1.7 |
| | 2 | -38 | -14 | 108 | -2 | -1 | 252 | 2.3 |
| | 3 | -44 | -32 | 100 | -16 | 114 | 203 | 2.0 |

| | | ¹² C: Peak Area 195→138 | | | Calc Conc (ng/L) |
|-------------------|---|---------------------------------------|---------|---------|---------------------|
| <i>Injection:</i> | | Wash #1 | Wash #2 | Wash #3 | Wash #3 |
| | 1 | -37 | -41 | 144 | n/d |
| | 2 | -38 | -14 | 108* | n/d |
| | 3 | -44 | -32 | 100* | n/d |

Methanol Extract Sample:

| Injection: | ¹³ C: Peak Area 198→140 | Calc Conc (ng/L) |
|------------|---------------------------------------|---------------------|
| 1 | -144 | n/d |
| 2 | 90* | n/d |
| 3 | -99 | n/d |

| | Peak Area 195→138 | Peak Area 195→110 | Ratio |
|---|----------------------|----------------------|-------|
| 1 | 199 | 73 | 0.4 |
| 2 | 224 | 679 | 3.0 |
| 3 | 159 | 69 | 0.4 |

79

| | Peak Area 195→138 | Calc Conc (ng/L) |
|---|----------------------|------------------|
| 1 | 199 | n/d |
| 2 | 224 | n/d |
| 3 | 159 | n/d |

Results:

| ¹³ C: Peak Area 198→140 Total (ng/L) | Abs Unc |
|--|---------|
| 29786 | 980 |
| Actual Conc (ng/L) | |
| 14.9 | 0.5 |

| ¹² C: Peak Area 195→138 Total (ng/L) |
|--|
| 0 |
| Actual Conc (ng/L) |
| 0 |

* Not above MDS

** Below LLOD

*** Below LLOQ

Method Blank + Internal Standard

Method Blank + Internal Std-1

Wash Samples:

| ¹³ C: Peak Area 198→140 | | | | |
|---------------------------------------|---------|---------|---------|--|
| Injection: | Wash #1 | Wash #2 | Wash #3 | |
| 1 | -183 | -208 | -44 | |
| 2 | -192 | -226 | -27 | |
| 3 | -158 | -193 | -121 | |

| ∞ Injection: | Peak Area 195→138 | | | Peak Area 195→110 | | | Ratio |
|--------------|----------------------|---------|---------|----------------------|---------|---------|---------|
| | Wash #1 | Wash #2 | Wash #3 | Wash #1 | Wash #2 | Wash #3 | Wash #3 |
| 1 | -32 | -30 | 59 | -21 | 58 | 298 | 5.1 |
| 2 | -25 | -27 | 75 | -13 | 52 | 290 | 3.9 |
| 3 | -24 | -32 | 79 | -15 | 65 | 296 | 3.7 |

| Injection: | ¹² C: Peak Area 195→138 | | | Calc Conc (ng/L) |
|------------|---------------------------------------|---------|---------|------------------|
| | Wash #1 | Wash #2 | Wash #3 | Wash #3 |
| 1 | -32 | -30 | 59* | n/d |
| 2 | -25 | -27 | 75* | n/d |
| 3 | -24 | -32 | 79* | n/d |

Methanol Extract Sample:

| Injection: | | ¹³ C: Peak Area 198→140 | Calc Conc (ng/L) |
|------------|--|---------------------------------------|------------------|
| 1 | | 163144 | 1285265 |
| 2 | | 164502 | 1295783 |
| 3 | | 167160 | 1316370 |
| | | Average= | 1299140 |
| | | Std Dev= | 15822 |

| | Peak Area 195→138 | Peak Area 195→110 | Ratio |
|---|----------------------|----------------------|-------|
| 1 | 471 | 892 | 1.9 |
| 2 | 501 | 933 | 1.9 |
| 3 | 496 | 950 | 1.9 |

| | Peak Area 195→138 | Calc Conc (ng/L) |
|---|----------------------|------------------|
| 1 | 471 | 12967 |
| 2 | 501 | 13049 |
| 3 | 496 | 13035 |
| | | Average= |
| | | 13017 |
| | | Std Dev= |
| | | 44 |

* Not above MDS
 ** Below LLOD
 *** Below LLOQ

Results:

¹³C: Peak Area 198→140

| Total (ng/L) | Std Dev | Spike Conc (ng/L) | % Rec | % Rel Unc | Spike Correction |
|---------------------|----------------|--------------------------|--------------|------------------|-------------------------|
| 1299140 | 15822 | 1000000 | 130 | 1 | 0.77 |

¹²C: Peak Area 195→138

| Total (ng/L) | Std Dev | % Rel Unc | |
|-------------------------------------|----------------|------------------|----------------|
| 13017 | 44 | 0.3 | |
| Spike Corrected Total (ng/L) | | | Abs Unc |
| 10020 | | 0.01 | 127 |
| Actual Conc (ng/L) | | | |
| 5.01 | | | 0.06 |

Method Blank + Internal Std-2

Wash Samples:

| ¹³ C: Peak Area 198→140 | | | | |
|---------------------------------------|---------|---------|---------|--|
| Injection: | Wash #1 | Wash #2 | Wash #3 | |
| 1 | -240 | -227 | -115 | |
| 2 | -251 | -204 | -135 | |
| 3 | -235 | -209 | -77 | |

| Peak Area 195→138 | | | | Peak Area 195→110 | | | Ratio | |
|----------------------|---------|---------|---------|----------------------|---------|---------|---------|--|
| Injection: | Wash #1 | Wash #2 | Wash #3 | Wash #1 | Wash #2 | Wash #3 | Wash #3 | |
| 1 | -52 | -40 | 170 | -12 | 102 | 235 | 1.4 | |
| 2 | -51 | -45 | 128 | -23 | 81 | 200 | 1.6 | |
| 3 | -52 | -32 | 142 | -11 | 70 | 206 | 1.5 | |

| ¹² C: Peak Area 195→138 | | | | | Calc Conc (ng/L) |
|---------------------------------------|---------|---------|---------|---------|------------------|
| Injection: | Wash #1 | Wash #2 | Wash #3 | Wash #3 | |
| 1 | -52 | -40 | 170 | n/d | |
| 2 | -51 | -45 | 128 | n/d | |
| 3 | -52 | -32 | 142 | n/d | |

Methanol Extract Sample:

| <i>Injection:</i> | ¹³ C: Peak Area 198→140 | Calc Conc (ng/L) |
|-------------------|---------------------------------------|------------------|
| 1 | 118621 | 940426 |
| 2 | 122976 | 974156 |
| 3 | 124904 | 989089 |
| | Average= | 967890 |
| | Std Dev= | 24929 |

| | Peak Area 195→138 | Peak Area 195→110 | Ratio |
|---|----------------------|----------------------|-------|
| 1 | 499 | 700 | 1.4 |
| 2 | 561 | 793 | 1.4 |
| 3 | 509 | 787 | 1.5 |

| | Peak Area 195→138 | Calc Conc (ng/L) |
|---|----------------------|------------------|
| 1 | 499 | 13043 |
| 2 | 561 | 13213 |
| 3 | 509 | 13071 |
| | Average= | 13109 |
| | Std Dev= | 91 |

* Not above MDS
 ** Below LLOD
 *** Below LLOQ

Results:

| ¹³C: Peak Area 198→140 | | | | | | |
|--|-------------------------------------|----------------|--------------------------|----------------|------------------|-------------------------|
| | Total (ng/L) | Std Dev | Spike Conc (ng/L) | % Rec | % Rel Unc | Spike Correction |
| | 967890 | 24924 | 1000000 | 97 | 3 | 1.03 |
| ¹²C: Peak Area 195→138 | | | | | | |
| | Total (ng/L) | Std Dev | % Rel Unc | | | |
| | 13109 | 91 | 0.7 | | | |
| | Spike Corrected Total (ng/L) | | | Abs Unc | | |
| | 13544 | 0.03 | | 361 | | |
| | Actual Conc (ng/L) | | | | Abs Unc | |
| | 6.8 | | | | 0.2 | |

85

Average Results for Blank Correction:

| ¹²C: Peak Area 195→138 | | | | |
|--|-----------------------|----------------|---------------------------|----------------|
| | Average (ng/L) | Abs Unc | Actual Conc (ng/L) | Abs Unc |
| | 11782 | 383 | 5.9 | 0.2 |

APPENDIX J
Check Standard Data

¹²C-
Caffeine

| Hour | Conc (ng/L) | Injection | Peak Area | | Ratio | Calc Conc (ng/L) | Std Dev | % Resp | % Rel Unc |
|------|----------------|-----------------|-----------|---------|-------|---------------------|---------|--------|-----------|
| | | | 195→138 | 195→110 | | | | | |
| 7 | 54000 | 1 | 22069 | 5569 | 0.25 | 72119 | 25858 | 134 | 30 |
| | | 2 | 21449 | 5307 | 0.25 | 70421 | | 130 | |
| | | 3 | 38103 | 10308 | 0.27 | 116032 | | 215 | |
| | | Average= | | | | | | 86190 | |
| 10 | 54000 | 1 | 13473 | 3085 | 0.23 | 48576 | 542 | 90 | 1 |
| | | 2 | 13854 | 3176 | 0.23 | 49620 | | 92 | |
| | | 3 | 13757 | 3266 | 0.24 | 49354 | | 91 | |
| | | Average= | | | | | | 49183 | |
| 13 | 54000 | 1 | 13839 | 3293 | 0.24 | 49579 | 953 | 92 | 2 |
| | | 2 | 13493 | 3256 | 0.24 | 48631 | | 90 | |
| | | 3 | 13143 | 3037 | 0.23 | 47672 | | 88 | |
| | | Average= | | | | | | 48627 | |
| 20 | 54000 | 1 | 54613 | 15537 | 0.28 | 161249 | 11905 | 299 | 8 |
| | | 2 | 48614 | 13559 | 0.28 | 144819 | | 268 | |
| | | 3 | 46164 | 13209 | 0.29 | 138109 | | 256 | |
| | | Average= | | | | | | 148059 | |

98

¹³C₃-Caffeine

| Hour | Conc (ng/L) | Injection | Peak Area 195→138 | Calc Conc (ng/L) | Std Dev | % Resp | % Rel Unc |
|------|-----------------|-----------|----------------------|------------------|---------|--------|-----------|
| 7 | 50000 | 1 | 7993 | 83589 | 2650 | 167 | 3 |
| | | 2 | 7355 | 78648 | | 157 | |
| | | 3 | 7888 | 82776 | | 166 | |
| | | | | | | 163 | |
| | Average= | | | 81671 | | | |
| 10 | 50000 | 1 | 4944 | 59974 | 3784 | 120 | 7 |
| | | 2 | 4826 | 59060 | | 118 | |
| | | 3 | 4045 | 53011 | | 106 | |
| | | | | | | | |
| | Average= | | | 57348 | | 115 | |
| 13 | 50000 | 1 | 4770 | 58626 | 910 | 117 | 2 |
| | | 2 | 4701 | 58092 | | 116 | |
| | | 3 | 4541 | 56852 | | 114 | |
| | | | | | | | |
| | Average= | | | 57857 | | 116 | |
| 20 | 50000 | 1 | 16760 | 151491 | 4927 | 303 | 3 |
| | | 2 | 15690 | 143204 | | 286 | |
| | | 3 | 15629 | 142731 | | 285 | |
| | | | | | | | |
| | Average= | | | 145809 | | 292 | |

APPENDIX K

Sampling Station Data

Wash Samples:

| Station A + ¹³C Spike | ¹³C: Peak Area 198→140 | | | Calc Conc (ng/L) | |
|---|--|---------|---------|-------------------------|---------|
| <i>Injection:</i> | Wash #1 | Wash #2 | Wash #3 | Wash #2 | Wash #3 |
| 1 | -219 | -48 | 263 | | 30663 |
| 2 | -212 | 43* | 306 | n/d | 31206 |
| 3 | -206 | -214 | 205 | | 29930 |
| | | | | Average= | 30599 |
| | | | | Std Dev= | 641 |

88

| | Peak Area 195-->138 | | | Peak Area 195-->110 | | | Ratio | |
|-------------------|-------------------------------|---------|---------|-------------------------------|---------|---------|--------------|---------|
| <i>Injection:</i> | Wash #1 | Wash #2 | Wash #3 | Wash #1 | Wash #2 | Wash #3 | Wash #2 | Wash #3 |
| 1 | -38 | 3438 | 1353 | -7 | 127 | 517 | 0.04 | 0.38 |
| 2 | -32 | 3463 | 1128 | 8 | 151 | 470 | 0.04 | 0.42 |
| 3 | -31 | 80 | 1275 | 1 | 9 | 461 | 0.11 | 0.36 |

| | ¹²C: Peak Area 195→138 | | | Calc Conc (ng/L) | |
|-------------------|--|---------|---------|-------------------------|---------|
| <i>Injection:</i> | Wash #1 | Wash #2 | Wash #3 | Wash #2 | Wash #3 |
| 1 | -38 | 3438 | 1353 | 15537 | 939*** |
| 2 | -32 | 3463 | 1128 | 15712 | n/d |
| 3 | -31 | 80* | 1275 | n/d | 393** |

Average= 15625 666
 Std Dev= 124 386

Methanol Extract Sample:

Injection:

| ¹³ C: Peak Area | | |
|----------------------------|---------|------------------|
| | 198→140 | Calc Conc (ng/L) |
| 1 | 81846 | 655596 |
| 2 | 80719 | 646867 |
| 3 | 79285 | 635760 |
| Average= | | 646074 |
| Std Dev= | | 9941 |

69

| Peak Area | | | |
|-----------|---------|---------|-------|
| | 195→138 | 195→110 | Ratio |
| 1 | 956 | 964 | 1.01 |
| 2 | 878 | 939 | 1.07 |
| 3 | 914 | 912 | 1.00 |

| Peak Area | | |
|-----------|---------|------------------|
| | 195→138 | Calc Conc (ng/L) |
| 1 | 956 | 14295 |
| 2 | 878 | 14081 |
| 3 | 914 | 14180 |
| Average= | | 14185 |
| Std Dev= | | 107 |

* Not above MDS
 ** Below LLOD
 *** Below LLOQ

Results:

¹³C: Peak Area 198→140

| Total (ng/L) | Abs Unc | Spike Conc (ng/L) | % Rec | % Rel Unc | Spike Correction |
|--------------|---------|-------------------|-------|-----------|------------------|
| 676674 | 9962 | 1000000 | 68 | 1.47 | 1.48 |

¹²C: Peak Area 195→138

| Total (ng/L) | Abs Unc | % Rel Unc |
|--------------------------------------|---------|-----------|
| 30476 | 419 | 1.38 |
| Spike Corrected Total (ng/L) | | |
| 45038 | 908 | 0.02 |
| Method Blank Corrected (ng/L) | | |
| 33256 | 985 | |
| Actual Conc (ng/L) | | |
| 16.6 | 0.5 | |

06

Wash Samples:

| Station B + ¹³C Spike | | ¹³C: Peak Area 198→140 | | | Calc Conc (ng/L) |
|---|---|--|---------|-----------------|-----------------------------|
| <i>Injection:</i> | | Wash #1 | Wash #2 | Wash #3 | Wash #3 |
| | 1 | -164 | -22 | 294 | 31055 |
| | 2 | -234 | -24 | 277 | 30840 |
| | 3 | -243 | -193 | 247 | 30460 |
| | | | | Average= | 30785 |
| | | | | Std Dev= | 301 |

| | | Peak Area 195→138 | | | Peak Area 195→110 | | | Ratio | |
|-------------------|---|------------------------------|---------|---------|------------------------------|---------|---------|--------------|---------|
| <i>Injection:</i> | | Wash #1 | Wash #2 | Wash #3 | Wash #1 | Wash #2 | Wash #3 | Wash #2 | Wash #3 |
| | 1 | -31 | 1276 | 706 | 6 | 101 | 412 | 0.08 | 0.58 |
| 91 | 2 | -21 | 1215 | 617 | 2 | 103 | 464 | 0.08 | 0.75 |
| | 3 | -15 | 21 | 604 | -4 | 5 | 487 | 0.24 | 0.81 |

| | | ¹²C: Peak Area 195→138 | | | Calc Conc (ng/L) | |
|-------------------|---|--|---------|---------|-----------------------------|---------|
| <i>Injection:</i> | | Wash #1 | Wash #2 | Wash #3 | Wash #2 | Wash #3 |
| | 1 | -31 | 1276 | 706 | 400** | n/d |
| | 2 | -21 | 1215 | 617 | n/d | n/d |
| | 3 | -15 | 21* | 604 | n/d | n/d |

Methanol Extract Sample:

| Injection: | ¹³ C: Peak Area | | Calc Conc (ng/L) |
|------------|----------------------------|--|------------------|
| | 198→140 | | |
| 1 | 71987 | | 579236 |
| 2 | 73297 | | 589382 |
| 3 | 74091 | | 595532 |
| | Average= | | 588050 |
| | Std Dev= | | 8229 |

| | Peak Area | Peak Area | Ratio |
|---|-----------|-----------|-------|
| | 195→138 | 195→110 | |
| 1 | 845 | 867 | 1.03 |
| 2 | 767 | 895 | 1.17 |
| 3 | 811 | 954 | 1.18 |

92

| | Peak Area | Calc Conc (ng/L) | |
|---|-----------------|------------------|-------|
| | 195→138 | | |
| 1 | 845 | 13991 | |
| 2 | 767 | 13777 | |
| 3 | 811 | 13898 | |
| | Average= | | 13889 |
| | Std Dev= | | 107 |

* Not above MDS
 ** Below LLOD
 *** Below LLOQ

Results:

¹³C: Peak Area 198→140

| Total (ng/L) | Abs Unc | Spike Conc (ng/L) | % Rec | % Rel Unc | Spike Correction |
|---------------------|----------------|--------------------------|--------------|------------------|-------------------------|
| 618835 | 8235 | 1000000 | 62 | 1.33 | 1.62 |

¹²C: Peak Area 195→138

| Total (ng/L) | Abs Unc | % Rel Unc |
|--------------------------------------|----------------|------------------|
| 13889 | 107 | 0.77 |
| Spike Corrected Total (ng/L) | | |
| 22443 | 345 | 0.02 |
| Method Blank Corrected (ng/L) | | |
| 10662 | 515 | |
| Actual Conc (ng/L) | | |
| 5.3 | 0.3 | |

Wash Samples:

| Station C + 13C Spike | 13C: Peak Area 198→140 | | | Calc Conc (ng/L) | |
|--------------------------|---------------------------|---------|---------|---------------------|---------|
| <i>Injection:</i> | Wash #1 | Wash #2 | Wash #3 | Wash #2 | Wash #3 |
| 1 | -218 | 5* | 252 | n/d | 30524 |
| 2 | -208 | 0* | 187 | n/d | 29702 |
| 3 | -192 | 21* | 185 | n/d | 29677 |
| | | | | Average= | 29968 |
| | | | | Std Dev= | 482 |

| | Peak Area 195→138 | | | Peak Area 195→110 | | | Ratio | | |
|-------------------|----------------------|---------|---------|----------------------|---------|---------|---------|---------|---------|
| <i>Injection:</i> | Wash #1 | Wash #2 | Wash #3 | Wash #1 | Wash #2 | Wash #3 | Wash #1 | Wash #2 | Wash #3 |
| 94 1 | -15 | 2740 | 1011 | 8 | 120 | 479 | n/d | 0.04 | 0.47 |
| 2 | -18 | 2728 | 1049 | 15 | 113 | 470 | n/d | 0.04 | 0.45 |
| 3 | 5 | 2691 | 999 | 10 | 112 | 478 | 2.02 | 0.04 | 0.48 |

| | 12C: Peak Area 195→138 | | | Calc Conc (ng/L) | | |
|-------------------|---------------------------|---------|---------|---------------------|---------|---------|
| <i>Injection:</i> | Wash #1 | Wash #2 | Wash #3 | Wash #1 | Wash #2 | Wash #3 |
| 1 | -15 | 2740 | 1011 | | 10650 | n/d |
| 2 | -18 | 2728 | 1049 | | 10566 | n/d |
| 3 | 5* | 2691 | 999 | n/d | 10307 | n/d |
| | | | | Average= | 10508 | |
| | | | | Std Dev= | 179 | |

Methanol Extract Sample:

| Injection: | ¹³ C: Peak Area 198→140 | Calc Conc (ng/L) |
|------------|---------------------------------------|---------------------|
| 1 | 86408 | 690929 |
| 2 | 87165 | 696792 |
| 3 | 83530 | 668639 |
| | Average= | 685453 |
| | Std Dev= | 14854 |

| | Peak Area 195→138 | Peak Area 195→110 | Ratio |
|---|----------------------|----------------------|-------|
| 1 | 1819 | 1164 | 0.64 |
| 2 | 1752 | 1208 | 0.69 |
| 3 | 1710 | 1171 | 0.68 |

95

| | Peak Area 195→138 | Calc Conc (ng/L) |
|---|----------------------|---------------------|
| 1 | 1819 | 16659 |
| 2 | 1752 | 16475 |
| 3 | 1710 | 16360 |
| | Average= | 16498 |
| | Std Dev= | 151 |

* Not above MDS
 ** Below LLOD
 *** Below LLOQ

Results:

¹³C: Peak Area 198→140

| Total (ng/L) | Error | Spike Conc (ng/L) | % Rec | % Rel Unc | Spike Correction |
|---------------------|--------------|--------------------------|--------------|------------------|-------------------------|
| 715421 | 14862 | 1000000 | 72 | 2.08 | 1.40 |

¹²C: Peak Area 195→138

| Total (ng/L) | Abs Unc | % Rel Unc |
|--------------------------------------|----------------|------------------|
| 27005 | 234 | 0.87 |
| Spike Corrected Total (ng/L) | | |
| 37748 | 850 | |
| Method Blank Corrected (ng/L) | | |
| 25966 | 932 | |
| Actual Conc (ng/L) | | |
| 13.0 | 0.5 | |

Wash Samples:

| Station D + 13C Spike | 13C: Peak Area 198→140 | Wash #1 | Wash #2 | Wash #3 | Calc Conc (ng/L) |
|--------------------------|---------------------------|---------|---------|---------|---------------------|
| <i>Injection:</i> | | | | | |
| | | Wash #1 | Wash #2 | Wash #3 | Wash #3 |
| 1 | -199 | | -34 | 37* | n/d |
| 2 | -198 | | -41 | 41* | n/d |
| 3 | -191 | | -151 | 61* | n/d |

| | Peak Area 195→138 | Wash #1 | Wash #2 | Wash #3 | Peak Area 195→110 | Wash #1 | Wash #2 | Wash #3 | Ratio | Wash #1 | Wash #2 | Wash #3 |
|-------------------|----------------------|---------|---------|---------|----------------------|---------|---------|---------|-------|---------|---------|---------|
| <i>Injection:</i> | | | | | | | | | | | | |
| | | Wash #1 | Wash #2 | Wash #3 | Wash #1 | Wash #2 | Wash #3 | | | Wash #1 | Wash #2 | Wash #3 |
| 1 | -13 | | 1092 | 514 | 8 | 70 | 458 | | | | 0.06 | 0.89 |
| 2 | -6 | | 1124 | 527 | 9 | 87 | 465 | | | | 0.08 | 0.88 |
| 3 | 9 | | 26 | 577 | 7 | 1 | 535 | 0.78 | | | 0.04 | 0.93 |

97

| | 12C: Peak Area 195→138 | Wash #1 | Wash #2 | Wash #3 | Calc Conc (ng/L) | Wash #1 | Wash #2 | Wash #3 |
|-------------------|---------------------------|---------|---------|---------|---------------------|---------|---------|---------|
| <i>Injection:</i> | | | | | | | | |
| | | Wash #1 | Wash #2 | Wash #3 | Wash #1 | Wash #2 | Wash #3 | |
| 1 | -13 | | 1092 | 514 | | n/d | n/d | |
| 2 | -6 | | 1124 | 527 | | n/d | n/d | |
| 3 | 9 | | 26* | 577 | n/d | n/d | n/d | |

Methanol Extract Sample:

| <i>Injection:</i> | ¹³ C: Peak Area 198→140 | Calc Conc (ng/L) |
|-------------------|---------------------------------------|---------------------|
| 1 | 69360 | 558889 |
| 2 | 71861 | 578260 |
| 3 | 73943 | 594385 |
| | Average= | 577178 |
| | Std Dev= | 17773 |

| | Peak Area 195→138 | Peak Area 195→110 | Ratio |
|---|----------------------|----------------------|-------|
| 1 | 600 | 780 | 1.30 |
| 2 | 602 | 790 | 1.31 |
| 3 | 700 | 731 | 1.04 |

| | Peak Area 195→138 | Calc Conc (ng/L) |
|---|----------------------|---------------------|
| 1 | 600 | 13320 |
| 2 | 602 | 13325 |
| 3 | 700 | 13594 |
| | Average= | 13413 |
| | Std Dev= | 157 |

86

* Not above MDS
 ** Below LLOD
 *** Below LLOQ

Results:

¹³C: Peak Area 198→140

| Total (ng/L) | Std Dev | Spike Conc (ng/L) | % Rec | % Rel Unc | Spike Correction |
|---------------------|----------------|--------------------------|--------------|------------------|-------------------------|
| 577178 | 17773 | 1000000 | 58 | 3.08 | 1.73 |

¹²C: Peak Area 195→138

| Total (ng/L) | Abs Unc | % Rel Unc |
|--------------------------------------|----------------|------------------|
| 13413 | 157 | 1.17 |
| Spike Corrected Total (ng/L) | | |
| 23239 | 765 | |
| Method Blank Corrected (ng/L) | | |
| 11457 | 856 | |
| Actual Conc (ng/L) | | |
| 5.7 | 0.4 | |

Wash Samples:

| Station E + ¹³ C Spike | ¹³ C: Peak Area 198→140 | | | Calc Conc (ng/L) | |
|-----------------------------------|---------------------------------------|---------|---------|---------------------|---------|
| | Wash #1 | Wash #2 | Wash #3 | Wash #2 | Wash #3 |
| <i>Injection:</i> | | | | | |
| 1 | -224 | 6* | 37* | n/d | n/d |
| 2 | -194 | -19 | 99* | | n/d |
| 3 | -182 | -9 | 64* | | n/d |

| <i>Injection:</i> | Peak Area 195→138 | | | Peak Area 195→110 | | | Ratio | |
|-------------------|----------------------|---------|---------|----------------------|---------|---------|---------|---------|
| | Wash #1 | Wash #2 | Wash #3 | Wash #1 | Wash #2 | Wash #3 | Wash #2 | Wash #3 |
| 100 | | | | | | | | |
| 1 | -24 | 2935 | 955 | 2 | 112 | 384 | 0.04 | 0.40 |
| 2 | -35 | 2877 | 941 | -3 | 114 | 403 | 0.04 | 0.43 |
| 3 | -19 | 2774 | 924 | -10 | 87 | 382 | 0.03 | 0.41 |

| <i>Injection:</i> | ¹² C: Peak Area 195→138 | | | Calc Conc (ng/L) | |
|-------------------|---------------------------------------|---------|---------|---------------------|---------|
| | Wash #1 | Wash #2 | Wash #3 | Wash #2 | Wash #3 |
| 1 | -24 | 2935 | 955 | 12015 | n/d |
| 2 | -35 | 2877 | 941 | 11609 | n/d |
| 3 | -19 | 2774 | 924 | 10888 | n/d |

Average= 11504
Std Dev= 571

Methanol Extract Sample:

Injection:

| | ¹³ C: Peak Area 198→140 | Calc Conc (ng/L) |
|---|---------------------------------------|---------------------|
| 1 | 51347 | 419375 |
| 2 | 52404 | 427561 |
| 3 | 51642 | 421659 |
| | Average= | 422865 |
| | Std Dev= | 4224 |

| | Peak Area 195→138 | Peak Area 195→110 | Ratio |
|---|----------------------|----------------------|-------|
| 1 | 1515 | 719 | 0.47 |
| 2 | 1478 | 663 | 0.45 |
| 3 | 1509 | 736 | 0.49 |

| | Peak Area 195→138 | Calc Conc (ng/L) |
|---|----------------------|---------------------|
| 1 | 1515 | 15826 |
| 2 | 1478 | 15725 |
| 3 | 1509 | 15810 |
| | Average= | 15787 |
| | Std Dev= | 54 |

101

* Not above MDS
 ** Below LLOD
 *** Below LLOQ

Results:

¹³C: Peak Area 198→140

| Total (ng/L) | Std Dev | Spike Conc (ng/L) | % Rec | % Rel Unc | Spike Correction |
|---------------------|----------------|--------------------------|--------------|------------------|-------------------------|
| 422865 | 4224 | 1000000 | 42 | 1.00 | 2.36 |

¹²C: Peak Area 195→138

| Total (ng/L) | Abs Unc | % Rel Unc |
|--------------------------------------|----------------|------------------|
| 27291 | 574 | 2.10 |
| Spike Corrected Total (ng/L) | | |
| 64538 | 1502 | |
| Method Blank Corrected (ng/L) | | |
| 52756 | 1550 | |
| Actual Conc (ng/L) | | |
| 26.4 | 0.8 | |

APPENDIX L

Sample Suite with Instrument Blank Corrected Data

| Sample Name | Vial | TIC Area | Peak Area 198-->140 | Peak Area 195-->138 | Peak Area 195-->110 |
|----------------------|------|----------|------------------------|------------------------|------------------------|
| 9010blank 0 | 1 | -300 | 22 | 36 | 5 |
| 9010blank 0 | 1 | -329 | 38 | 18 | 7 |
| 9010blank 0 | 1 | -352 | 33 | 14 | 2 |
| 9010blank 0 | 1 | -335 | 30 | 18 | 9 |
| 9010blank 0 | 1 | -366 | 21 | 20 | 5 |
| 9010blank 1 | 1 | -348 | 32 | 17 | 3 |
| 9010blank 1 | 1 | -359 | 26 | 14 | 3 |
| 9010blank 1 | 1 | -357 | 25 | 15 | 2 |
| ¹² C cal1 | 2 | 218 | | 371 | 95 |
| ¹² C cal2 | 3 | 3507 | | 2984 | 750 |
| ¹² C cal3 | 4 | 10409 | | 8276 | 2121 |
| ¹² C cal4 | 5 | 28169 | | 22466 | 5756 |
| ¹² C cal4 | 5 | 25195 | | 19923 | 5114 |
| ¹² C cal4 | 5 | 28441 | | 22314 | 5817 |
| ¹² C cal5 | 6 | 40125 | | 32089 | 8107 |
| ¹² C cal6 | 7 | 107010 | | 86236 | 21510 |
| ¹² C cal7 | 8 | 217687 | | 173526 | 41410 |
| ¹² C cal8 | 9 | 745511 | | 593664 | 139649 |
| 9010blank 1 | 1 | -262 | 31 | 78 | 34 |
| 9010blank 1 | 1 | -324 | 34 | 41 | 18 |
| 9010blank 1 | 1 | -340 | 23 | 0 | 0 |
| 9010blank 1 | 1 | -330 | 51 | 0 | 0 |
| 9010blank 1 | 1 | -338 | 29 | 30 | 13 |
| ¹³ C cal1 | 10 | -215 | -113 | | |
| ¹³ C cal2 | 11 | 883 | 972 | | |
| ¹³ C cal3 | 12 | 2639 | 2700 | | |
| ¹³ C cal4 | 13 | 6659 | 6568 | | |
| ¹³ C cal4 | 13 | 7048 | 6886 | | |
| ¹³ C cal4 | 13 | 6817 | 6644 | | |
| ¹³ C cal5 | 14 | 10657 | 10361 | | |
| ¹³ C cal6 | 15 | 22552 | 22433 | | |
| ¹³ C cal7 | 16 | 54077 | 52470 | | |
| ¹³ C cal8 | 17 | 132204 | 132457 | | |
| 9010blank 2 | 18 | -314 | 74 | 0 | 0 |
| 9010blank 2 | 18 | -392 | 0 | 0 | 0 |
| 9010blank 2 | 18 | -348 | 48 | 0 | 0 |
| 9010blank 2 | 18 | -346 | 36 | 0 | 0 |
| 9010blank 2 | 18 | -362 | 20 | 0 | 0 |
| 9010blank 3 | 22 | -347 | 30 | 0 | 0 |
| 9010blank 3 | 22 | -354 | 27 | 0 | 0 |
| 9010blank 3 | 22 | -357 | 19 | 0 | 0 |
| methodblank | 23 | 1734 | 214 | 372 | 1115 |
| methodblank | 23 | -170 | -115 | -7 | -11 |
| methodblank | 23 | -51 | -63 | -7 | -11 |
| 9010blank 3 | 22 | -345 | 32 | 0 | 0 |

| | | | | | |
|---------------------------|----|--------|--------|-------|-------|
| 9010blank 3 | 22 | -363 | 28 | 0 | 0 |
| 9010blank 3 | 22 | -350 | 43 | 0 | 0 |
| STA5 | 24 | 86353 | 81846 | 956 | 964 |
| STA5 | 24 | 84288 | 80719 | 878 | 939 |
| STA5 | 24 | 84314 | 79285 | 914 | 912 |
| 9010blank 4 | 25 | -265 | 111 | 0 | 0 |
| 9010blank 4 | 25 | -294 | 75 | 0 | 0 |
| 9010blank 4 | 25 | -302 | 69 | 0 | 0 |
| STA6 | 26 | 77497 | 71987 | 845 | 867 |
| STA6 | 26 | 76553 | 73297 | 767 | 895 |
| STA6 | 26 | 76580 | 74091 | 811 | 954 |
| 9010blank 4 | 25 | -299 | 82 | 20 | 0 |
| 9010blank 4 | 25 | -334 | 52 | 0 | 0 |
| 9010blank 4 | 25 | -305 | 59 | 18 | 0 |
| methodblank+spike-1 | 27 | 171520 | 163144 | 471 | 892 |
| methodblank+spike-1 | 27 | 172628 | 164502 | 501 | 933 |
| methodblank+spike-1 | 27 | 173000 | 167160 | 496 | 950 |
| 9010blank 5 | 28 | 1674 | 1547 | 65 | 22 |
| 9010blank 5 | 28 | -311 | 67 | 11 | 5 |
| 9010blank 5 | 28 | -335 | 38 | 0 | 0 |
| STA7 | 29 | 92852 | 86408 | 1819 | 1164 |
| STA7 | 29 | 94720 | 87165 | 1752 | 1208 |
| STA7 | 29 | 93357 | 83530 | 1710 | 1171 |
| 9010blank 5 | 28 | -317 | 67 | 0 | 0 |
| 9010blank 5 | 28 | -339 | 47 | 0 | 0 |
| 9010blank 5 | 28 | -340 | 44 | 0 | 0 |
| STA8 | 30 | 73665 | 69360 | 600 | 780 |
| STA8 | 30 | 78066 | 71861 | 602 | 790 |
| STA8 | 30 | 77940 | 73943 | 700 | 731 |
| 9010blank 6 | 31 | 8613 | 8141 | 348 | 202 |
| 9010blank 6 | 31 | 1373 | 1369 | 93 | 42 |
| 9010blank 6 | 31 | -309 | 82 | 0 | 0 |
| methodblank+spike-2 | 32 | 121544 | 118621 | 499 | 700 |
| methodblank+spike-2 | 32 | 129920 | 122976 | 561 | 793 |
| methodblank+spike-2 | 32 | 129335 | 124904 | 509 | 787 |
| 9010blank 7 | 33 | 1708 | 1883 | 47 | 48 |
| 9010blank 7 | 33 | 638 | 837 | 41 | 30 |
| 9010blank 7 | 33 | -179 | 151 | 20 | 8 |
| ¹² C checkcal4 | 5 | 68950 | | 22069 | 5569 |
| ¹² C checkcal4 | 5 | 63003 | | 21449 | 5307 |
| ¹² C checkcal4 | 5 | 59869 | | 38103 | 10308 |
| ¹³ C checkcal4 | 13 | 7827 | 7993 | | |
| ¹³ C checkcal4 | 13 | 7346 | 7355 | | |
| ¹³ C checkcal4 | 13 | 7730 | 7888 | | |
| 9010blank 6 | 31 | 1585 | 1412 | 334 | 124 |
| 9010blank 6 | 31 | -303 | 77 | 16 | 6 |
| 9010blank 6 | 31 | 953 | 953 | 235 | 85 |
| STA11 | 34 | 56544 | 51347 | 1515 | 719 |
| STA11 | 34 | 56311 | 52404 | 1478 | 663 |
| STA11 | 34 | 55351 | 51642 | 1509 | 736 |
| 9010blank 8 | 35 | 2346 | 2124 | 165 | 77 |
| 9010blank 8 | 35 | 913 | 1153 | 94 | 45 |

| | | | | | |
|---------------------------|----|-------|------|-------|------|
| 9010blank 8 | 35 | 399 | 657 | 66 | 22 |
| methodblankwash1 | 36 | -160 | -116 | -37 | -4 |
| methodblankwash1 | 36 | -212 | -152 | -38 | -2 |
| methodblankwash1 | 36 | -187 | -123 | -44 | -16 |
| 9010blank 8 | 35 | -66 | 245 | 43 | 22 |
| STA5wash1 | 37 | -256 | -219 | -38 | -7 |
| STA5wash1 | 37 | -254 | -212 | -32 | 8 |
| STA5wash1 | 37 | -261 | -206 | -31 | 1 |
| 9010blank 8 | 35 | -180 | 161 | 32 | 17 |
| STA6wash1 | 38 | -200 | -164 | -31 | 6 |
| STA6wash1 | 38 | -263 | -234 | -21 | 2 |
| STA6wash1 | 38 | -292 | -243 | -15 | -4 |
| 9010blank 8 | 35 | -232 | 90 | 35 | 12 |
| methodblank+spike-1 wash1 | 39 | -259 | -183 | -32 | -21 |
| methodblank+spike-1 wash1 | 39 | -251 | -192 | -25 | -13 |
| methodblank+spike-1 wash1 | 39 | -208 | -158 | -24 | -15 |
| 9010blank 8 | 35 | -249 | 78 | 47 | 15 |
| STA7wash1 | 40 | -227 | -218 | -15 | 8 |
| STA7wash1 | 40 | -193 | -208 | -18 | 15 |
| STA7wash1 | 40 | -195 | -192 | 5 | 10 |
| 9010blank 8 | 35 | -240 | 70 | 42 | 14 |
| STA8wash1 | 41 | -219 | -199 | -13 | 8 |
| STA8wash1 | 41 | -251 | -198 | -6 | 9 |
| STA8wash1 | 41 | -210 | -191 | 9 | 7 |
| 9010blank 7 | 33 | -291 | 72 | 52 | 21 |
| 9010blank 7 | 33 | -293 | 56 | 54 | 28 |
| 9010blank 7 | 33 | -240 | 99 | 53 | 24 |
| ¹² C checkcal4 | 5 | 16413 | | 13473 | 3085 |
| ¹² C checkcal4 | 5 | 17003 | | 13854 | 3176 |
| ¹² C checkcal4 | 5 | 16943 | | 13757 | 3266 |
| ¹³ C checkcal4 | 13 | 5119 | 4944 | | |
| ¹³ C checkcal4 | 13 | 4889 | 4826 | | |
| ¹³ C checkcal4 | 13 | 4146 | 4045 | | |
| 9010blank 8 | 35 | -265 | 52 | 50 | 35 |
| 9010blank 8 | 35 | -242 | 63 | 57 | 19 |
| 9010blank 8 | 35 | -246 | 59 | 49 | 24 |
| methodblank+spike-2 wash1 | 42 | -270 | -240 | -52 | -12 |
| methodblank+spike-2 wash1 | 42 | -346 | -251 | -51 | -23 |
| methodblank+spike-2 wash1 | 42 | -343 | -235 | -52 | -11 |
| 9010blank 8 | 35 | -240 | 52 | 46 | 21 |
| STA11wash1 | 43 | -256 | -224 | -24 | 2 |
| STA11wash1 | 43 | -250 | -194 | -35 | -3 |
| STA11wash1 | 43 | -247 | -182 | -19 | -10 |
| 9010blank 9 | 44 | -300 | 40 | 34 | 18 |
| methodblankwash2 | 45 | -155 | -231 | -41 | 116 |
| methodblankwash2 | 45 | -247 | -209 | -14 | -1 |
| methodblankwash2 | 45 | -215 | -215 | -32 | 114 |
| 9010blank 9 | 44 | -254 | 46 | 78 | 37 |
| STA5wash2 | 46 | 3664 | -48 | 3438 | 127 |
| STA5wash2 | 46 | 3577 | 43 | 3463 | 151 |
| STA5wash2 | 46 | -1 | -214 | 80 | 9 |
| 9010blank 9 | 44 | -181 | 89 | 93 | 41 |

| | | | | | |
|---------------------------|----|--------|------|--------|-------|
| STA6wash2 | 47 | 1386 | -22 | 1276 | 101 |
| STA6wash2 | 47 | 1439 | -24 | 1215 | 103 |
| STA6wash2 | 47 | -127 | -193 | 21 | 5 |
| 9010blank 9 | 44 | -214 | 83 | 72 | 27 |
| methodblank+spike-1 wash2 | 48 | -207 | -208 | -30 | 58 |
| methodblank+spike-1 wash2 | 48 | -229 | -226 | -27 | 52 |
| methodblank+spike-1 wash2 | 48 | -195 | -193 | -32 | 65 |
| 9010blank 9 | 44 | -238 | 69 | 52 | 25 |
| STA7wash2 | 49 | 2984 | 5 | 2740 | 120 |
| STA7wash2 | 49 | 2859 | 0 | 2728 | 113 |
| STA7wash2 | 49 | 2835 | 21 | 2691 | 112 |
| 9010blank 7 | 33 | -260 | 61 | 59 | 14 |
| 9010blank 7 | 33 | -255 | 63 | 60 | 14 |
| 9010blank 7 | 33 | -247 | 88 | 63 | 15 |
| ¹² C checkcal4 | 5 | 17144 | | 13839 | 3293 |
| ¹² C checkcal4 | 5 | 16838 | | 13493 | 3256 |
| ¹² C checkcal4 | 5 | 16246 | | 13143 | 3037 |
| ¹³ C checkcal4 | 13 | 4836 | 4770 | | |
| ¹³ C checkcal4 | 13 | 4807 | 4701 | | |
| ¹³ C checkcal4 | 13 | 4658 | 4541 | | |
| 9010blank 9 | 44 | -267 | 74 | 47 | 19 |
| 9010blank 9 | 44 | -250 | 56 | 47 | 21 |
| 9010blank 9 | 44 | -250 | 80 | 54 | 20 |
| STA8wash2 | 50 | 1178 | -34 | 1092 | 70 |
| STA8wash2 | 50 | 1319 | -41 | 1124 | 87 |
| STA8wash2 | 50 | -48 | -151 | 26 | 1 |
| 9010blank 9 | 44 | -298 | 62 | 49 | 12 |
| methodblank+spike-2 wash2 | 51 | -213 | -227 | -40 | 102 |
| methodblank+spike-2 wash2 | 51 | -201 | -204 | -45 | 81 |
| methodblank+spike-2 wash2 | 51 | -201 | -209 | -32 | 70 |
| 9010blank 9 | 44 | -303 | 61 | 40 | 16 |
| STA11wash2 | 52 | 3184 | 6 | 2935 | 112 |
| STA11wash2 | 52 | 3072 | -19 | 2877 | 114 |
| STA11wash2 | 52 | 3084 | -9 | 2774 | 87 |
| 9010blank 9 | 44 | -253 | 53 | 102 | 23 |
| 9010blank 9 | 44 | -200 | 47 | 81 | 21 |
| 9010blank 1 | 1 | -237 | 51 | 67 | 25 |
| 9010blank 1 | 1 | -278 | 36 | 57 | 16 |
| 9010blank 1 | 1 | -254 | 57 | 56 | 22 |
| ¹² C cal1 | 2 | 165 | | 326 | 73 |
| ¹² C cal2 | 3 | 2038 | | 1875 | 453 |
| ¹² C cal3 | 4 | 6201 | | 5092 | 1181 |
| ¹² C cal4 | 5 | 13490 | | 11037 | 2477 |
| ¹² C cal4 | 5 | 13178 | | 10857 | 2427 |
| ¹² C cal4 | 5 | 13043 | | 10626 | 2439 |
| ¹² C cal5 | 6 | 20825 | | 17332 | 3829 |
| ¹² C cal6 | 7 | 43514 | | 35811 | 7809 |
| ¹² C cal7 | 8 | 95706 | | 78663 | 16909 |
| ¹² C cal8 | 9 | 282353 | | 232520 | 49228 |
| 9010blank 1 | 1 | 87 | 42 | 365 | 111 |
| 9010blank 1 | 1 | 131 | 37 | 381 | 128 |
| 9010blank 1 | 1 | -6 | 41 | 368 | 131 |

| | | | | | |
|---------------------------|----|-------|-------|-------|-------|
| 9010blank 1 | 1 | -110 | 48 | 196 | 91 |
| 9010blank 1 | 1 | -52 | 40 | 221 | 117 |
| ¹³ C cal1 | 10 | -11 | -153 | | |
| ¹³ C cal2 | 11 | 527 | 318 | | |
| ¹³ C cal3 | 12 | 1352 | 1191 | | |
| ¹³ C cal4 | 13 | 3141 | 2711 | | |
| ¹³ C cal4 | 13 | 2914 | 2715 | | |
| ¹³ C cal4 | 13 | 2991 | 2662 | | |
| ¹³ C cal5 | 14 | 6074 | 6035 | | |
| ¹³ C cal6 | 15 | 13450 | 13238 | | |
| ¹³ C cal7 | 16 | 33080 | 32352 | | |
| ¹³ C cal8 | 17 | 80407 | 80445 | | |
| 9010blank 10 | 53 | -41 | 210 | 90 | 75 |
| 9010blank 10 | 53 | -75 | 131 | 90 | 63 |
| 9010blank 10 | 53 | -50 | 194 | 93 | 53 |
| 9010blank 10 | 53 | -86 | 143 | 84 | 54 |
| 9010blank 10 | 53 | -85 | 157 | 70 | 56 |
| methodblankwash3 | 54 | 972 | 280 | 144 | 251 |
| methodblankwash3 | 54 | 707 | 171 | 108 | 252 |
| methodblankwash3 | 54 | 517 | 130 | 100 | 203 |
| 9010blank 10 | 53 | 204 | 391 | 137 | 94 |
| STA5wash3 | 55 | 2407 | 263 | 1353 | 517 |
| STA5wash3 | 55 | 2106 | 306 | 1128 | 470 |
| STA5wash3 | 55 | 2075 | 205 | 1275 | 461 |
| 9010blank 10 | 53 | 256 | 404 | 145 | 78 |
| STA6wash3 | 56 | 1607 | 294 | 706 | 412 |
| STA6wash3 | 56 | 1409 | 277 | 617 | 464 |
| STA6wash3 | 56 | 1453 | 247 | 604 | 487 |
| 9010blank 10 | 53 | 41 | 236 | 139 | 61 |
| methodblank+spike-1 wash3 | 57 | 256 | -44 | 59 | 298 |
| methodblank+spike-1 wash3 | 57 | 376 | -27 | 75 | 290 |
| methodblank+spike-1 wash3 | 57 | 288 | -121 | 79 | 296 |
| 9010blank 10 | 53 | 23 | 195 | 131 | 56 |
| STA7wash3 | 58 | 1804 | 252 | 1011 | 479 |
| STA7wash3 | 58 | 1816 | 187 | 1049 | 470 |
| STA7wash3 | 58 | 1841 | 185 | 999 | 478 |
| 9010blank 10 | 53 | -50 | 206 | 83 | 66 |
| STA8wash3 | 59 | 1182 | 37 | 514 | 458 |
| STA8wash3 | 59 | 1089 | 41 | 527 | 465 |
| STA8wash3 | 59 | 1235 | 61 | 577 | 535 |
| 9010blank 7 | 33 | -16 | 197 | 136 | 48 |
| 9010blank 7 | 33 | -66 | 160 | 125 | 61 |
| 9010blank 7 | 33 | -34 | 150 | 118 | 55 |
| ¹² C checkcal4 | 5 | 70088 | | 54613 | 15537 |
| ¹² C checkcal4 | 5 | 63322 | | 48614 | 13559 |
| ¹² C checkcal4 | 5 | 59756 | | 46164 | 13209 |
| ¹³ C checkcal4 | 13 | 17081 | 16760 | | |
| ¹³ C checkcal4 | 13 | 16245 | 15690 | | |
| ¹³ C checkcal4 | 13 | 15900 | 15629 | | |
| 9010blank 10 | 53 | -113 | 118 | 135 | 56 |
| 9010blank 10 | 53 | -59 | 119 | 169 | 38 |
| 9010blank 10 | 53 | -124 | 103 | 126 | 48 |

| | | | | | |
|---------------------------|----|------|------|-----|-----|
| methodblank+spike-2 wash3 | 60 | 357 | -115 | 170 | 235 |
| methodblank+spike-2 wash3 | 60 | 129 | -135 | 128 | 200 |
| methodblank+spike-2 wash3 | 60 | 194 | -77 | 142 | 206 |
| 9010blank 10 | 53 | -115 | 93 | 135 | 65 |
| STA11wash3 | 61 | 1417 | 37 | 955 | 384 |
| STA11wash3 | 61 | 1363 | 99 | 941 | 403 |
| STA11wash3 | 61 | 1468 | 64 | 924 | 382 |
| 9010blank 10 | 53 | -64 | 97 | 193 | 69 |
| methodblank 1-4 | 23 | 117 | -144 | 199 | 73 |
| methodblank 1-5 | 23 | 1046 | 90 | 224 | 679 |
| methodblank 1-6 | 23 | 131 | -99 | 159 | 69 |

APPENDIX M

Sample Suite with Raw Instrument Data

| Sample Name | Vial | TIC Area | Peak Area 198-->140 | Peak Area 195-->138 | Peak Area 195-->110 |
|----------------------|------|----------|---------------------|---------------------|---------------------|
| 9010blank 0 | 1 | 92 | 22 | 36 | 5 |
| 9010blank 0 | 1 | 63 | 38 | 18 | 7 |
| 9010blank 0 | 1 | 40 | 33 | 14 | 2 |
| 9010blank 0 | 1 | 57 | 30 | 18 | 9 |
| 9010blank 0 | 1 | 26 | 21 | 20 | 5 |
| 9010blank 1 | 1 | 44 | 32 | 17 | 3 |
| 9010blank 1 | 1 | 33 | 26 | 14 | 3 |
| 9010blank 1 | 1 | 35 | 25 | 15 | 2 |
| ¹² C cal1 | 2 | 610 | | 443 | 126 |
| ¹² C cal2 | 3 | 3899 | | 3056 | 781 |
| ¹² C cal3 | 4 | 10801 | | 8348 | 2152 |
| ¹² C cal4 | 5 | 28561 | | 22538 | 5787 |
| ¹² C cal4 | 5 | 25587 | | 19995 | 5145 |
| ¹² C cal4 | 5 | 28833 | | 22386 | 5848 |
| ¹² C cal5 | 6 | 40517 | | 32161 | 8138 |
| ¹² C cal6 | 7 | 107402 | | 86308 | 21541 |
| ¹² C cal7 | 8 | 218079 | | 173598 | 41441 |
| ¹² C cal8 | 9 | 745903 | | 593736 | 139680 |
| 9010blank 1 | 1 | 130 | 31 | 78 | 34 |
| 9010blank 1 | 1 | 68 | 34 | 41 | 18 |
| 9010blank 1 | 1 | 52 | 23 | 0 | 0 |
| 9010blank 1 | 1 | 62 | 51 | 0 | 0 |
| 9010blank 1 | 1 | 54 | 29 | 30 | 13 |
| ¹³ C cal1 | 10 | 177 | 160 | | |
| ¹³ C cal2 | 11 | 1275 | 1245 | | |
| ¹³ C cal3 | 12 | 3031 | 2973 | | |
| ¹³ C cal4 | 13 | 7051 | 6841 | | |
| ¹³ C cal4 | 13 | 7440 | 7159 | | |
| ¹³ C cal4 | 13 | 7209 | 6917 | | |
| ¹³ C cal5 | 14 | 11049 | 10634 | | |
| ¹³ C cal6 | 15 | 22944 | 22706 | | |
| ¹³ C cal7 | 16 | 54469 | 52743 | | |
| ¹³ C cal8 | 17 | 132596 | 132730 | | |
| 9010blank 2 | 18 | 78 | 74 | 0 | 0 |
| 9010blank 2 | 18 | 0 | 0 | 0 | 0 |
| 9010blank 2 | 18 | 44 | 48 | 0 | 0 |
| 9010blank 2 | 18 | 46 | 36 | 0 | 0 |
| 9010blank 2 | 18 | 30 | 20 | 0 | 0 |
| 9010blank 3 | 22 | 45 | 30 | 0 | 0 |
| 9010blank 3 | 22 | 38 | 27 | 0 | 0 |
| 9010blank 3 | 22 | 35 | 19 | 0 | 0 |
| methodblank | 23 | 2126 | 487 | 444 | 1146 |
| methodblank | 23 | 222 | 158 | 65 | 20 |
| methodblank | 23 | 341 | 210 | 65 | 20 |
| 9010blank 3 | 22 | 47 | 32 | 0 | 0 |

| | | | | | |
|---------------------------|----|--------|--------|-------|-------|
| 9010blank 3 | 22 | 29 | 28 | 0 | 0 |
| 9010blank 3 | 22 | 42 | 43 | 0 | 0 |
| STA5 | 24 | 86745 | 82119 | 1028 | 995 |
| STA5 | 24 | 84680 | 80992 | 950 | 970 |
| STA5 | 24 | 84706 | 79558 | 986 | 943 |
| 9010blank 4 | 25 | 127 | 111 | 0 | 0 |
| 9010blank 4 | 25 | 98 | 75 | 0 | 0 |
| 9010blank 4 | 25 | 90 | 69 | 0 | 0 |
| STA6 | 26 | 77889 | 72260 | 917 | 898 |
| STA6 | 26 | 76945 | 73570 | 839 | 926 |
| STA6 | 26 | 76972 | 74364 | 883 | 985 |
| 9010blank 4 | 25 | 93 | 82 | 20 | 0 |
| 9010blank 4 | 25 | 58 | 52 | 0 | 0 |
| 9010blank 4 | 25 | 87 | 59 | 18 | 0 |
| methodblank+spike-1 | 27 | 171912 | 163417 | 543 | 923 |
| methodblank+spike-1 | 27 | 173020 | 164775 | 573 | 964 |
| methodblank+spike-1 | 27 | 173392 | 167433 | 568 | 981 |
| 9010blank 5 | 28 | 2066 | 1547 | 65 | 22 |
| 9010blank 5 | 28 | 81 | 67 | 11 | 5 |
| 9010blank 5 | 28 | 57 | 38 | 0 | 0 |
| STA7 | 29 | 93244 | 86681 | 1891 | 1195 |
| STA7 | 29 | 95112 | 87438 | 1824 | 1239 |
| STA7 | 29 | 93749 | 83803 | 1782 | 1202 |
| 9010blank 5 | 28 | 75 | 67 | 0 | 0 |
| 9010blank 5 | 28 | 53 | 47 | 0 | 0 |
| 9010blank 5 | 28 | 52 | 44 | 0 | 0 |
| STA8 | 30 | 74057 | 69633 | 672 | 811 |
| STA8 | 30 | 78458 | 72134 | 674 | 821 |
| STA8 | 30 | 78332 | 74216 | 772 | 762 |
| 9010blank 6 | 31 | 9005 | 8141 | 348 | 202 |
| 9010blank 6 | 31 | 1765 | 1369 | 93 | 42 |
| 9010blank 6 | 31 | 83 | 82 | 0 | 0 |
| methodblank+spike-2 | 32 | 121936 | 118894 | 571 | 731 |
| methodblank+spike-2 | 32 | 130312 | 123249 | 633 | 824 |
| methodblank+spike-2 | 32 | 129727 | 125177 | 581 | 818 |
| 9010blank 7 | 33 | 2100 | 1883 | 47 | 48 |
| 9010blank 7 | 33 | 1030 | 837 | 41 | 30 |
| 9010blank 7 | 33 | 213 | 151 | 20 | 8 |
| ¹² C checkcal4 | 5 | 69342 | | 22141 | 5600 |
| ¹² C checkcal4 | 5 | 63395 | | 21521 | 5338 |
| ¹² C checkcal4 | 5 | 60261 | | 38175 | 10339 |
| ¹³ C checkcal4 | 13 | 8219 | 8266 | | |
| ¹³ C checkcal4 | 13 | 7738 | 7628 | | |
| ¹³ C checkcal4 | 13 | 8122 | 8161 | | |
| 9010blank 6 | 31 | 1977 | 1412 | 334 | 124 |
| 9010blank 6 | 31 | 89 | 77 | 16 | 6 |
| 9010blank 6 | 31 | 1345 | 953 | 235 | 85 |
| STA11 | 34 | 56936 | 51620 | 1587 | 750 |
| STA11 | 34 | 56703 | 52677 | 1550 | 694 |
| STA11 | 34 | 55743 | 51915 | 1581 | 767 |
| 9010blank 8 | 35 | 2738 | 2124 | 165 | 77 |
| 9010blank 8 | 35 | 1305 | 1153 | 94 | 45 |

| | | | | | |
|---------------------------|----|-------|------|-------|------|
| 9010blank 8 | 35 | 791 | 657 | 66 | 22 |
| methodblankwash1 | 36 | 232 | 157 | 35 | 27 |
| methodblankwash1 | 36 | 180 | 121 | 34 | 29 |
| methodblankwash1 | 36 | 205 | 150 | 28 | 15 |
| 9010blank 8 | 35 | 326 | 245 | 43 | 22 |
| STA5wash1 | 37 | 136 | 54 | 34 | 24 |
| STA5wash1 | 37 | 138 | 61 | 40 | 39 |
| STA5wash1 | 37 | 131 | 67 | 41 | 32 |
| 9010blank 8 | 35 | 212 | 161 | 32 | 17 |
| STA6wash1 | 38 | 192 | 109 | 41 | 37 |
| STA6wash1 | 38 | 129 | 39 | 51 | 33 |
| STA6wash1 | 38 | 100 | 30 | 57 | 27 |
| 9010blank 8 | 35 | 160 | 90 | 35 | 12 |
| methodblank+spike-1 wash1 | 39 | 133 | 90 | 40 | 10 |
| methodblank+spike-1 wash1 | 39 | 141 | 81 | 47 | 18 |
| methodblank+spike-1 wash1 | 39 | 184 | 115 | 48 | 16 |
| 9010blank 8 | 35 | 143 | 78 | 47 | 15 |
| STA7wash1 | 40 | 165 | 55 | 57 | 39 |
| STA7wash1 | 40 | 199 | 65 | 54 | 46 |
| STA7wash1 | 40 | 197 | 81 | 77 | 41 |
| 9010blank 8 | 35 | 152 | 70 | 42 | 14 |
| STA8wash1 | 41 | 173 | 74 | 59 | 39 |
| STA8wash1 | 41 | 141 | 75 | 66 | 40 |
| STA8wash1 | 41 | 182 | 82 | 81 | 38 |
| 9010blank 7 | 33 | 101 | 72 | 52 | 21 |
| 9010blank 7 | 33 | 99 | 56 | 54 | 28 |
| 9010blank 7 | 33 | 152 | 99 | 53 | 24 |
| ¹² C checkcal4 | 5 | 16805 | | 13545 | 3116 |
| ¹² C checkcal4 | 5 | 17395 | | 13926 | 3207 |
| ¹² C checkcal4 | 5 | 17335 | | 13829 | 3297 |
| ¹³ C checkcal4 | 13 | 5511 | 5217 | | |
| ¹³ C checkcal4 | 13 | 5281 | 5099 | | |
| ¹³ C checkcal4 | 13 | 4538 | 4318 | | |
| 9010blank 8 | 35 | 127 | 52 | 50 | 35 |
| 9010blank 8 | 35 | 150 | 63 | 57 | 19 |
| 9010blank 8 | 35 | 146 | 59 | 49 | 24 |
| methodblank+spike-2 wash1 | 42 | 122 | 33 | 20 | 19 |
| methodblank+spike-2 wash1 | 42 | 46 | 22 | 21 | 8 |
| methodblank+spike-2 wash1 | 42 | 49 | 38 | 20 | 20 |
| 9010blank 8 | 35 | 152 | 52 | 46 | 21 |
| STA11wash1 | 43 | 136 | 49 | 48 | 33 |
| STA11wash1 | 43 | 142 | 79 | 37 | 28 |
| STA11wash1 | 43 | 145 | 91 | 53 | 21 |
| 9010blank 9 | 44 | 92 | 40 | 34 | 18 |
| methodblankwash2 | 45 | 237 | 42 | 31 | 147 |
| methodblankwash2 | 45 | 145 | 64 | 58 | 30 |
| methodblankwash2 | 45 | 177 | 58 | 40 | 145 |
| 9010blank 9 | 44 | 138 | 46 | 78 | 37 |
| STA5wash2 | 46 | 4056 | 225 | 3510 | 158 |
| STA5wash2 | 46 | 3969 | 316 | 3535 | 182 |
| STA5wash2 | 46 | 391 | 59 | 152 | 40 |
| 9010blank 9 | 44 | 211 | 89 | 93 | 41 |

| | | | | | |
|---------------------------|----|--------|------|--------|-------|
| STA6wash2 | 47 | 1778 | 251 | 1348 | 132 |
| STA6wash2 | 47 | 1831 | 249 | 1287 | 134 |
| STA6wash2 | 47 | 265 | 80 | 93 | 36 |
| 9010blank 9 | 44 | 178 | 83 | 72 | 27 |
| methodblank+spike-1 wash2 | 48 | 185 | 65 | 42 | 89 |
| methodblank+spike-1 wash2 | 48 | 163 | 47 | 45 | 83 |
| methodblank+spike-1 wash2 | 48 | 197 | 80 | 40 | 96 |
| 9010blank 9 | 44 | 154 | 69 | 52 | 25 |
| STA7wash2 | 49 | 3376 | 278 | 2812 | 151 |
| STA7wash2 | 49 | 3251 | 273 | 2800 | 144 |
| STA7wash2 | 49 | 3227 | 294 | 2763 | 143 |
| 9010blank 7 | 33 | 132 | 61 | 59 | 14 |
| 9010blank 7 | 33 | 137 | 63 | 60 | 14 |
| 9010blank 7 | 33 | 145 | 88 | 63 | 15 |
| ¹² C checkcal4 | 5 | 17536 | | 13911 | 3324 |
| ¹² C checkcal4 | 5 | 17230 | | 13565 | 3287 |
| ¹² C checkcal4 | 5 | 16638 | | 13215 | 3068 |
| ¹³ C checkcal4 | 13 | 5228 | 5043 | | |
| ¹³ C checkcal4 | 13 | 5199 | 4974 | | |
| ¹³ C checkcal4 | 13 | 5050 | 4814 | | |
| 9010blank 9 | 44 | 125 | 74 | 47 | 19 |
| 9010blank 9 | 44 | 142 | 56 | 47 | 21 |
| 9010blank 9 | 44 | 142 | 80 | 54 | 20 |
| STA8wash2 | 50 | 1570 | 239 | 1164 | 101 |
| STA8wash2 | 50 | 1711 | 232 | 1196 | 118 |
| STA8wash2 | 50 | 344 | 122 | 98 | 32 |
| 9010blank 9 | 44 | 94 | 62 | 49 | 12 |
| methodblank+spike-2 wash2 | 51 | 179 | 46 | 32 | 133 |
| methodblank+spike-2 wash2 | 51 | 191 | 69 | 27 | 112 |
| methodblank+spike-2 wash2 | 51 | 191 | 64 | 40 | 101 |
| 9010blank 9 | 44 | 89 | 61 | 40 | 16 |
| STA11wash2 | 52 | 3576 | 279 | 3007 | 143 |
| STA11wash2 | 52 | 3464 | 254 | 2949 | 145 |
| STA11wash2 | 52 | 3476 | 264 | 2846 | 118 |
| 9010blank 9 | 44 | 139 | 53 | 102 | 23 |
| 9010blank 9 | 44 | 192 | 47 | 81 | 21 |
| 9010blank 1 | 1 | 155 | 51 | 67 | 25 |
| 9010blank 1 | 1 | 114 | 36 | 57 | 16 |
| 9010blank 1 | 1 | 138 | 57 | 56 | 22 |
| ¹² C cal1 | 2 | 557 | | 398 | 104 |
| ¹² C cal2 | 3 | 2430 | | 1947 | 484 |
| ¹² C cal3 | 4 | 6593 | | 5164 | 1212 |
| ¹² C cal4 | 5 | 13882 | | 11109 | 2508 |
| ¹² C cal4 | 5 | 13570 | | 10929 | 2458 |
| ¹² C cal4 | 5 | 13435 | | 10698 | 2470 |
| ¹² C cal5 | 6 | 21217 | | 17404 | 3860 |
| ¹² C cal6 | 7 | 43906 | | 35883 | 7840 |
| ¹² C cal7 | 8 | 96098 | | 78735 | 16940 |
| ¹² C cal8 | 9 | 282745 | | 232592 | 49259 |
| 9010blank 1 | 1 | 479 | 42 | 365 | 111 |
| 9010blank 1 | 1 | 523 | 37 | 381 | 128 |
| 9010blank 1 | 1 | 386 | 41 | 368 | 131 |

| | | | | | |
|---------------------------|----|-------|-------|-------|-------|
| 9010blank 1 | 1 | 282 | 48 | 196 | 91 |
| 9010blank 1 | 1 | 340 | 40 | 221 | 117 |
| ¹³ C cal1 | 10 | 381 | 120 | | |
| ¹³ C cal2 | 11 | 919 | 591 | | |
| ¹³ C cal3 | 12 | 1744 | 1464 | | |
| ¹³ C cal4 | 13 | 3533 | 2984 | | |
| ¹³ C cal4 | 13 | 3306 | 2988 | | |
| ¹³ C cal4 | 13 | 3383 | 2935 | | |
| ¹³ C cal5 | 14 | 6466 | 6308 | | |
| ¹³ C cal6 | 15 | 13842 | 13511 | | |
| ¹³ C cal7 | 16 | 33472 | 32625 | | |
| ¹³ C cal8 | 17 | 80799 | 80718 | | |
| 9010blank 10 | 53 | 351 | 210 | 90 | 75 |
| 9010blank 10 | 53 | 317 | 131 | 90 | 63 |
| 9010blank 10 | 53 | 342 | 194 | 93 | 53 |
| 9010blank 10 | 53 | 306 | 143 | 84 | 54 |
| 9010blank 10 | 53 | 307 | 157 | 70 | 56 |
| methodblankwash3 | 54 | 1364 | 553 | 216 | 282 |
| methodblankwash3 | 54 | 1099 | 444 | 180 | 283 |
| methodblankwash3 | 54 | 909 | 403 | 172 | 234 |
| 9010blank 10 | 53 | 596 | 391 | 137 | 94 |
| STA5wash3 | 55 | 2799 | 536 | 1425 | 548 |
| STA5wash3 | 55 | 2498 | 579 | 1200 | 501 |
| STA5wash3 | 55 | 2467 | 478 | 1347 | 492 |
| 9010blank 10 | 53 | 648 | 404 | 145 | 78 |
| STA6wash3 | 56 | 1999 | 567 | 778 | 443 |
| STA6wash3 | 56 | 1801 | 550 | 689 | 495 |
| STA6wash3 | 56 | 1845 | 520 | 676 | 518 |
| 9010blank 10 | 53 | 433 | 236 | 139 | 61 |
| methodblank+spike-1 wash3 | 57 | 648 | 229 | 131 | 329 |
| methodblank+spike-1 wash3 | 57 | 768 | 246 | 147 | 321 |
| methodblank+spike-1 wash3 | 57 | 680 | 152 | 151 | 327 |
| 9010blank 10 | 53 | 415 | 195 | 131 | 56 |
| STA7wash3 | 58 | 2196 | 525 | 1083 | 510 |
| STA7wash3 | 58 | 2208 | 460 | 1121 | 501 |
| STA7wash3 | 58 | 2233 | 458 | 1071 | 509 |
| 9010blank 10 | 53 | 342 | 206 | 83 | 66 |
| STA8wash3 | 59 | 1574 | 310 | 586 | 489 |
| STA8wash3 | 59 | 1481 | 314 | 599 | 496 |
| STA8wash3 | 59 | 1627 | 334 | 649 | 566 |
| 9010blank 7 | 33 | 376 | 197 | 136 | 48 |
| 9010blank 7 | 33 | 326 | 160 | 125 | 61 |
| 9010blank 7 | 33 | 358 | 150 | 118 | 55 |
| ¹² C checkcal4 | 5 | 70480 | | 54685 | 15568 |
| ¹² C checkcal4 | 5 | 63714 | | 48686 | 13590 |
| ¹² C checkcal4 | 5 | 60148 | | 46236 | 13240 |
| ¹³ C checkcal4 | 13 | 17473 | 17033 | | |
| ¹³ C checkcal4 | 13 | 16637 | 15963 | | |
| ¹³ C checkcal4 | 13 | 16292 | 15902 | | |
| 9010blank 10 | 53 | 279 | 118 | 135 | 56 |
| 9010blank 10 | 53 | 333 | 119 | 169 | 38 |
| 9010blank 10 | 53 | 268 | 103 | 126 | 48 |

| | | | | | |
|---------------------------|----|------|-----|------|-----|
| methodblank+spike-2 wash3 | 60 | 749 | 158 | 242 | 266 |
| methodblank+spike-2 wash3 | 60 | 521 | 138 | 200 | 231 |
| methodblank+spike-2 wash3 | 60 | 586 | 196 | 214 | 237 |
| 9010blank 10 | 53 | 277 | 93 | 135 | 65 |
| STA11wash3 | 61 | 1809 | 310 | 1027 | 415 |
| STA11wash3 | 61 | 1755 | 372 | 1013 | 434 |
| STA11wash3 | 61 | 1860 | 337 | 996 | 413 |
| 9010blank 10 | 53 | 328 | 97 | 193 | 69 |
| methodblank 1-4 | 23 | 509 | 129 | 271 | 104 |
| methodblank 1-5 | 23 | 1438 | 363 | 296 | 710 |
| methodblank 1-6 | 23 | 523 | 174 | 231 | 100 |