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Development of an LC-MS/MS Method for the Characterization of Halogenated Furanones in Drinking Water

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Abstract

Development of an LC-MS/MS Method for the Characterization of Halogenated Furanones in Drinking Water

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Halogenated furanones are chlorine disinfection by-products (DBPs) that have been shown to be direct acting mutagens and are considered potential human carcinogens. These high priority DBPs are among the by-products to be analyzed for the USEPA's Integrated Disinfection By-product Research Project (Four Lab Study) developed to evaluate the toxicity of the complex mixture of DBPs present in treated water. The traditional method for characterization of halogenated furanones involves liquid-liquid extraction (LLE) and a time intensive derivatization step for gas chromatography

analysis. In order to remove the derivatization step and provide a more efficient method to characterize halofuranones in the Four Lab Study's water sample, a liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed, using electrospray ionization. Halofuranones MX, MBA, BMX-1 and BMX-3 were successfully quantified through LC-ESI-MS/MS at the ng/L level in treated water samples. Method linearity was observed ($r^2 \geq 0.98$) with accurate and reproducible results. Compared to current detection methods, this LC-MS/MS method provides a faster yet highly sensitive and selective method to quantify halofuranones at the ng/L level.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
BMX-1	3-chloro-4-bromochloromethyl-5-hydroxy-2(5H)-furanone
BMX-2	3-chloro-4-dibromomethyl-5-hydroxy-2(5H)-furanone
BMX-3	3-bromo-4-dibromomethyl-5-hydroxy-2(5H)-furanone
CE	Collision energy
Cl_2	Free Chlorine
DAD	Diode array detector
DBPs	Disinfection by-products
DIW	Deionized water (18MΩ)
DOC	Dissolved organic carbon
ESI	Electrospray ionization
FA	Formic acid
FV	Fragmentor voltage
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IDL	Instrument detection limit
LC	Liquid chromatography
LLE	Liquid-liquid extraction
MBA	Mucobromic acid
MBTFA	N-methyl-bis-trifluoroacetamide
MCA	Mucochloric acid
MeOH	Methanol

LIST OF ABBREVIATIONS (continued)

MRM	Multiple reaction monitoring
MS/MS	tandem mass spectrometry
MX	3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone
NH_2Cl	Chloramine
NOM	Natural organic matter
PI	Product ion
RP	Reversed phase
SIM	Selected ion monitoring
SOP	Standard operating procedure
SPE	Solid phase extraction
UFC	Uniform formation conditions
XAD	Absorbent polymer resin

1. INTRODUCTION

1.1 Disinfection Byproducts

Municipal drinking water is commonly disinfected through addition of chlorine (Cl_2) or chloroamine (NH_2Cl). Natural organic matter (NOM) present in drinking water sources can react with chlorine to form byproducts that are potentially harmful to humans. The presence of bromide in drinking water sources, as a result of salt water intrusion or proximity to sea water, can also lead to the formation of brominated disinfection byproducts (DBPs)[1].

More than 500 byproducts have been identified[2] since 1976 when the DBP chloroform was found to be carcinogenic, and a widespread concern for DBPs began[3]. Additional studies discussed by Simmons et al[4] have shown that DBPs may be associated with bladder cancer, and exhibit reproductive and developmental toxicity. However, these studies include exposure to a single byproduct, or a mixture with few DBPs rather than the complex mixture to which people are most likely to be exposed[4].

Through the collaboration of four National Laboratories of the US EPA's Office of Research and Development, the Integrated Disinfection Byproducts Mixtures Research Project (the 4Lab Study) was initiated[4]. It is a project intended to evaluate reproductive and developmental toxicological effects associated with DBP mixtures through an animal model. The completion of the project would be carried out in a series of three phases. The first phase is focused on determination of appropriate water treatment and concentration

parameters. In the second stage, application of these methods at a larger scale is addressed, as well as initial *in vivo* and *in vitro* studies along with chemical analysis of DBPs. Finally, after revising methods and research from the first two stages, DBPs are characterized and toxicological data is gathered in the third phase. The objective of the current stage of the 4Lab Study is to develop integrated chemical and toxicological assessments of water concentrates, which require efficient, sensitive and selective DBP detection methods for samples with complex matrices. In order to effectively and efficiently characterize DBPs in complex samples, detection methods need to be developed or optimized.

1.2 Halogenated Furanones

The halogenated furanone 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone, known as Mutagen X (MX), was identified in the 1980's in the effluent of bleach liquors from pulp mills, and later detected in drinking water[5]. Studies have found that it is formed in the presence humic acids and chlorine, accounting for up to 67% of the mutagenicity observed in chlorinated drinking water according to Smeds et al[6], and up to 76% according to Myllykangas et al[7]. It was shown to cause DNA damage in human fibroblasts, where migration of DNA in a comet assay was observed at concentrations of 5.44 mg/L[8]. Mucochloric acid and brominated hydroxyl furanones are MX-analogs that have also been detected in drinking water and observed to be genotoxicants[9,10]. In fact, mutagenicity of treated waters was observed to increase with the addition of bromide and thus with the presence of brominated MX analogs[7]. Additionally, when comparing the

overall mutagenicity contribution of MX between waters with and without bromide, MX's contribution decreased significantly with presence of bromide, indicating that its brominated analogs were more potent mutagens. A nationwide DBP occurrence study conducted in the US showed that MX and analogs are present in drinking waters at levels higher than previously reported[1]. In plant distribution systems, MX was detected at levels up to 300 ng/L versus the previously reported 90 ng/L, and BMX-1 was detected up to 140 ng/L.

MX (pKa=5.3[11]) and analogs are non-volatile and thermally labile, and their chemical stability is dependent on the pH in water samples, where their analytical detection in the closed-ring forms (Figure 1) is optimal at pH2. Such aspects make characterization of MX and analogs in drinking waters challenging. Mutagen X and its analogs are considered high priority DBPs by the United States Environmental Protection Agency[12], and more focus has been placed in improving detection methods.

1.3 Detection Methods

The most common analysis methods of MX, BMX-1, BMX-2, BMX-3, MBA and MCA include (1) concentration through solid phase, liquid-liquid extraction (LLE) or XAD resins, (2) derivatization, (3) separation through gas chromatography, and (4) detection by mass spectrometry or electron capture detection[1,13,9,14]. One of the main challenges that arises from these detection methods is the need to derivatize, which has been the focus for optimization in much research. Derivatization methods of halofuranones often

involve methylation, propylation or acylation of their hydroxyl group with acid and organic solvents, a very time intensive process. Onstad and Weinberg[15] refined a derivatization method using a boron trifluoride methanol (BF_3/MeOH) complex to methylate halofuranones, which required a four hour reaction time at 70ºC. Other challenges include lack of specificity or sensitivity of the methylated ions in the mass spectrum, formation of interfering by-products (acetals), loss of analyte during neutralization and back extraction, and transferring of acid by-products to the GC column that may affect the instrument[13]. Kubwabo et al.[13] derivatized MX with N-methyl-bis-trifluoroacetamide (MBTFA) eliminating the use of acids, and thus neutralization and back extraction of the acylated MX. However, acylation still requires a two hour incubation step at a high temperature, and the method was not evaluated for other halofuranones.

Mitigation of matrix effects observed with GC analysis of MX, BMX-1, BMX-2 and BMX-3 was evaluated by Rantakokko et al.[16]. A sample preparation method was proposed and shown to mitigate matrix effects affecting GC peak size and shape, and therefore, accuracy of quantification. Their tandem solid phase extraction method removed humic and fulvic acids from water samples by passing them through a trifunctional C_{18} (tC_{18}) column, prior to retention of halofuranones on an Oasis HLB column.

Kubwabo et al[13] compared a modified version of Rantakokko's tandem SPE method to liquid-liquid extraction, single-cartridge SPE, and XAD extraction. The tandem SPE

yielded an MX average recovery of 124%, much higher compared to liquid-liquid extraction, XAD extraction, and single-cartridge solid phase extraction methods. Additionally, tandem SPE reduced the need for a larger sample size and the use of large quantities of solvents used in the LLE method, which necessitated a total of 750mL (three 250mL extractions per sample) of methyl tert-butyl ether (MTBE). Even in the refined method of Onstad and Weinberg[15] where only 100 mL of MTBE were used per sample (two 50 mL extractions) for LLE, solvent volume is still large compared to the 20 mL used during tandem SPE. Despite sample clean up improvements, extracts require derivatization for GC analysis. It would therefore be advantageous to develop a method that removes the derivatization step, thereby eliminating its challenges and improving characterization of halogenated furanones in drinking water.

1.4 Potential for the development of an LC-MS/MS method

Separation of halogenated furanones through liquid chromatography is the most reasonable alternative to gas chromatography as it would remove the need for derivatization. High performance liquid chromatography (HPLC) has been previously used for fractionation[17] and halofuranone stability analysis when coupled with UV-detection[18]. Still, sensitivity and specificity tend to be low when using UV-detection compared to GC methods using mass spectrometry or electron capture detection.

Coupling mass spectrometry with HPLC could provide a more sensitive method given that the analytes can be successfully ionized. For non-volatile, polar, and thermally labile compounds like halogenated furanones, a “soft ionization” technique such as electrospray

ionization (ESI) would be the best option. However, this type of ionization has not been previously investigated for halofuranones.

In order to maintain halogenated furanones in their closed-ring forms throughout the HPLC column, the pH of the mobile phase must remain at approximately pH2, below their pKa. Having this background matrix at a low pH may be advantageous when ionizing through ESI as it may promote the generation of protonated closed-ring ions. Evidence of this potential ionization is demonstrated by Crotti et al[19] who were able to ionize five-membered lactones (Figure 2) similar to MX and its analogs through liquid chromatography tandem mass spectrometry (LC-MS/MS), using ESI with collision induced dissociation. The main lactone fragments observed were generated by neutral CO and H_2O losses, revealing the potential halofuranone fragments generated through ESI.

The unique ion fragments produced for each halogenated furanone through LC-MS/MS will offer a greater method specificity than LC coupled with a diode array detector (UV-detection). Specifically, bromine and chlorine-containing organic compounds produce predictable mass spectral patterns that can be used to confirm the presence of a compound containing those atoms. The observed pattern is the result of the presence of two natural stable isotopes of bromine (^{79}Br and ^{81}Br) and chlorine (^{35}Cl and ^{37}Cl) (Figure 3). Each mass spectral line in a given pattern will be present at a specific abundance relative to the other spectral lines (Table 1)[20]. This abundance ratio of these lines was previously used by Petrovic et al[21] to confirm the presence of brominated and chlorinated

nonylphenols in water and sludge samples using LC-ESI-MS/MS, and could be used to increase the specificity of the proposed halofuranone LC-MS/MS method.

1.5 Specific Aims

There are more than 500 disinfection byproducts (DBPs) that have been identified with different chemical structures and properties. A number of DBPs have been shown to be carcinogenic, or associated with adverse reproductive and developmental health outcomes. The complexity of the formation and stability of DBPs, such as halogenated furanones, and the presence of natural organic matter, have made identification and quantification challenging. Understanding the health effects of the mixture of DBPs in drinking water relies on adequate detection and quantification of the fraction of DBPs present. In order to facilitate DBP characterization for future toxicological studies, our proposed work will focus on method development and refinement.

The long-term goal of the proposed study herein is to develop a method for the detection of the DBPs halogenated furanones in drinking water containing highly concentrated natural organic matter. It is a method intended to be utilized for the EPA's Integrated Disinfection Byproducts Mixtures Research Project (the 4Lab Study). Specifically, the objective is to create an analytical method to quantify the halofuranones 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone known as MX, mucochloric acid (MCA), mucobromic acid (MBA), 3-chloro-4-bromochloromethyl-5-hydroxy-2(5H)-furanone (BMX-1), 3-chloro-4-dibromomethyl-5-hydroxy-2(5H)-furanone (BMX-2), and 3-bromo-4-dibromomethyl-5-hydroxy-2(5H)-furanone (BMX-3). This method is intended to minimize the use of hazardous chemicals, reduce the number of analytical steps, and increase sensitivity and selectivity. We will pursue this study in two Specific Aims:

1. Develop an analytical method to characterize six halogenated furanones. Previous methods included derivatization steps in order to separate halofuranones through gas chromatography. Our intent is to concentrate, separate and detect the analytes through tandem solid phase extraction (SPE), liquid chromatography and tandem mass spectrometry (LC-MS/MS) respectively, thereby removing the need for derivatization, reducing the number of analytical steps, and the use of solvent/acid mixtures.
2. Apply and validate the resulting method from the first aim to chlorinated water samples from Lake Washington. Samples will be chlorinated under Uniform Formation Conditions[22] after addition of bromide. Adjustments to the protocol will be made if necessary, and a standard operating procedure (SOP) will be written for the finalized method.

The proposed work is intended to provide a novel method to characterize halogenated furanones, through which selective and sensitive results can be achieved in fewer steps than the gas chromatography methods currently used. As a result, the large number of water samples needed for the EPA's fourteen week 4lab study will be processed faster with the use of less hazardous chemicals.

2. EXPERIMENTAL SECTION

2.1 Reagents and Chemicals

Closed-ring MX (97% purity) and brominated MX analogs BMX-1 (98%), BMX-2 (98%) and BMX-3 (98%) were purchased from Toronto Research Chemicals (North York, ON, Canada), and commercially available closed-ring MCA (99%) and MBA (99%) were obtained from Sigma-Aldrich (St. Louis, MO). High purity organic solvents (methanol, acetone, and acetonitrile) for buffers, extractions, and dilutions were obtained from Fisher Scientific (Fair Lawn, NJ) Optima grade. High purity formic acid (FA) for buffers was obtained from Fisher Scientific (Fair Lawn, NJ) Optima LC/MS grade. Water samples were acidified with ACS Reagent grade 36.5-38% hydrochloric acid (JT Baker, Phillipsburg, NJ). Buffers and chemicals used for chlorination of Lake Washington samples were prepared with sodium bromide (99.99%, Alfa Aesar, Ward Hill, MA), laboratory grade sodium hypochlorite (4-6%, Fisher Scientific, Fair Lawn, NJ), analytical grade boric acid (EMD Chemicals, Gibbstown, NJ) and Sigma Ultra grade sodium hydroxide (≥98%, Sigma-Aldrich, St. Louis, MO). ACS reagent grade L-ascorbic acid (≥99%, Sigma-Aldrich, St. Louis, MO) was used to quench residual chlorine in treated samples. Organic-free, ultrapure 18.2MΩ deionized water (DIW) was used for all buffers, extractions, and dilutions (Barnstead Easypure, Dubuke, IA).

2.2 Safety

All procedures were carried out under a functional fume hood to prevent exposure to chemicals. Personal protective equipment including lab coat, gloves, and goggles were

worn at all times. Volumetric glassware utilized to make halofuranone standard solutions was rinsed at least three times with acetonitrile to remove residual halogenated furanones. All other glassware containing residue of halogenated furanones was soaked in detergent for at least three days, scrubbed and rinsed three times with DIW. Stocks and standard solutions were stored at -20°C. Degradation was observed for some methanolic mucobromic acid secondary stocks stored in silinized amber glass vials for more than one month. Degradation of MBA stocks was monitored by LC-DAD and new solutions were made if necessary.

2.3 Method Optimization

2.3.1 Liquid Chromatography

Separation conditions for MCA, MBA, MX, BMX-1, BMX-2, and BMX-3 were optimized using a Phenomenex (Torrance, CA) Synergy Polar-RP HPLC column (250mm x 2mm, 4µm particle size) with an Agilent (Palo Alto, CA) 1100 Series LC system equipped with a diode array detector. The full UV spectrum (Figure S.5 to Figure S.11) was acquired for each compound to determine the wavelength of maximum absorbance. Mobile phase was composed of eluent A (0.25% formic acid in DIW) and eluent B (0.25% formic acid in organic solvent). Methanol and acetonitrile were each evaluated as eluent B for separation of halofuranones. Isocratic and linear gradient elutions were tested with flow rates of 200 and 300 µL/min. Standards of halofuranones were dissolved in methanol or a mixture of 50/50 MeOH/DIW (0.25% FA) at concentrations ranging from 5 ng/mL to 250 µg/L (only MX and BMX's at the µg/L

level due to higher UV detection limits) prior to injection on the column. Injection volume effects were evaluated at 10, 25 and 30 µL.

2.3.2 Sample Preparation

Extraction of halogenated furanones from water samples was conducted following Rantakokko et al[16] and Kubwabo et al[13], where samples are acidified prior to extraction with the Sep-pak Plus tC18 (400mg) and Oasis HLB Plus (225mg) cartridges set in train (Waters; Wexford, Ireland). MCA was spiked at 10 ng/mL in 500 mL of acidified DIW (pH2) containing L-ascorbic acid (62 mg/L) to reproduce the Rantakokko et al[16] pre-concentration method. To aid extraction efficiency given halofuranone pKa values of 5.3 or less, acidification of sample was assessed with formic acid or HCl. Samples were pumped through cartridges at a flow rate of 1-5 mL/min through a vacuum manifold, followed by an additional 100 mL of pH2 DIW prior to drying the Oasis HLB cartridge under vacuum for 30-60 min. Methanol, acetonitrile, and acetone were each evaluated as eluents. Extracts were evaporated to near-dryness under a stream of nitrogen at 30ºC, and subsequently reconstituted in a mixture of 50/50 MeOH/DIW (0.25% FA) and filtered through a 0.2 µm PTFE filter.

To evaluate the adapted pre-concentration method of Kubwabo et al[13], 3 L of ultrapure water were acidified to pH2 (12N HCl) and spiked with L-ascorbic acid (62 mg/L). Two 500 mL aliquots were set aside to use as method blanks, and three 500 mL aliquots were each spiked with MCA and MBA at 100 ng/L, and MX and BMXs at 100 ng/mL to achieve UV-detectable levels in extracts. Samples were pumped through tC18 and Oasis

HLB cartridges set in train on a vacuum manifold with flow rates between 1-10mL/min, followed by an additional 100 mL of pH2 DIW (12N HCl). The Oasis HLB cartridge was dried under vacuum for 15 min prior to eluting analytes with 10 mL of acetone. Solvent was evaporated from extracts to near-dryness under a stream of nitrogen at 30ºC, to ultimately reconstitute in 500 µL of a 50/50 MeOH/DIW (0.25%FA) buffer. Extracts were filtered through a 0.2µm ID PTFE filter.

2.3.3 Mass Spectrometry Detection

Mass spectrometry optimization was carried out with the Agilent (Palo Alto, CA) G6460 LC-MS/MS system. Multiple reaction monitoring (MRM) Parent-Product ion transitions were initially optimized for the six analytes with Agilent MassHunter Workstation Optimizer Software (version B.06.00), by directly injecting 10 µL of individual analyte standards into the system at concentrations between 200 ng/mL and10 µg/mL. Fragmentor voltage (FV) and collision energy (CE) are automatically optimized by the software in a four step process: 1) Fragmentor voltage is optimized for precursor ions during MS/MS Selected Ion Monitoring (SIM) acquisition mode, 2) Product ions are monitored during Product Ion (PI) acquisition mode, 3) Collision energy for the detected product ions is optimized through MRM mode, 4) Optimized CE for product ions is validated through PI acquisition mode. Positive and negative electrospray ionization (ESI) were evaluated along with two mobile phases composed of 50-70% ultrapure water (0.25% FA) and 30-50% ACN (0.25%FA) or MeOH (0.25%FA). Individual analyte standards were generated from methanolic stocks and dissolved in a mixture of DIW and

organic solvent containing formic acid, consistent with the mobile phase composition evaluated. All other MS parameters were kept constant and at the recommended default setting.

Final optimization of fragmentor voltage and collision energy for each halofuranone was conducted manually with the G6460 LC-MS/MS system using the Synergy Polar-RP column and optimized chromatographic conditions. Individual standards of each analyte were generated in a mixture of 50/50 ACN/DIW (0.25% FA) at a concentration of 200 ng/mL from methanolic stocks. Fragmentor voltage was optimized for each individual analyte standard through MS/MS Full Scan acquisition mode. Product ions were monitored at different collision energies through PI acquisition mode. The optimized FV and CE were validated through MRM acquisition mode. All other MS parameters were kept constant for each analyte at the recommended default settings, with exception of BMX-2 for which the gas temperature was evaluated at 300 and 350ºC during Full Scan acquisition mode.

2.4 Method Validation

2.4.1 Collection and Chlorination of Samples

2.4.1.1 Chlorine Demand Study and DOC analysis

A surface water sample was collected from Lake Washington on June 2015 in a gallon amber glass bottle and stored at 4ºC. On the day of chlorine demand study, sample was allowed to equilibrate to room temperature prior to chlorination following Uniform

Formation Conditions (UFC)[22]. Three headspace-free 1L amber glass bottles were spiked individually with bromide (0.2 mg/L) using a stock solution of sodium bromide (2000 mg/L) in DIW. Hypochlorite-borate buffer (pH8) was added to each 1L sample after addition of borate buffer (pH8) and bromide, to yield final free Cl_2 concentrations of 3, 4 and 6.25 mg/L. Samples were incubated in the dark at 19ºC for 24±1 hr. Residual free Cl_2 was measured with the Hach DR/890 Portable Colorimeter Method 10102 (Loveland, CO). Residual Cl_2 measurements were graphed as a function of Cl_2 dose to extrapolate the chlorine dose necessary to yield a 1 mg/L free Cl_2 residual. An exponential regression was used to determine the dose for 1 mg/L residual since the 3 mg/L point was below samples' dissolved organic carbon concentration (3.65 mg/L DOC), where chlorine consumption may not behave linearly in relation to higher Cl_2 doses (Figure 4). A 10mL aliquot of water sample was filtered through a GF/F grade (47mm) glass microfiber filter for DOC analysis with the Dionex Ultimate 300 UHPLC (Thermo Fisher, Sunnyvale, California). A carbon standard of 1000ppm was diluted with DIW to generate four DOC calibrants (2, 4, 6, and 8 mg/L).

2.4.1.2 Chlorination of Samples

Two additional surface water samples were collected each in gallon-sized amber glass bottles on June 2015 at the same sampling location as the chlorine demand study. Samples were stored at 4ºC for no more than 48 hours, and allowed to equilibrate to room temperature on the day of chlorination following UFC[22]. Aliquots of 1 L or 500 mL were individually spiked with bromide (0.2 mg/L) and then chlorinated (4.87 mg/L Cl_2). Residual Cl_2 was measured after a contact time of 24±1hr and quenched with L-ascorbic

acid (62 mg/L). Samples were stored at 4°C if extraction was not conducted immediately after quenching.

2.4.2 Extraction of Halofuranones from Treated Samples.

Analytes were extracted from samples within 48 hours of quenching residual chlorine. Samples were allowed to equilibrate to room temperature prior to filtration through a GF/F grade glass microfiber filter under vacuum. pH was individually adjusted (pH2) for 500 mL sample aliquots in 500 mL volumetric flasks with 400 µL of 12N HCl. Aliquots were each spiked with a stock mixture of MBA, MX, BMX-1, and BMX-3 at different levels (50, 75, 100, 200, and 300 ng/L) to generate a standard addition curve. Duplicate extractions were conducted for matrix spikes of 50, 75, 200, and 300 ng/L, and triplicate extractions for matrix spike of 100 ng/L. Extractions were conducted with the evaluated pre-concentration method from Kubwabo et al[13] (see section *2.3.2 Sample Preparation*) on three separate days following the scheme in Figure 5. For these extracts, the eluent (acetone) was spiked with MCA (internal standard) at a concentration of 5 ng/mL. Relative to chlorinated halofuranones, closed-ring brominated MX analogs have been shown to be predominantly formed in treated waters containing high bromide levels. MCA is therefore a more suitable internal standard than MBA for this study.

2.4.3 Data Analysis

Rantakokko et al[16] found that quantitation of MX and BMXs was reasonably accurate when using a matrix-matched calibration, which required the use of blank matrix samples. Due to the unavailability of matrix-matched blank samples in this study,

standard addition calibration was chosen to curtail possible matrix effects. The relative analyte/MCA peak areas were plotted as a function of spike concentration to generate standard addition curves. Linearity, accuracy and precision of method were assessed based on the linear regression of the curves. External calibration curves were generated for each analyte to determine instrument detection limits (IDLs). IDLs were determined as $\frac{3 \times SD}{m}$ where *SD* is the standard deviation of at least seven replicates of a standard at a concentration near the limit of detection, and *m* is the slope of the external calibration curve[23]. Replicate injections were analyzed for 35% of samples.

3. RESULTS AND DISCUSSION

3.1 LC-MS/MS Method

3.1.1 LC and SPE Conditions obtained through LC-DAD

Optimal resolution of analytes using the Synergy Polar-RP HPLC column was obtained using a gradient of 28% eluent B (0.25% FA in ACN) and 72% eluent A (0.25% FA in ultrapure water), increased linearly in 20 minutes to 45% eluent B (0.25% FA in ACN) and 55% eluent A (0.25% FA in ultrapure water) with a flow rate of 300 µL/min (Figure 6). Isocratic elution with 35/65 ACN/DIW (0.25% FA) with the same flow rate did not resolve BMX-2 and BMX-3 peaks. An injection volume of 10 µL was determined to provide optimal peak shape, removing the broadening and fronting of peaks using higher injection volumes.

The tandem solid phase extraction method adapted by Kubwabo et al[13] was followed with a few modifications. 10 mL of acetone were used to elute analytes from the Oasis HLB cartridge, compared to the 15 mL used by Kubwabo et al. Extracts were reconstituted in 500 µL of a solvent mixture of 50/50 ACN/DIW (0.25%FA) instead of 250 µL of derivatizing agent (Figure 7). Preliminary optimization data using LC-DAD showed that good reproducible recoveries are obtained for MCA, MBA, MX, BMX-1, BMX-2, and BMX-3 using this extraction method (76-101% with %RSD <5, Table 2) compared to other tandem SPE and liquid-liquid extraction methods (Table 3).

3.1.2 Electrospray Ionization MS/MS

Ionization of all analytes with the exception of BMX-2 was observed using acetonitrile as the mobile phase in positive ionization mode. Data obtained through MassHunter Workstation Optimizer with BMX-2 direct injection (no LC column) during initial optimization of MS/MS parameters, showed that BMX-2 may be ionized in the positive ESI mode using methanol in the mobile phase. However, this was not further investigated. Optimal fragmentor voltages, collision energies, and MRM transitions for MCA, MBA, MX, BMX-1 and BMX-3 are presented in Table 4 and Table 5. Gas temperature, gas flow, nebulizer pressure, and capillary voltage were maintained at 350 ºC, 11 L/min, 30 psi and 4000 V, respectively. Quantitation and confirmation transitions for each analyte were only acquired at the pertinent compound retention time window (Figure 8).

3.2 Method Validation

3.2.1 Impact of Coeluting Compounds

A peak was observed to coelute with MCA in treated samples near the expected 6.76±0.06 min retention time (based on external calibrants' retention time). The vertices of the coeluting peaks occur at approximately 6.7 and 7.0 minutes (Figure 9, Figure 10). The chosen quantitation and confirmation product ion peaks for each analyte represent two isotopic mass spectral lines of brominated and chlorinated compounds. The abundance ratio of the two lines was used to confirm the identity of the compounds eluting at 6.7 and 7.0 minutes. The identity of the peak eluting at 6.0 minutes in all samples including the method blank was not evaluated.

Three samples were selected to evaluate MCA quantitation and confirmation ion peak ratios (*m/z* 151, and *m/z* 152.9) at 6.7 and 7.0 minutes (Table 6). On average, the ratios at 6.7 and 7.0 minutes differed from the theoretical value by 96 and 19% respectively. Both values exceeded the accepted 10% difference, and the correct MCA peak could not be distinguished. Consequently, the sum of the area of both peaks was used to normalize peak areas of MBA, MX, BMX-1 and BMX-3 in all samples. It is worth noting that this coeluting peak was not observed in method blanks (Figure 11), indicating the presence of sample matrix effects.

Additionally, a small peak was observed at 10.57 minutes in the treated sample from day 2 extractions, eluting slightly earlier than the expected 10.71±0.08 min for MX (Figure

9). The ratio of the quantitation (*m/z* 199) and confirmation (*m/z* 201) product ion peaks was 45% lower than the expected 1.03 ratio, indicating that the eluting compound was not MX.

3.2.2 Formation of Halogenated Furanones in Treated Samples

Two peaks were observed eluting at the retention times of MBA and BMX-3 only in the treated sample from day 2 extractions (Figure 9), suggesting formation of MBA and BMX-3 in samples from day 2. This would not be unlikely given that in the presence of bromide, brominated rather than chlorinated halofuranones are predominantly formed in treated water[1]. It is also possible that slight temperature, pH, chlorination and bromide addition differences between the three extraction days, and between sample bottles within each day, varied enough to produce MBA and BMX-3 only in day 2 samples.

The additional signal of MBA was not consistent across all matrix spiked samples. Only four of the five treated samples from day 2 produced the additional MBA signal (sample spiked with 75 ng/L of analyte did not have an additional signal consistent with other samples). Measured chlorination conditions cannot explain such observation, and though the method blank from day 2 did not produce a signal indicating contamination of MBA, the possibility of contamination cannot be ruled out. For BMX-3, it is more likely that it was in fact formed after chlorination of samples because the additional signal was consistent across all samples.

In order to ensure that the signals observed were in fact of MBA and BMX-3, abundance ratios of confirmation and quantitation peaks were calculated and compared to expected isotopic peak ratios. The abundance ratio of the MBA confirmation (*m/z* 238.9) and quantitation (*m/z* 240.9) product ion peaks was 33% higher than the expected 0.51 ratio, indicating the possibility that the observed signal corresponds to another compound. However, it was noticed that even in MBA external calibrants at concentrations of 75 ng/mL and lower, the difference between the expected and observed peak ratios exceeded the acceptable 10% error. This may be due to the lower abundance of the mass spectral line chosen as the confirmation peak compared to that of the quantitation peak. That is, the confirmation peak may have a much lower detection sensitivity than that of the quantitation peak, explaining the significant peak ratio discrepancies observed only at low concentrations.

In contrast, the isotopic spectral lines chosen as confirmation and quantitation peaks for BMX-3 have similar abundances, and possibly similar detection sensitivities. The observed peak ratios $\left(\frac{m/z\ 332.9}{m/z\ 334.9}\right)$ of low concentration BMX-3 external calibrants were very similar to the expected ratios. Additionally, peak ratio of the potential BMX-3 signal in the treated sample from day two extractions, was less than 10% different from the expected value.

3.2.3 Standard Addition Calibration and Matrix Effects

MBA peak area of the treated sample from day 2 extractions was subtracted from the peak area of all day 2 matrix spikes, except matrix spike of 75ng/mL for which the observed signal reflected the spiked concentration. Two standard addition curves were generated for MBA: 1) using the data from all extractions during days 1-3, including day 2 corrected data (Figure 12), and 2) excluding data from day 2 (Figure 13). Similarly, two standard addition curves were generated for BMX-3: 1) excluding day 2 extractions, and 2) correcting data by subtracting the signal from day 2 treated sample from all day 2 matrix spikes.

To compare the two standard addition curves generated, concentrations of MBA and BMX-3 from day 2 treated sample were extrapolated from the standard additions' linear regressions using the relative peak areas (Table 7). The low percent relative difference between the calculated concentrations indicate that the method is robust, and supports the use of the corrected data. Additionally, MBA, MX, BMX-1 and BMX-3 standard addition curves were compared to external calibration curves. Slopes of the standard addition curves of all analytes were much smaller than those of external calibration curves, indicating signal suppression potentially due to matrix effects (Figure 12 to Figure 21). Standard addition and external calibration curves generated with peak area, rather than relative peak area, as a function of concentration were compared for each analyte to determine if slope suppression was an artifact of normalizing standard addition curve peak areas with the summation of the two coeluting peaks at the retention time of

MCA. Slope suppression, though to a lesser degree, was still observed for all analytes with the exception of MBA (Figure 22 to Figure 25).

3.2.4 Linearity, Accuracy, and Reproducibility of LC-MS/MS Method

Linearity of method was observed for all compounds at the ng/L level (1000:1 concentration factor) with r^2 values of 0.98 for MBA (Figure 12) and BMX-1 (Figure 14), and 0.99 for MX (Figure 15) and BMX-3 (Figure 16). Accuracy of method was verified by extrapolating the concentration of matrix spikes from the standard addition linear regression. An error of <20% (expected versus calculated concentrations) was observed for all analytes in each matrix spike sample, with the exception of MBA and MX. Errors of 22 and 23% were observed for MBA and MX respectively in the lowest matrix spike samples (50ng/L). Relative standard deviations of ≤25% show good day-to-day reproducibility across sample batches (Table 8).

3.2.5 Detection Limits

Instrument detection limits were determined for analytes using external calibration curves (Figure 17, Figure 18, Figure 19, Figure 20, and Figure 21). Practical limits of detection (1000:1 concentration factor) for MCA, MBA, MX, BMX-1, and BMX-3 were determined to be 35, 7, 10, 6, and 2 ng/L respectively using a 10 µL injection, which are comparable to those obtained by GC-MS methods (Table 9).

4. CONCLUSIONS

A novel method was developed for the characterization of halogenated furanones, demonstrating for the first time their detection through electrospray ionization tandem mass spectrometry. Though ionization of BMX-2 was not achieved through optimization of collision energy, fragmentor voltage, and gas temperature with acetonitrile as the mobile phase, this study provides a blueprint for the focus of future work in determining the appropriate ionization parameters.

The developed LC-MS/MS method proved to be reproducible and accurate when analyzing chlorinated water samples, though absolute method recoveries were not determined. After considering the 1000:1 concentration factor, instrument detection limits ranged from 2-35ng/L which are comparable to current GC detection methods. Only MCA specificity was affected by coeluting peaks, while the use of isotopic peak abundance ratios proved useful when confirming identity of peaks eluting near MX retention times. However, detection sensitivities of isotopic peaks can differ, affecting the calculated peak ratios and may thus provide misleading results. Their use to confirm compound identity should be further investigated. An alternative internal standard that will not be affected by coeluting compounds should also be considered for future work. Still, the developed LC-MS/MS method is less time intensive than current analysis methods, and provides a faster, sensitive and selective means to detect halofuranones at the ng/L level.

TABLES

Table 1. Bromine and Chlorine Isotopic Peak Abundances. M= monoisotopic peak[20]

Compound	No. of Cl & Br atoms	% Abundance of Isotopic Peaks			
		M	M+2	M+4	M+6
MCA	Cl_2	100	64	10	
MX	Cl_3	100	96	31	3
MBA	Br_2	51	100	49	
BMX-3	Br_3	34	100	97	32
BMX-1	$BrCl_2$	62	100	45	6
BMX-2	Br_2Cl	44	100	70	13

Table 2. SPE optimization recovery results obtained with UV detection @245nm. Mean concentration represents the average of sample triplicates, determined by external calibration (Table S.1). Reported instrument detection limit (IDL) is based on a 500 concentration factor.

	Mean Sample Concentration		Expected Concentration		SD	%RSD	%Recovery	Reported IDL
MCA	38	ng/L	50	ng/L	1	2.7	76	10 ng/L
MBA	40				1	2.9	81	
MX	47.8	µg/L	50.0	µg/L	0.9	1.9	96	40 µg/L
BMX-1	50				2	4.4	101	
BMX-2	45.5				0.2	0.4	91	
BMX-3	48				1	2.4	95	

Table 3. Comparison of analyte recoveries across extraction methods. *Recoveries of open ring forms

	% Recoveries			
Analyte	Onstad and Weinberg[15] LLE	Rantakokko et al[16] tandem SPE	Kubwabo et al[13] tandem SPE	Presented Method tandem SPE
MBA	-	-	-	81
MCA	82	-	-	76
MX	89	70-98	124	96
BMX-1	73*	72-126	-	101
BMX-2	75*	95-111	-	91
BMX-3	87*	102-123	-	95

Table 4. Finalized fragmentor voltages and collision energies for positive ionization MRM transitions

Compound	Fragmentor (V)	Collision Energy (V)
MCA	50	5
MBA	55	10
MX	60	5
BMX-1	70	5
BMX-3	100	5

Table 5. Finalized Positive Ionization MRM transitions for LC-MS/MS method.

Compound	MRM Quantitation Transitions		MRM Confirmation Transitions		Retention Time
	Precursor ion (*m/z*)	Product ion (*m/z*)	Precursor ion (*m/z*)	Product ion (*m/z*)	min (SD)
MCA	169 $[MH]^+$	151 $[MH\text{-}H_2O]^+$	171 $[MH+2]^+$	152.9 $[MH+2\text{-}H_2O]^+$	6.76 (0.06) *
MBA	258.9 $[MH+2]^+$	240.9 $[MH+2\text{-}H_2O]^+$	256.9 $[MH]^+$	238.9 $[MH\text{-}H_2O]^+$	7.97 (0.06)
MX	217 $[MH]^+$	199 $[MH\text{-}H_2O]^+$	219 $[MH+2]^+$	201 $[MH+2\text{-}H_2O]^+$	10.71 (0.08)
BMX-1	263 $[MH+2]^+$	244.7 $[MH+2\text{-}H_2O]^+$	261 $[MH]^+$	242.9 $[MH\text{-}H_2O]^+$	11.71 (0.09)
BMX-3	350.8 $[MH+2]^+$	332.9 $[MH+2\text{-}H_2O]^+$	352.8 $[MH+4]^+$	334.9 $[MH+4\text{-}H_2O]^+$	13.30 (0.09)

*Retention time for MCA is based on external calibrants only.

Table 6. Confirmation of MCA identity in coeluting peaks from samples and matrix spikes. MS= matrix spiked sample

Sample	RT (min)	Prod. Ion (*m/z*)	Abundance	Ratio	Expected Ratio	% Error
MS 100ng/L Day 1	6.7	151	3712	3.5	1.5	127
		152.9	1064			
	7.0	151	2060	1.4	1.5	8
		152.9	1453			
Sample Day2	6.7	151	3193	2.8	1.5	84
		152.9	1131			
	7.0	151	1450	1.0	1.5	32
		152.9	1386			
MS 300ng/L Day 1	6.7	151	4094	2.8	1.5	80
		152.9	1482			
	7.0	151	2004	1.3	1.5	17
		152.9	1568			

Table 7. MBA and BMX-3 concentration in treated sample from day 2 extractions using two calibration methods. Average concentration represents the mean of analytical replicates.

Analyte	Method	Average Calculated Conc. (ng/L)	SD	%RSD	%Relative Difference
BMX-3	Corrected regression[1]	32	3	8	11
	Days 1 & 3 regression	28	3	10	
MBA	Corrected regression	149	10	7	0.3
	Days 1 & 3 regression	148	10	7	

[1] Corrected Regression: Signal of treated sample from day 2 extractions subtracted from signal of matrix spikes from day 2 extractions

Table 8. %RSD and %Error of matrix spikes. n=2 or 3

Analyte	Matrix Spike Level (ng/L)	Calc. Mean Concentration Added (ng/L)	SD	%RSD	%Error
MBA	50	61	10	17	22
	75	87	17	19	16
	100	103	25	25	3
	200	197	4	2	2
	300	281	36	13	6
MX	50	62	9	14	23
	75	82	16	20	10
	100	103	20	19	3
	200	198	3	1	1
	300	303	29	9	1
BMX-1	50	46	1	3	8
	75	78	7	9	4
	100	106	24	23	6
	200	204	9	4	2
	300	270	20	7	10
BMX-3	50	48	7	14	4
	75	74	5	6	1
	100	110	21	19	10
	200	209	5	2	5
	300	285	15	5	5

Table 9. Comparison of practical detection limits across methods.

	Detection limits (ng/L)			
Analyte	Onstad and Weinberg[15] GC-ECD	Rantakokko et al[16] GC-MS	Kubwabo et al[13] GC-MS	Presented Method LC-MS/MS
MBA	-	-	-	7
MCA	20	-	-	35
MX	20	≤0.4*	1.9	10
BMX-1	20	≤0.4*	-	6
BMX-2	20	≤0.4*	-	-
BMX-3	20	≤0.4*	-	2

A dash (-) indicates detection limit was not reported

*Extrapolated from limit of quantitation

FIGURES

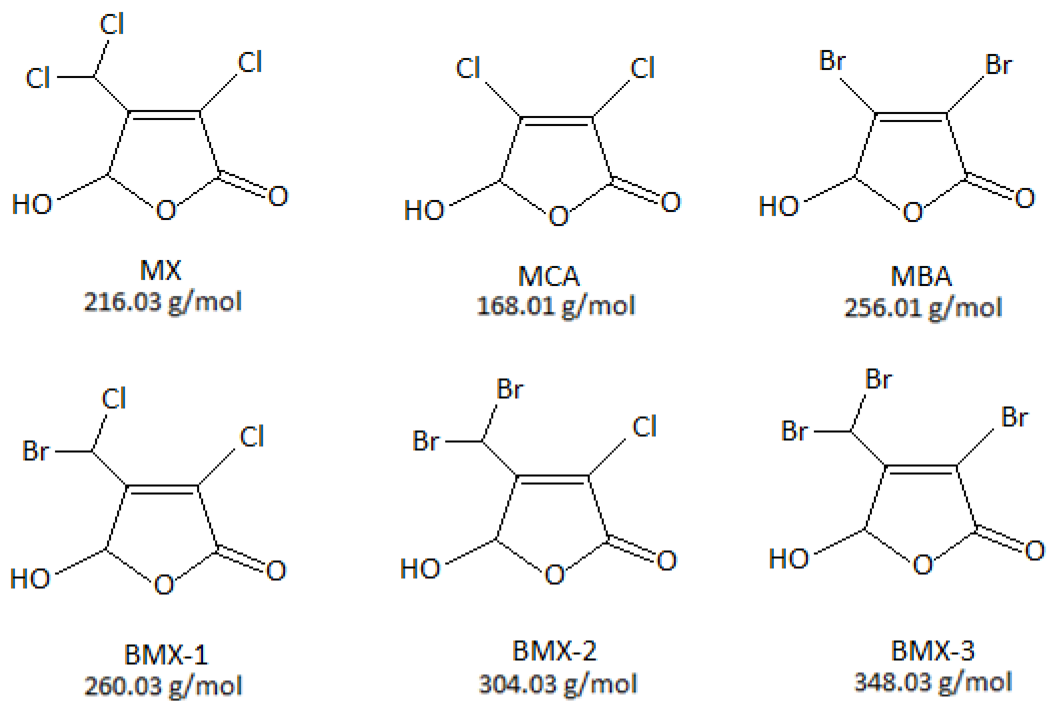

Figure 1. Structures of MX, Mucochloric acid, Mucobromic acid, and brominated analogs BMX-1, BMX-2, and BMX-3. Monoisotopic masses are presented below each structure.

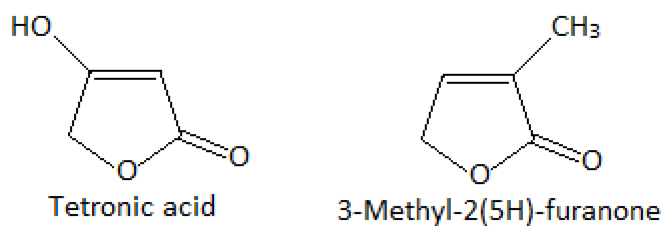

Figure 2. Ionized lactones by Crotti et al[19]

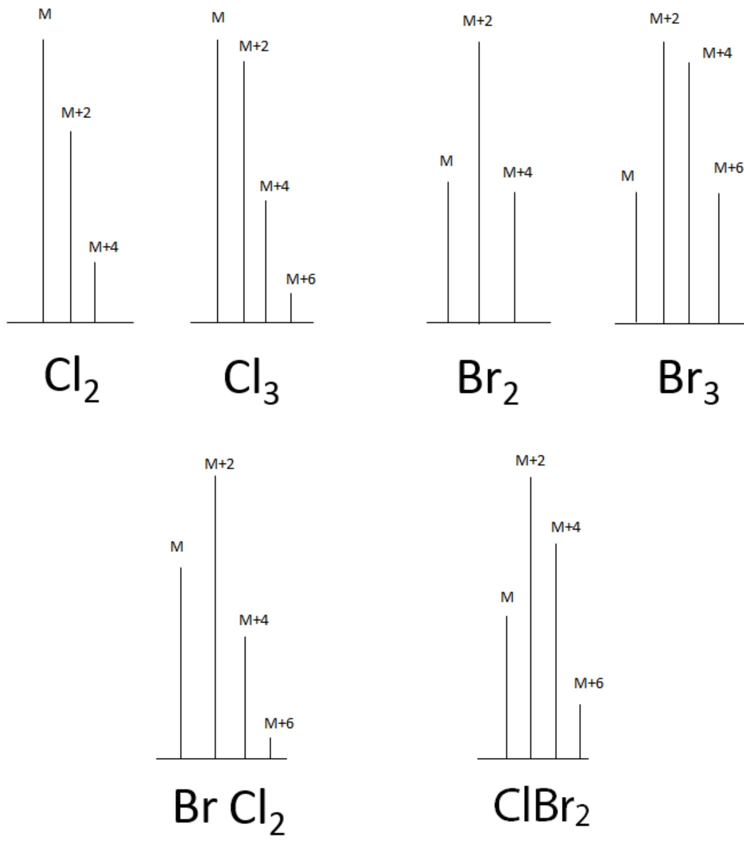

Figure 3. Bromine and chlorine isotopic peak patterns. M= monoisotopic peak

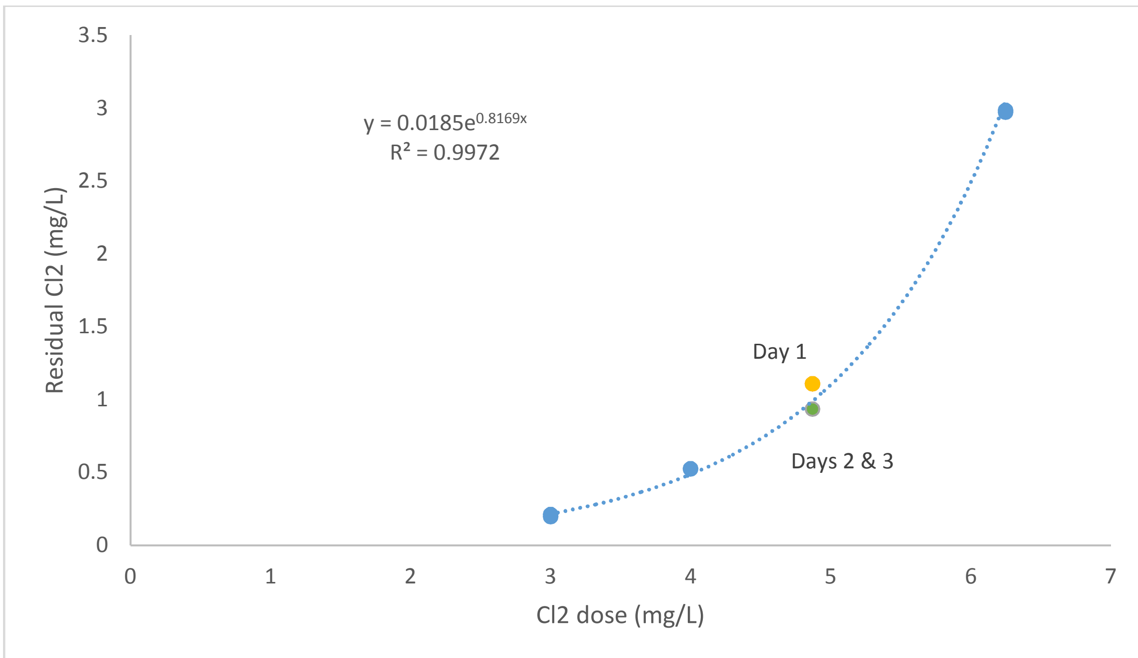

Figure 4. Chlorine demand study. Residual chlorine averages of treated samples from days 1-3 for method validation are shown.

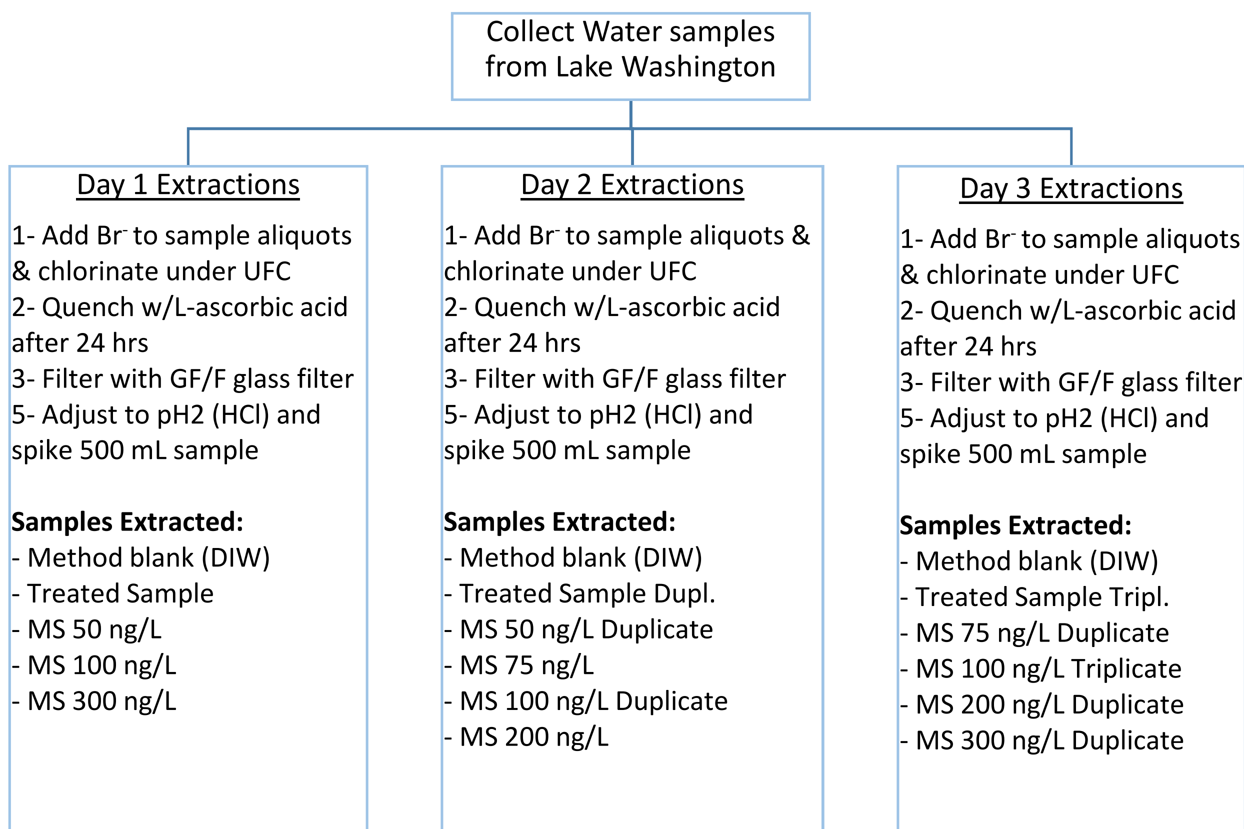

Figure 5. Chlorination and extraction scheme for Lake Washington water samples. MS=matrix spike

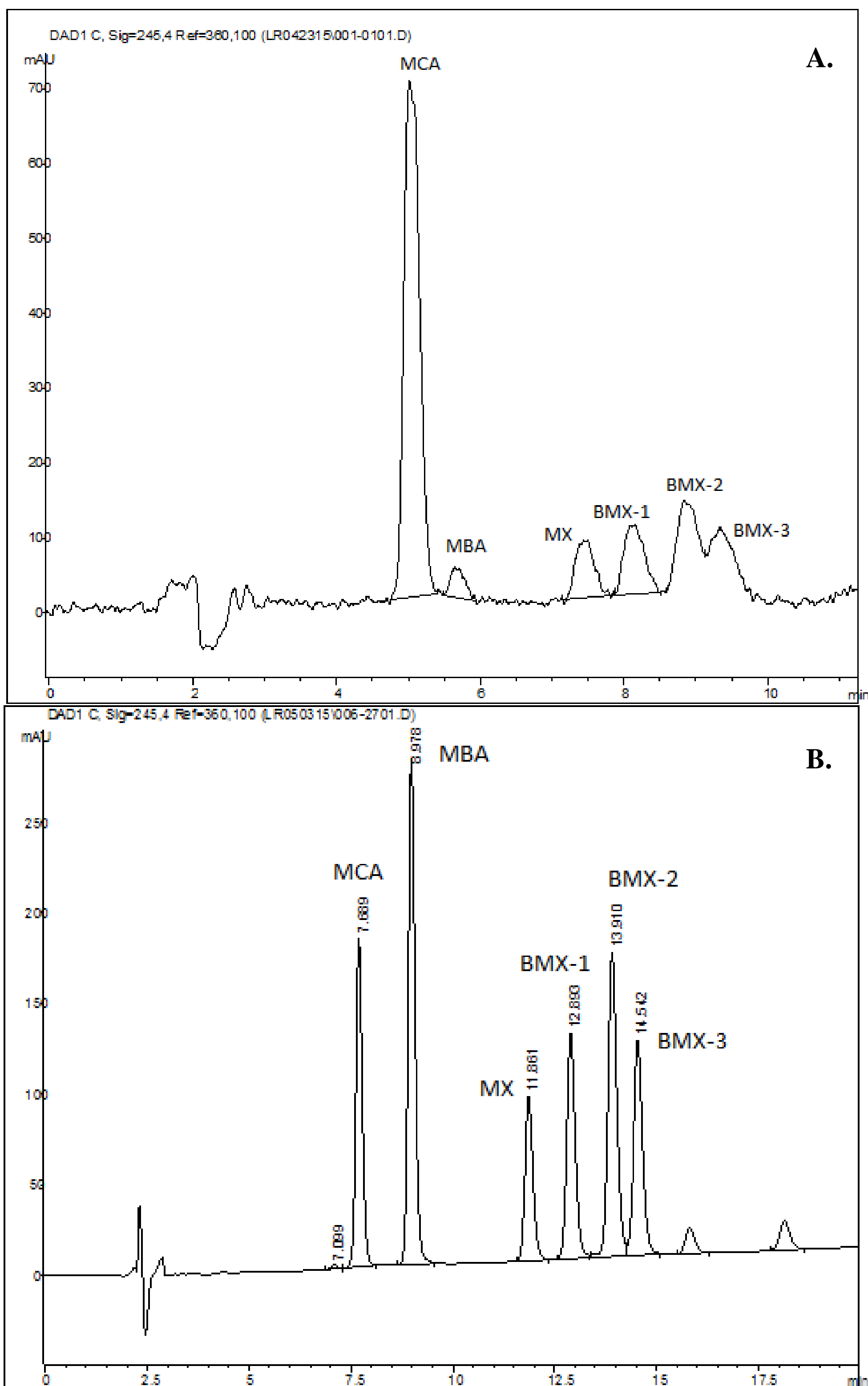

Figure 6. LC-DAD analysis of halofuranones **A. (Top image)** isocratic elution (35/65 ACN/DIW 0.25%FA) of std. mixture of MCA and MBA (500ng/mL), and MX and BMX's (100mg/L). Degradation of MBA was observed. **B. (Bottom image)** Linear gradient elution of std. mixture of MCA and MBA at 150ng/mL, and MX and BMX's at 150 mg/L.

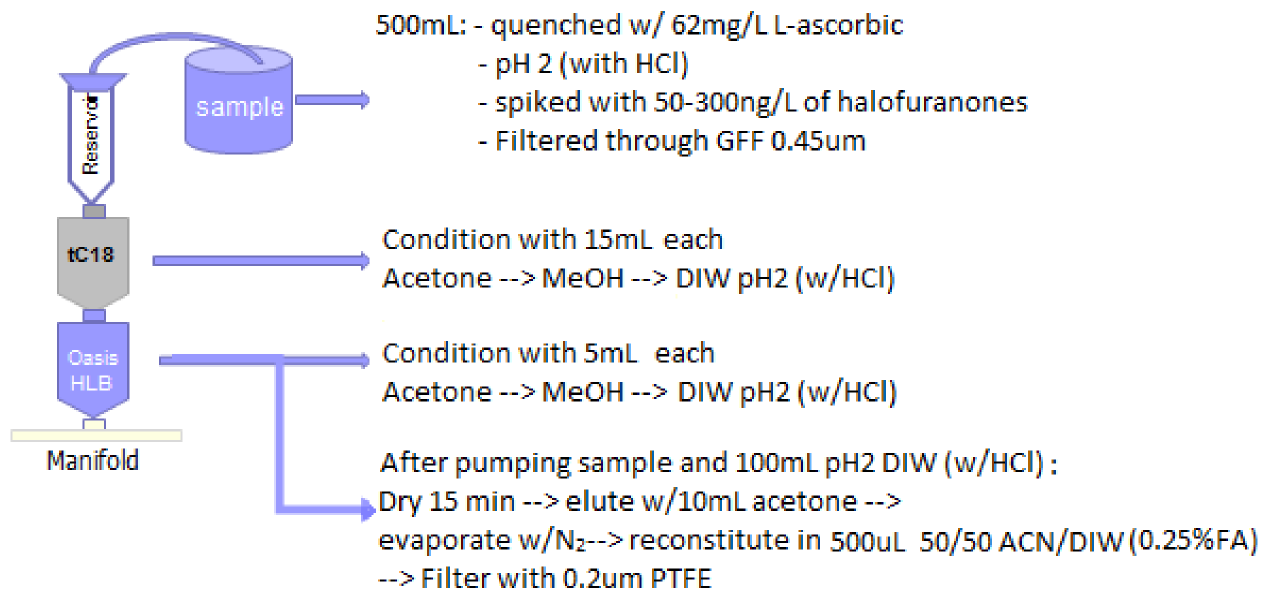

Figure 7. Finalized SPE protocol.

Figure 8. External calibrant (100ng/mL) MRM chromatogram. **A. (Top image)** Total ion chromatogram (TIC). **B. (Bottom image)** Overlaid extracted ion chromatograms (EIC) of quantitation and confirmation product ions.

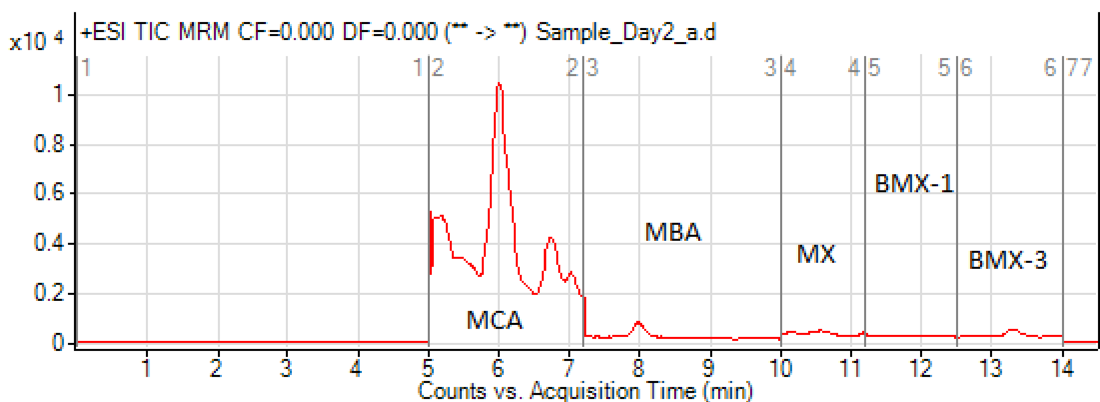

Figure 9. Chromatogram of treated sample (no spike) plus internal standard MCA (100ng/mL in extract) from day 2 extractions. Acquisition time windows for each analyte are divided by numbered vertical lines.

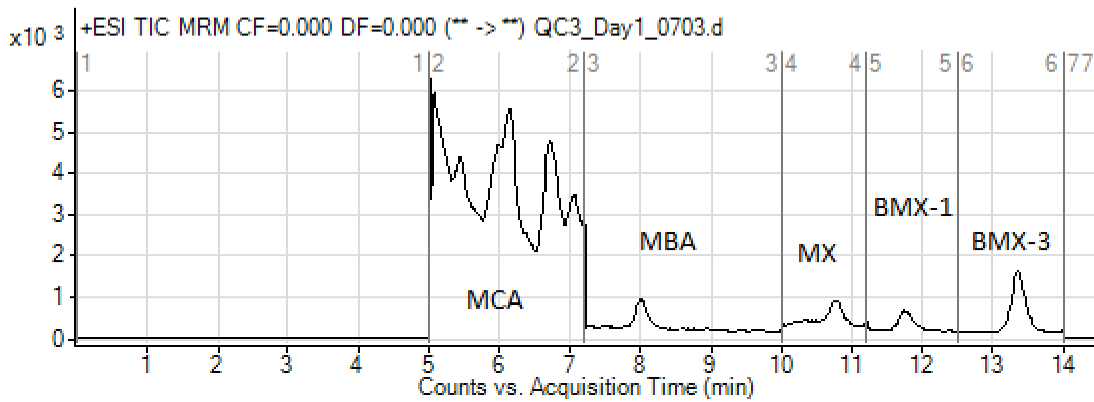

Figure 10. LC-MS/MS chromatogram of treated sample spiked with 100ng/L of MBA, MX, BMX-1 and BMX-3, and internal standard MCA (100ng/mL in extract).

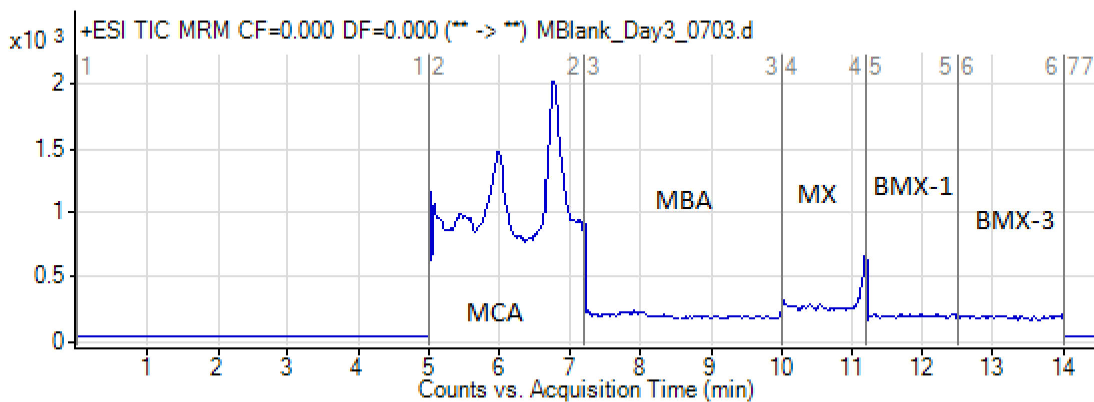

Figure 11. LC-MS/MS chromatogram of method blank (DIW) from day 3 extractions. MCA present as internal standard at 100ng/mL in extract

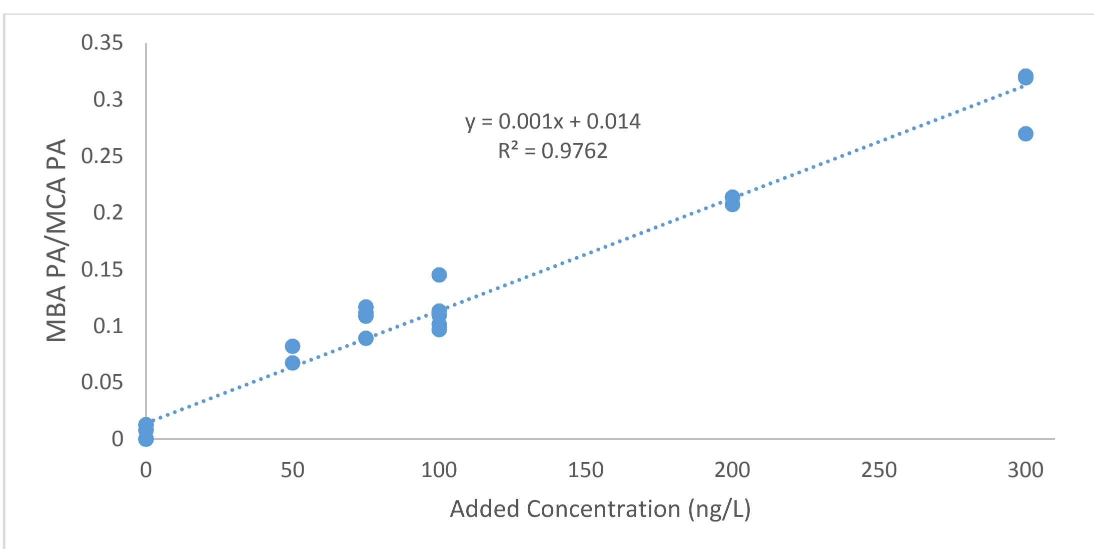

Figure 12. MBA Std Addition curve including corrected Day 2 data. PA=peak area

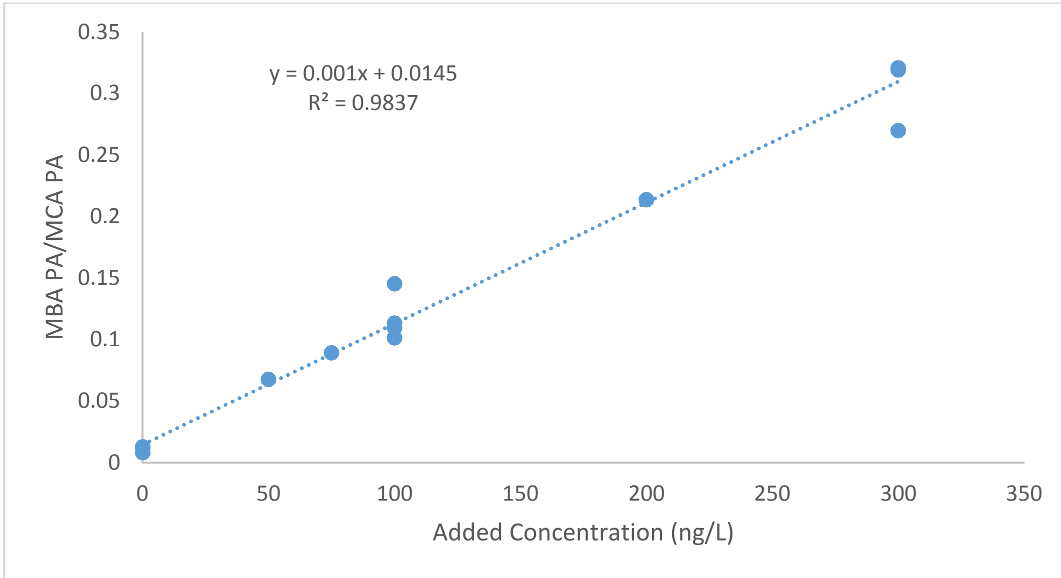

Figure 13. MBA Std Addition curve excluding day 2 data. PA=peak area

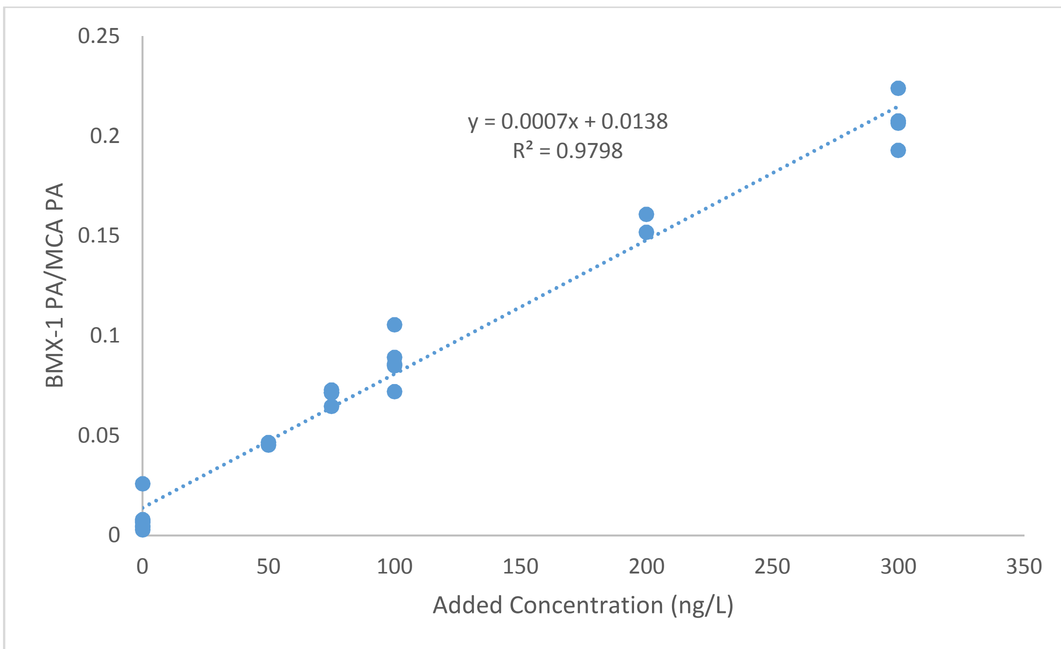

Figure 14. BMX-1 Std Addition curve of days 1-3 data. PA=peak area

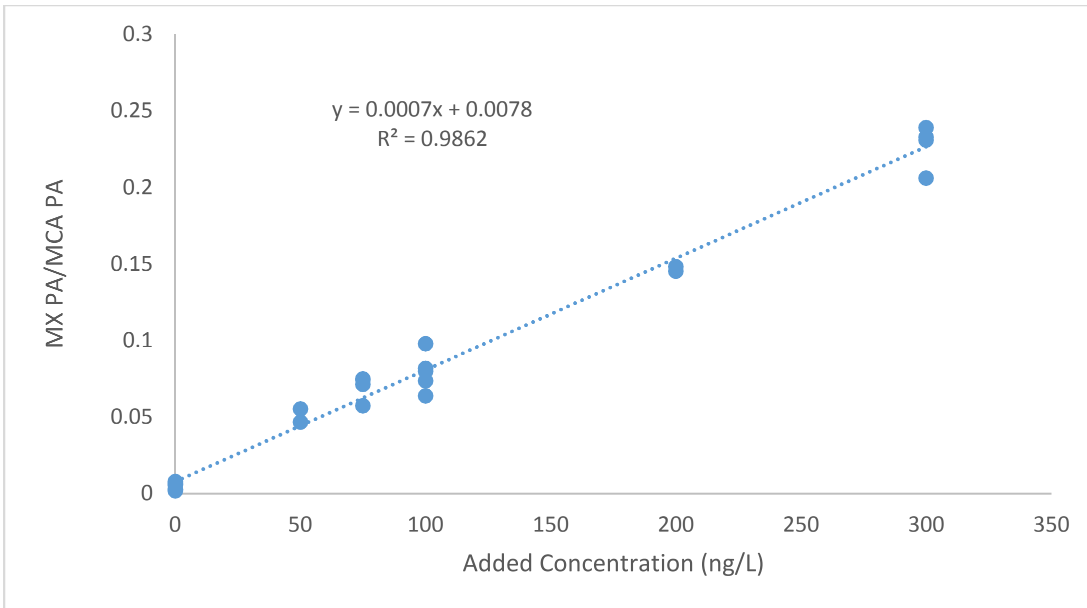

Figure 15. MX Std Addition curve of days 1-3 data. PA=peak area

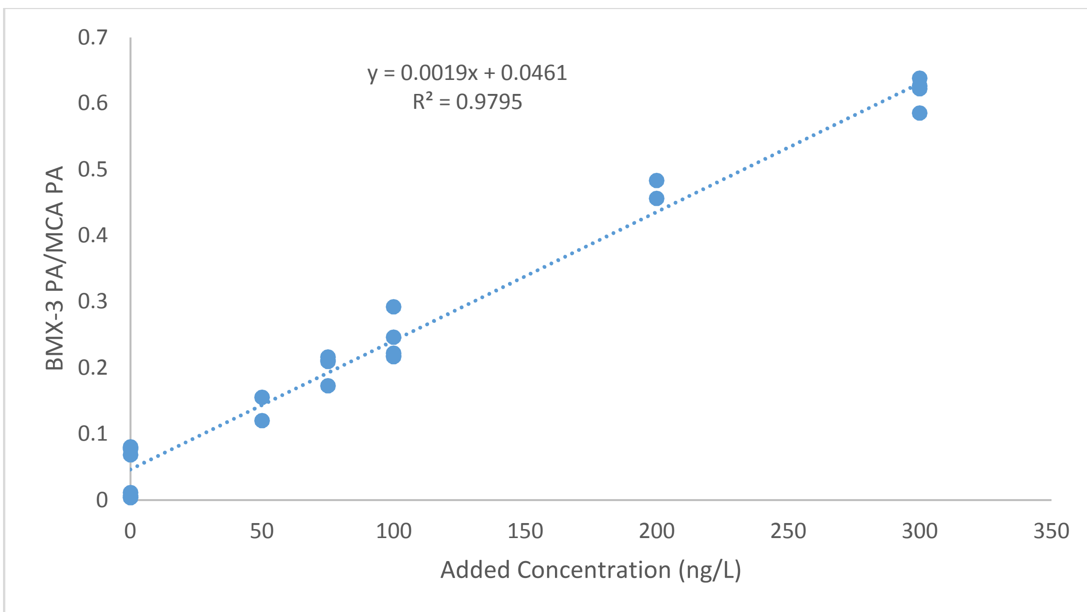

Figure 16. BMX-3 Std Addition curve of days 1-3 data. PA=peak area

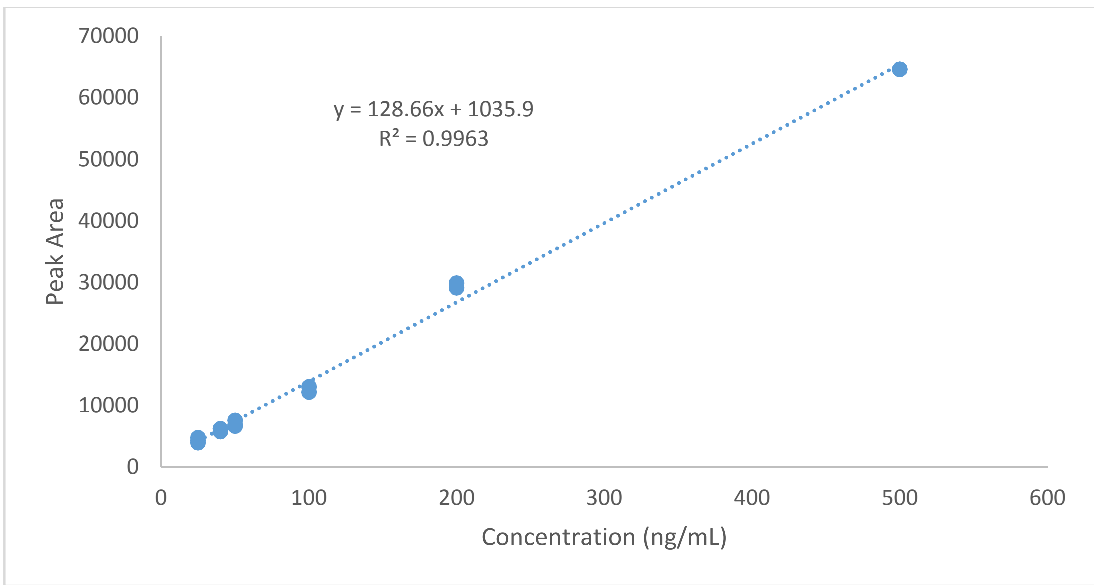

Figure 17. MCA external calibration curve by peak area.

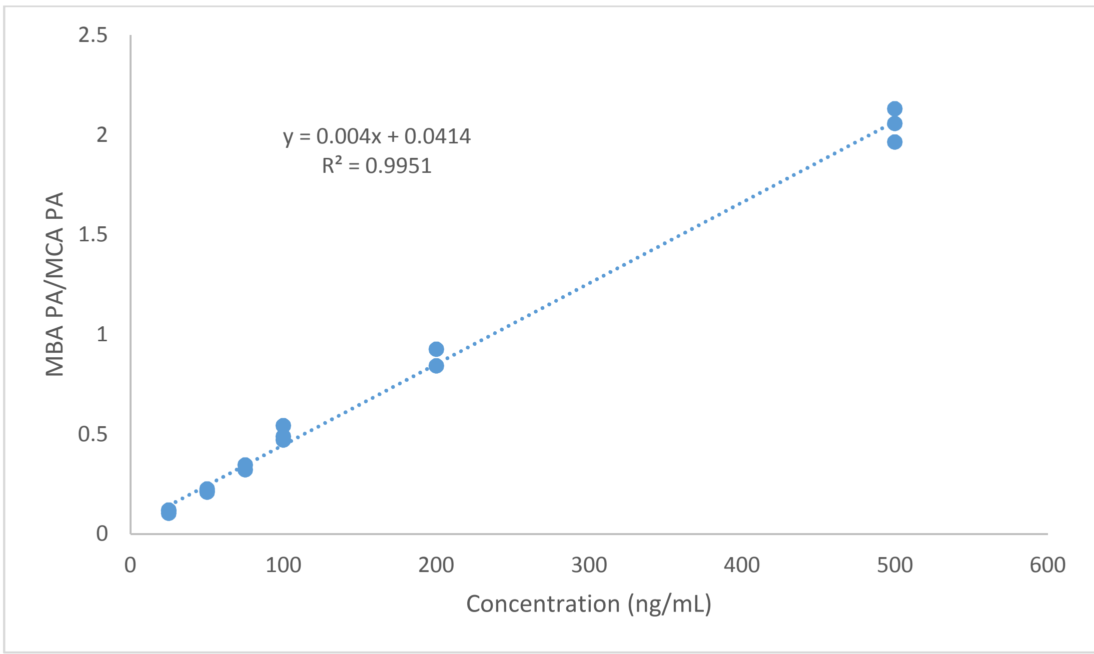

Figure 18. MBA external calibration curve. PA=peak area

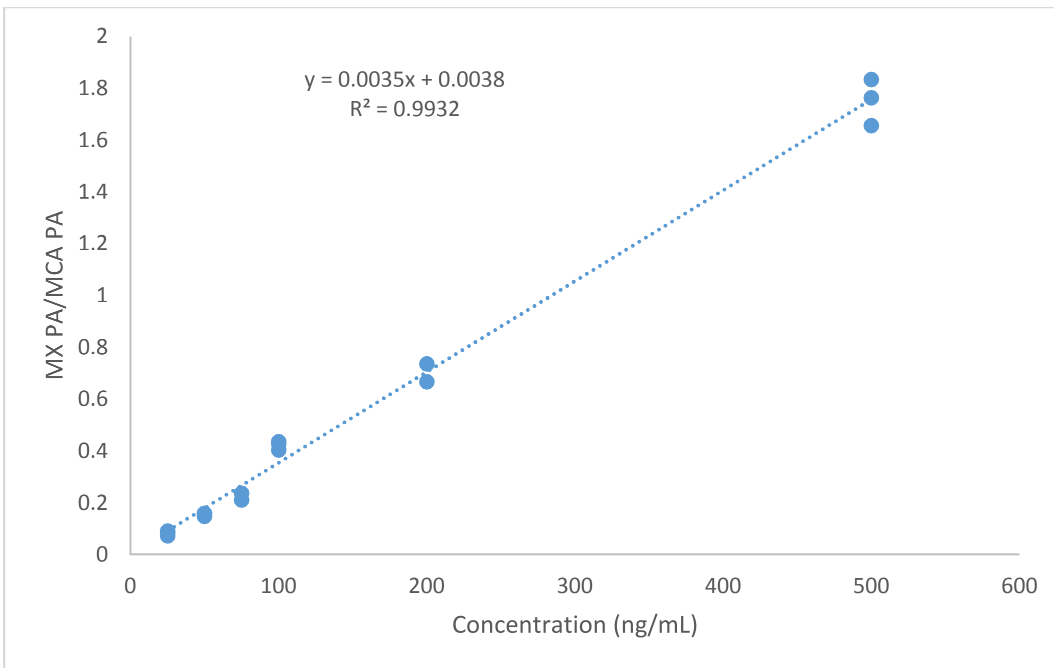

Figure 19. MX external calibration curve. PA=peak area

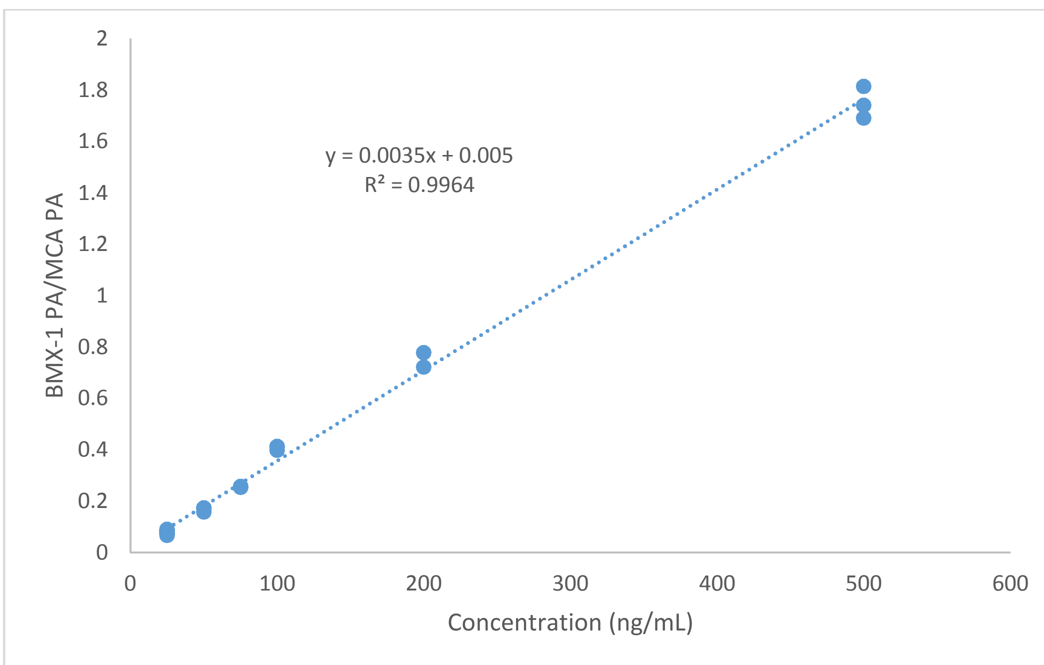

Figure 20. BMX-1 external calibration curve. PA=peak area

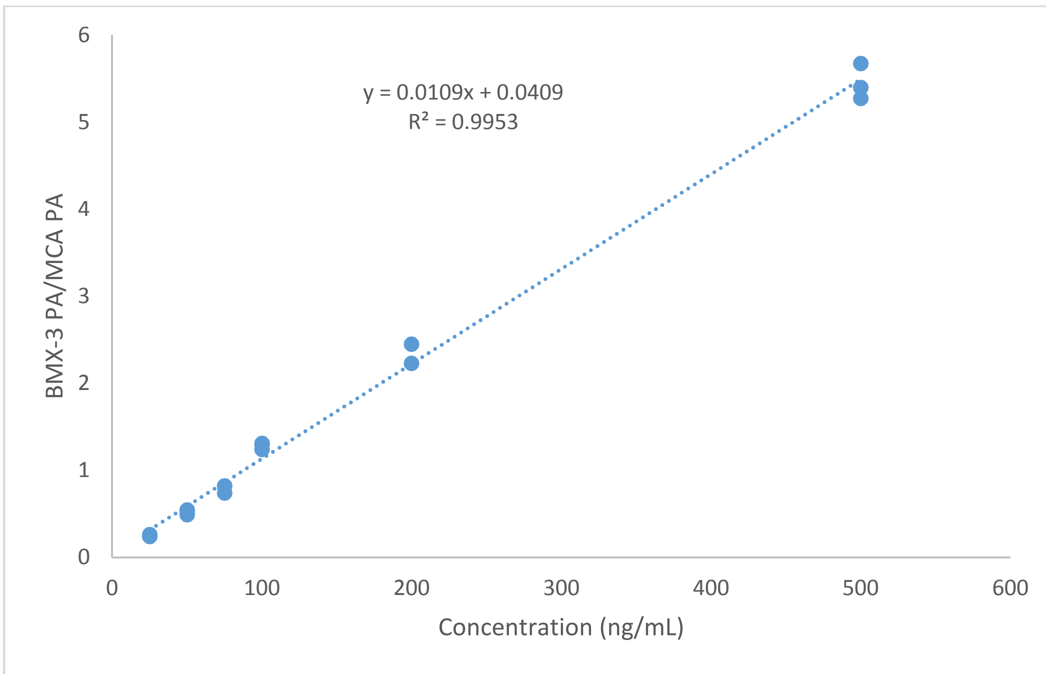

Figure 21. BMX-3 external calibration curve. PA=peak area

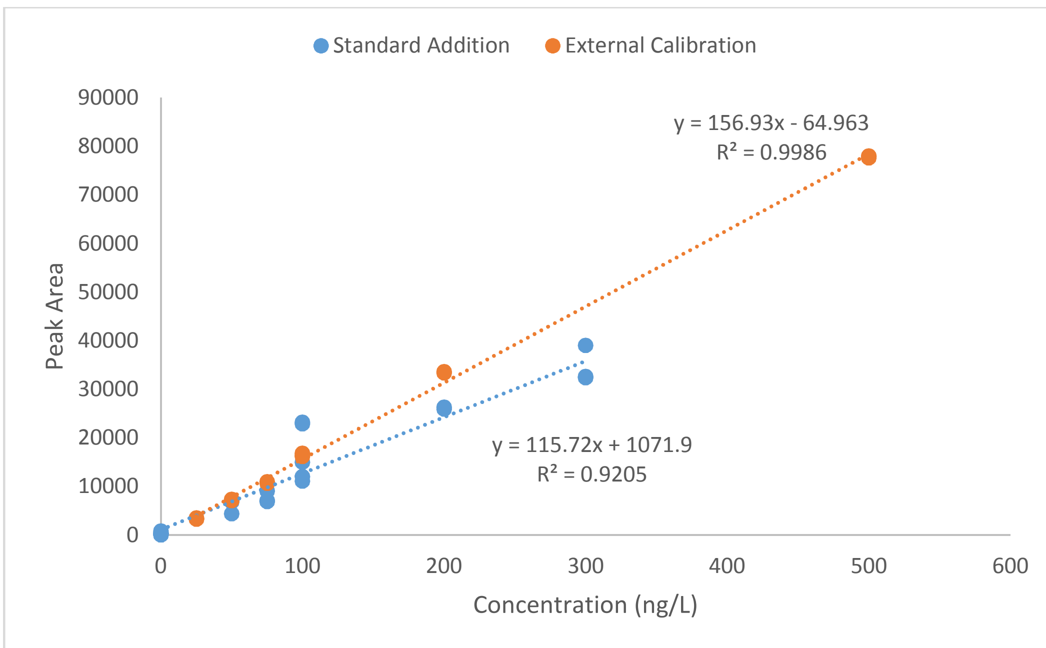

Figure 22. BMX-3 external and corrected standard addition calibration curves by peak area.

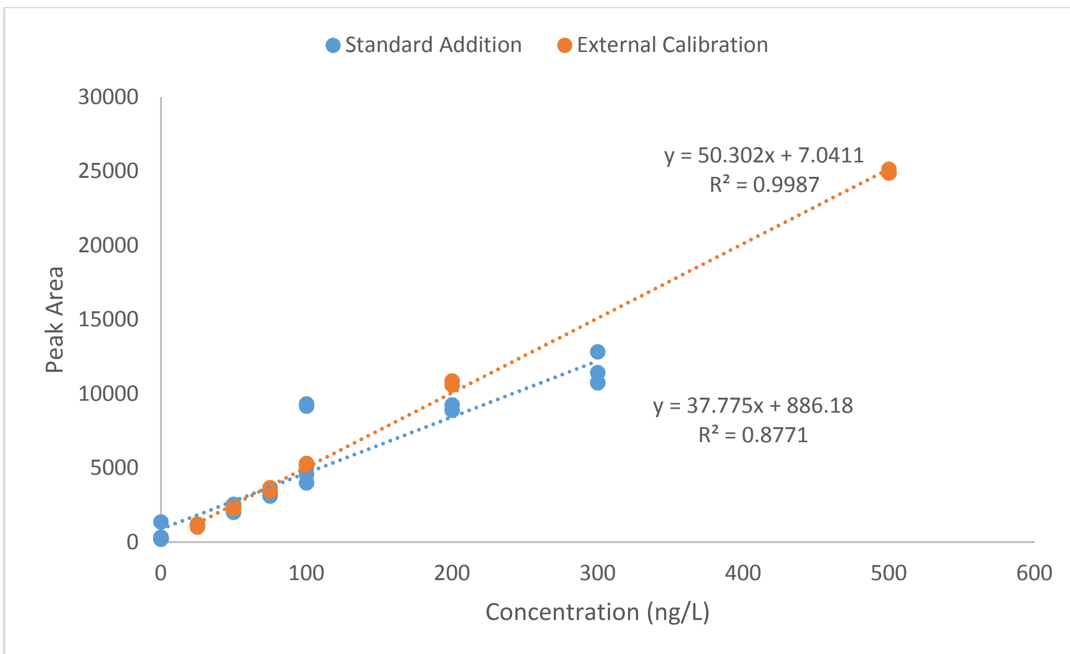

Figure 23. BMX-1 external and standard addition calibration curves by peak area.

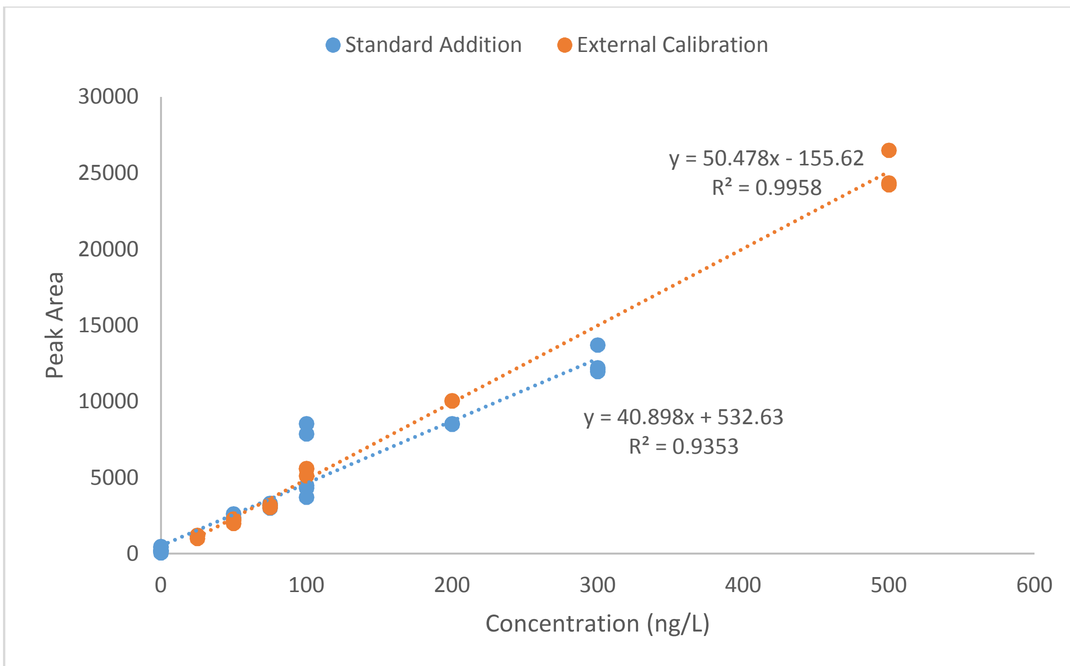

Figure 24. MX external and standard addition calibration curves by peak area.

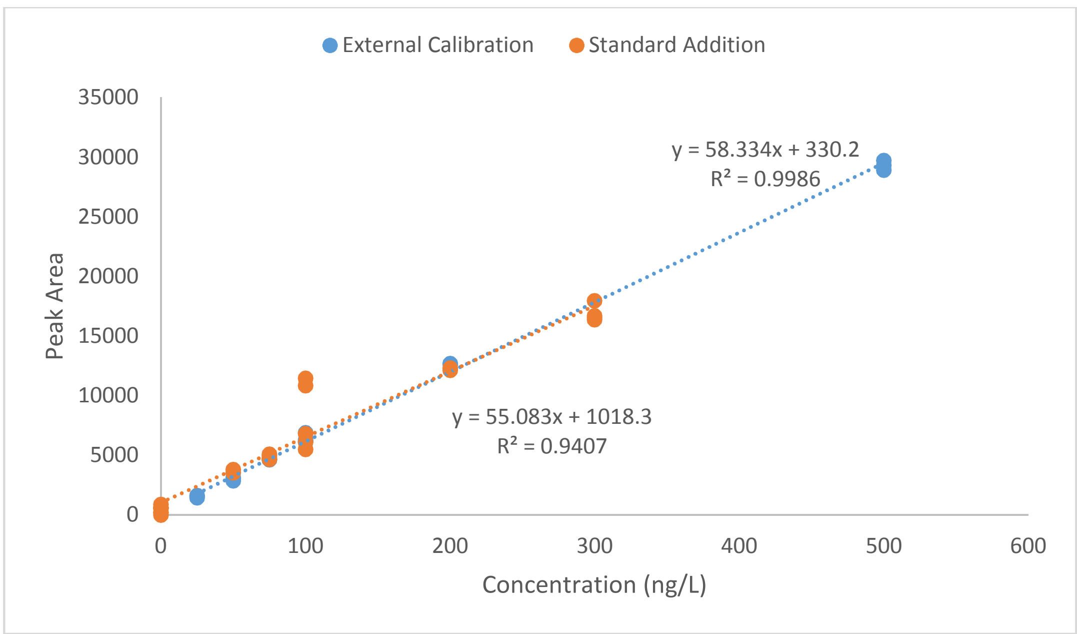

Figure 25. MBA external and corrected standard addition calibration curves by peak area.

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SUPPLEMENTARY DATA

Table S.1. External calibration curves used for SPE optimization .

Compound	Regression Equation	r^2	Concentration Range
MCA	y = 31.094x + 40.976	0.998	5-150 ng/mL
MBA	y = 51.729x + 35.562	0.999	
MX	y = 19.933x + 25.553	0.996	20-150 mg/L
BMX-1	y = 29.119x - 39.202	0.998	
BMX-2	y = 40.694x + 18.009	0.998	
BMX-3	y = 31.92x - 211.83	0.983	

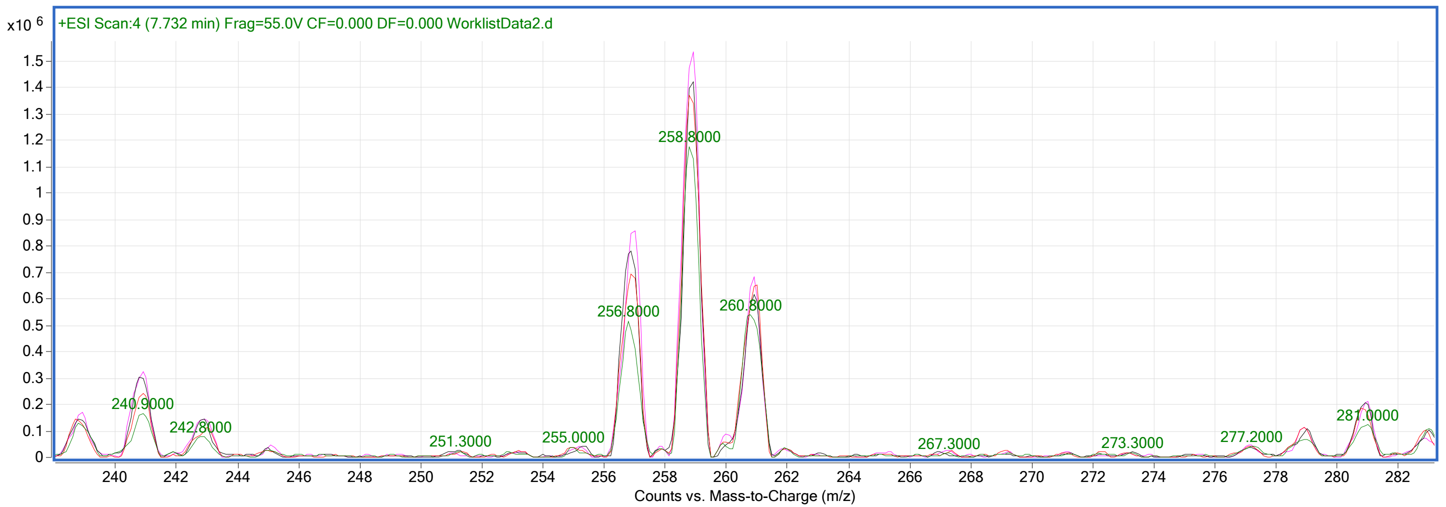

Figure S.1. Full scan mass spectra of MBA (200 ng/mL). Fragmentor voltages: 55-70V

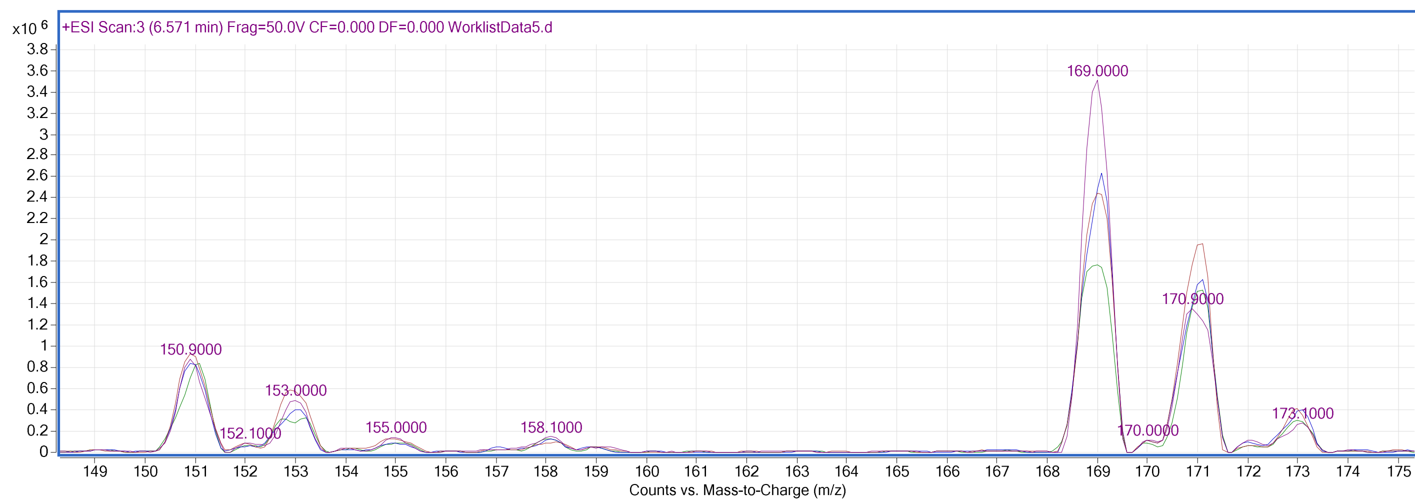

Figure S.2. Full scan mass spectra of MCA (200 ng/mL). Fragmentor voltages: 40-55V

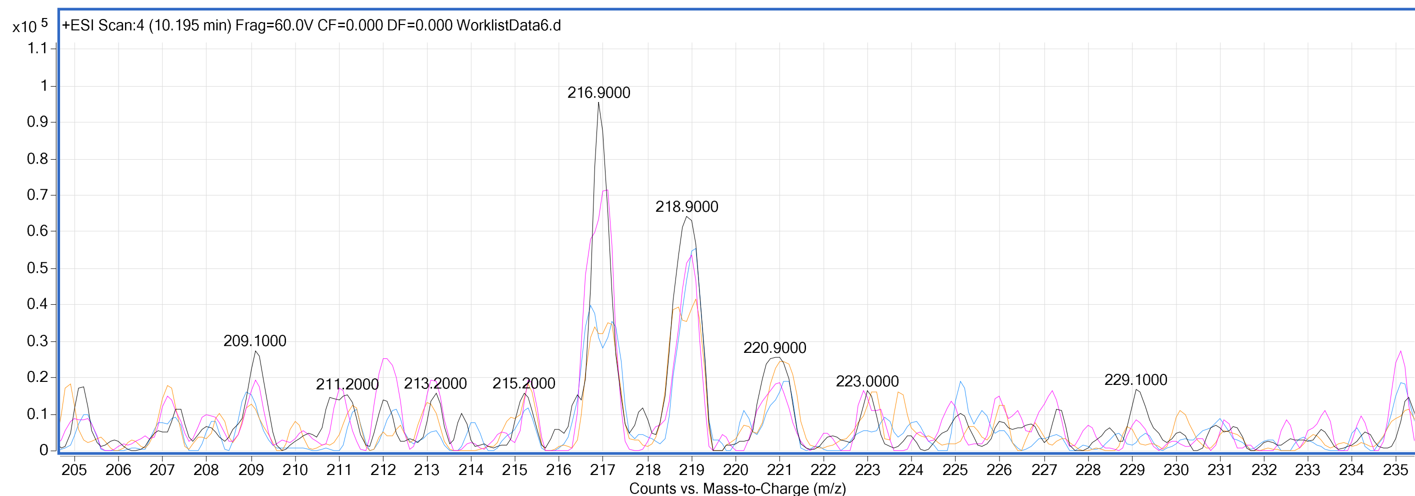

Figure S.3. Full scan mass spectra of MX (200 ng/mL). Fragmentor voltages: 20-60V

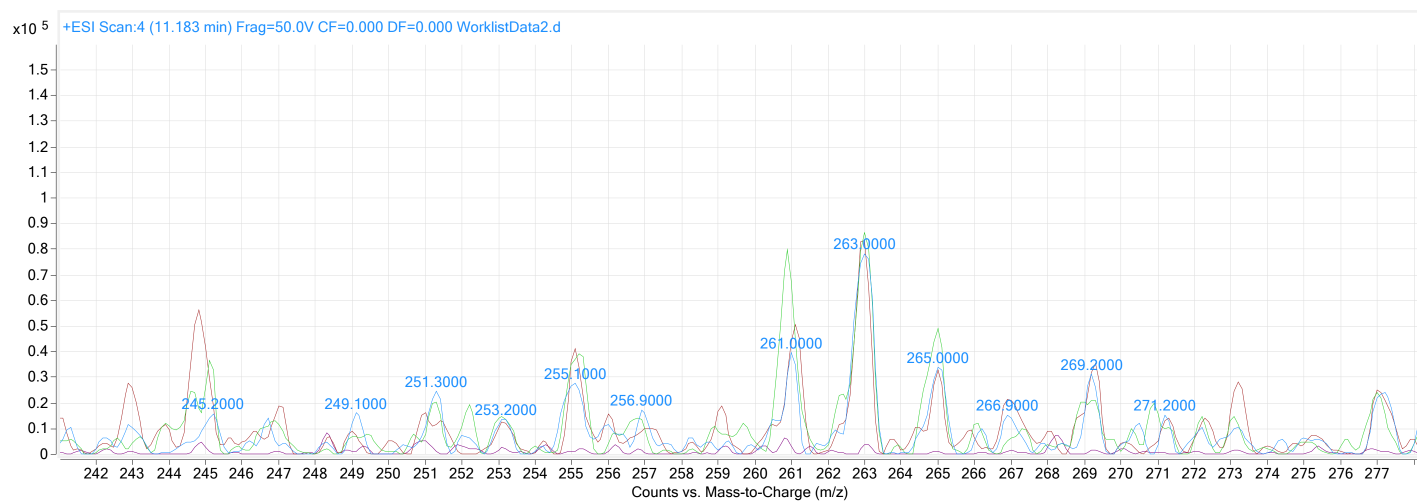

Figure S.4. Full scan mass spectra of BMX-1 (200 ng/mL). Fragmentor voltages: 50-200V

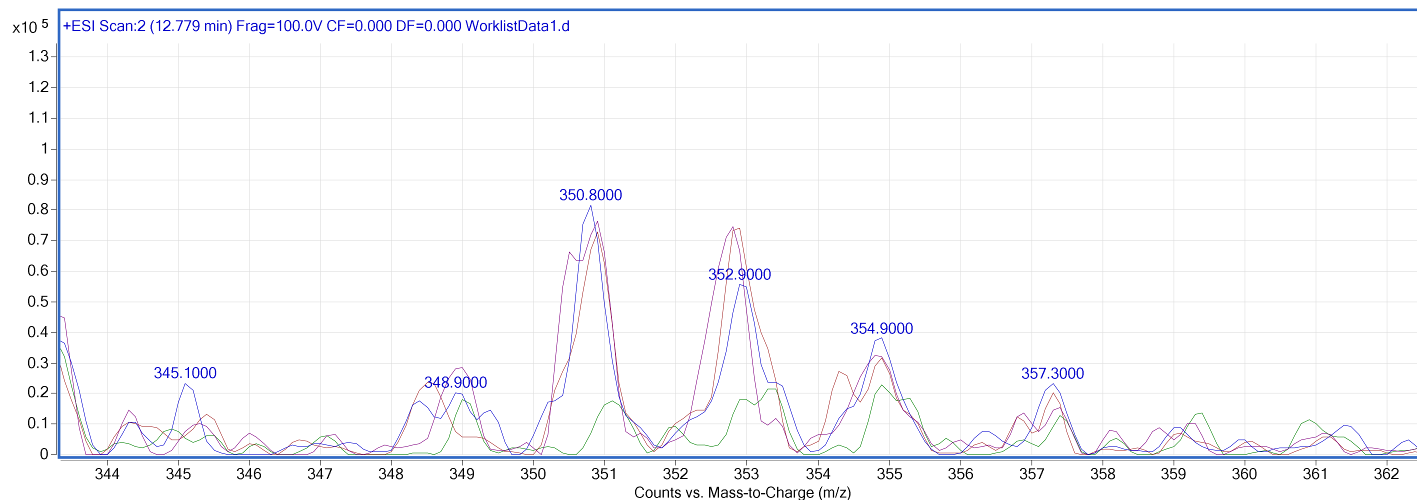

Figure S.5. Full scan mass spectra of BMX-3 (200 ng/mL). Fragmentor voltages: 50-200V

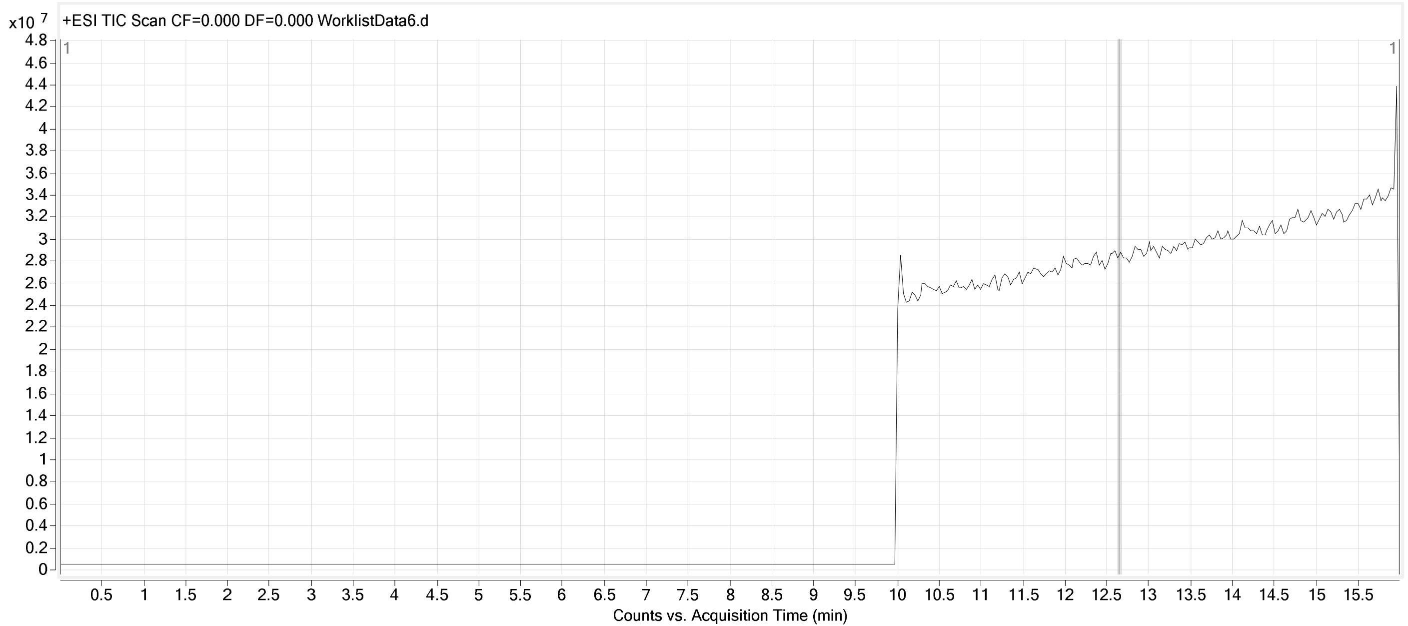

Figure S.6. Total ion chromatogram. Full scan acquisition of BMX-2 at 200 ng/mL. Fragmentor voltages: 0, 150, 200 and 250 V.

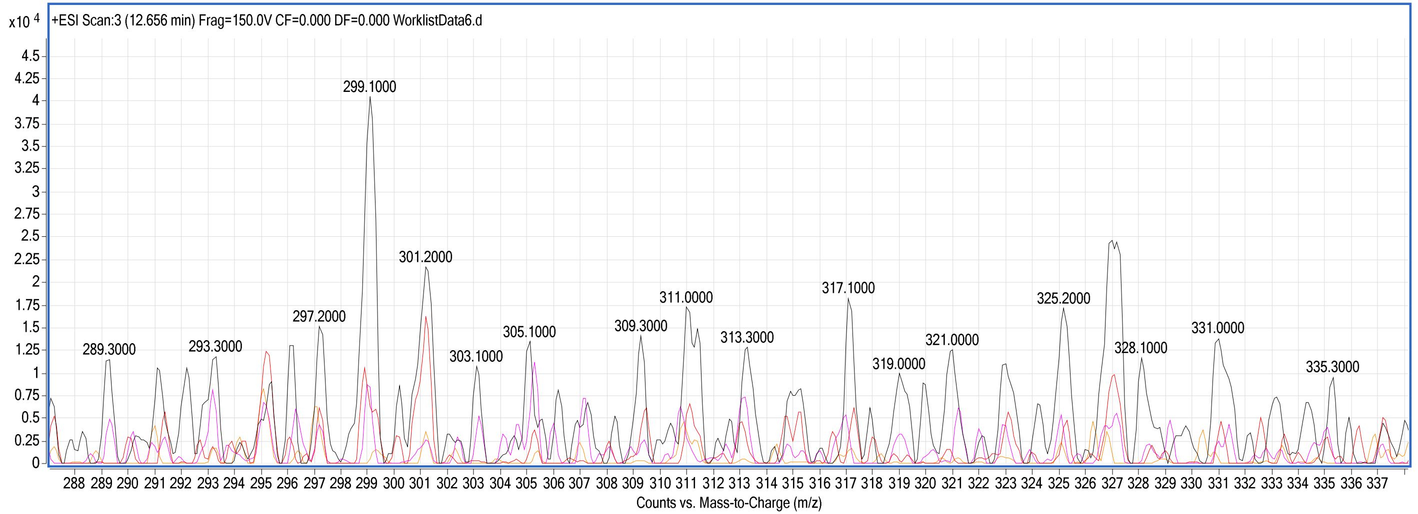

Figure S.7. Full scan acquisition mass spectra of BMX-2 at 200 ng/mL. Fragmentor voltages: 0, 150, 200 and 250 V.

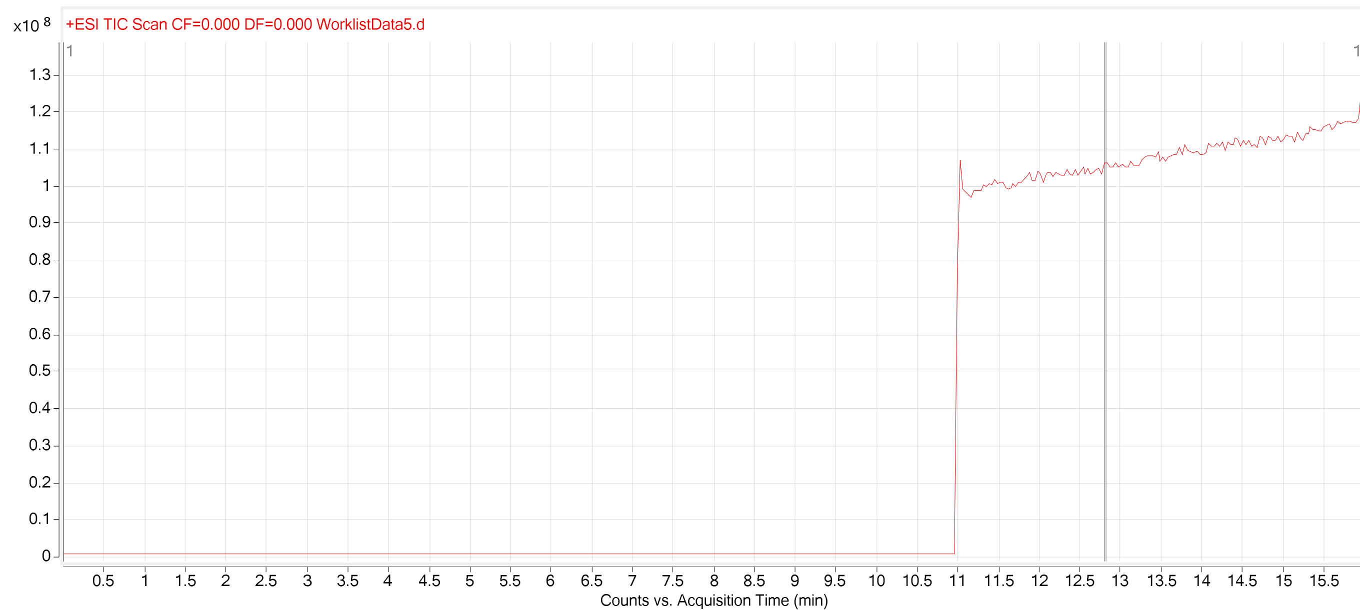

Figure S.8. Total ion chromatogram. Full scan acquisition of BMX-2 at 200 ng/mL. Fragmentor voltages: 50, 70, 85 and 100 V.

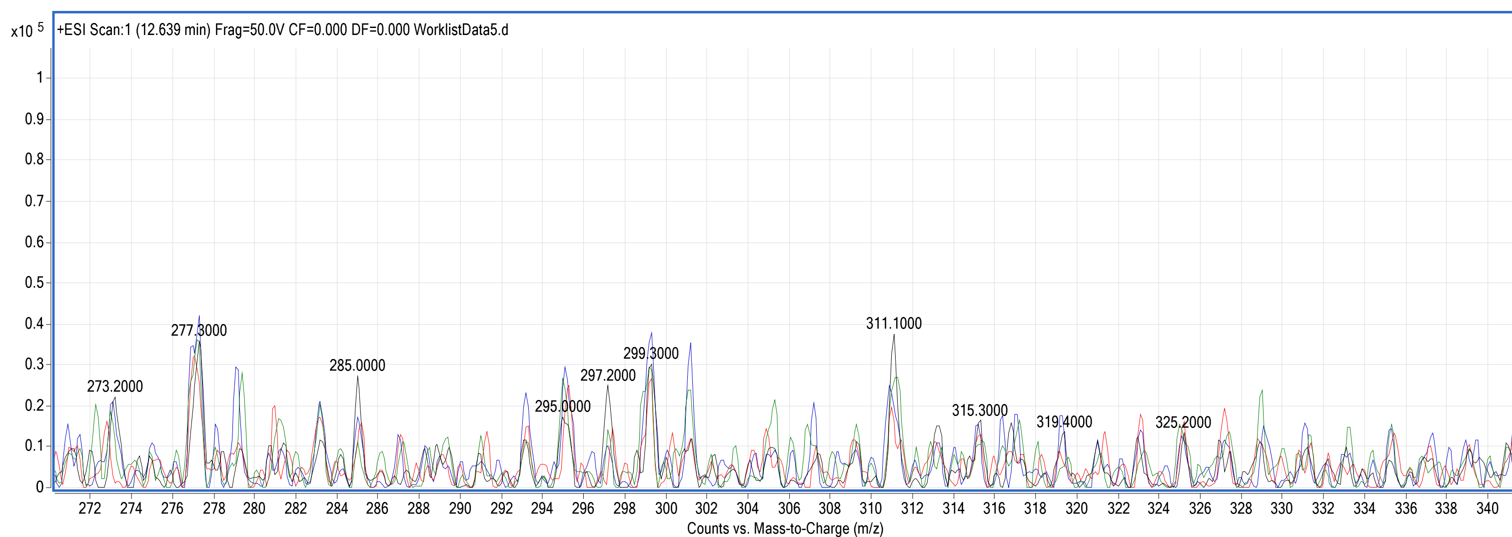

Figure S.9. Full scan acquisition mass spectra of BMX-2 at 200 ng/mL. Fragmentor voltages: 50, 70, 85 and 100 V.

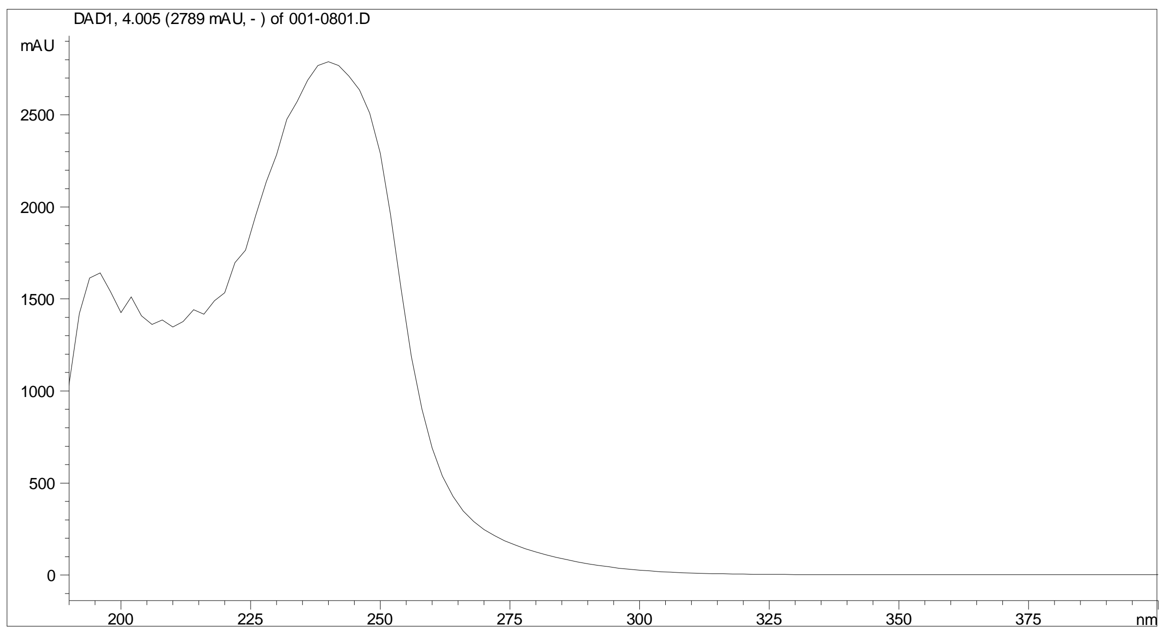

Figure S.10. MCA (10 ppm) spectrum measured by LC-DAD.

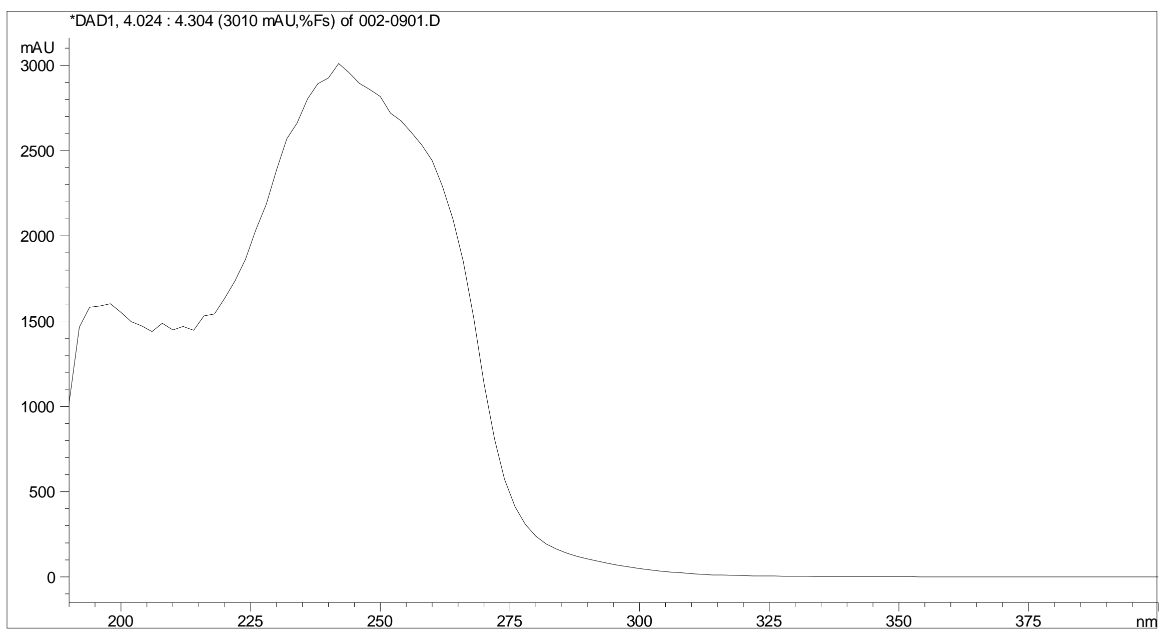

Figure S.11. MBA (10 ppm) spectrum measured by LC-DAD.

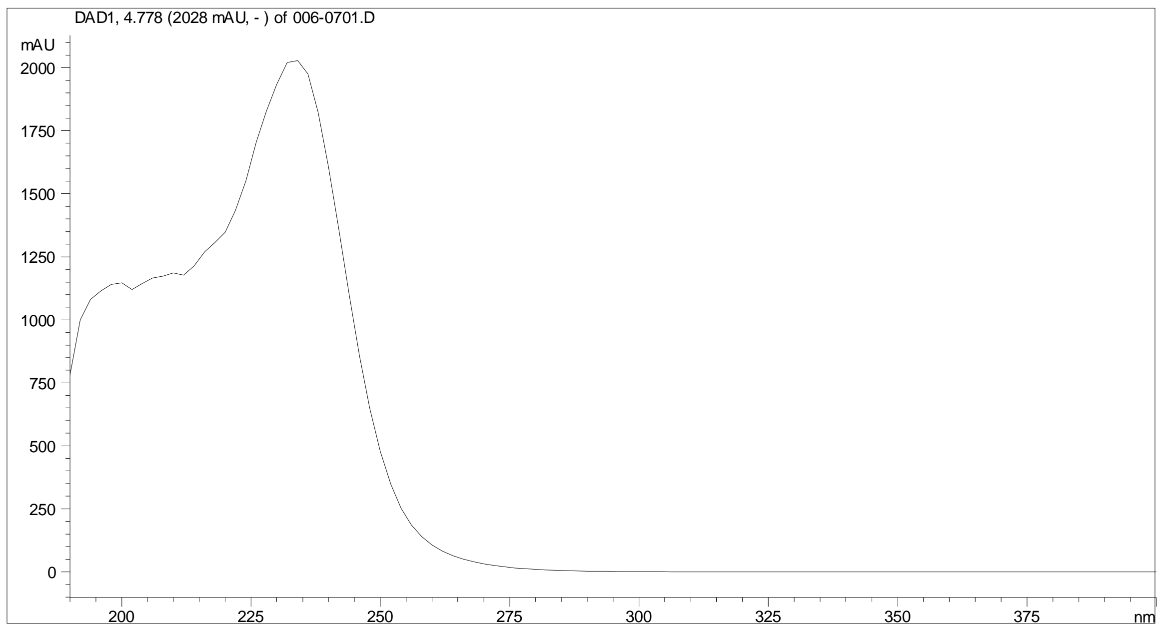

Figure S.12. MX (250 ppm) spectrum measured by LC-DAD.

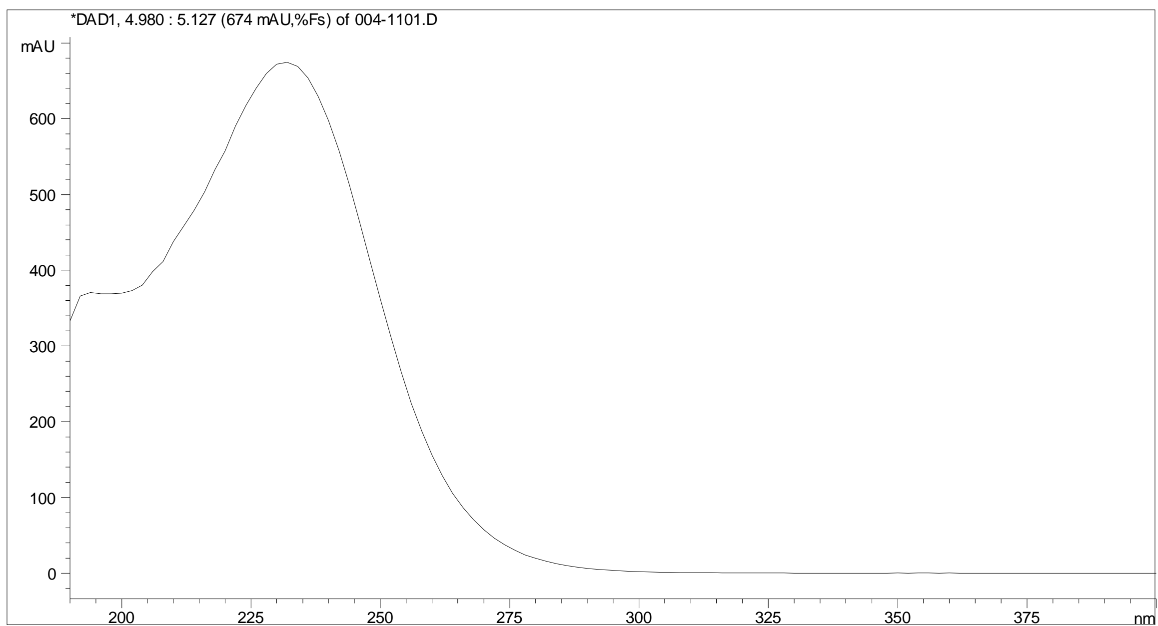

Figure S.13. BMX-1 (250 ppm) spectrum measured by LC-DAD.

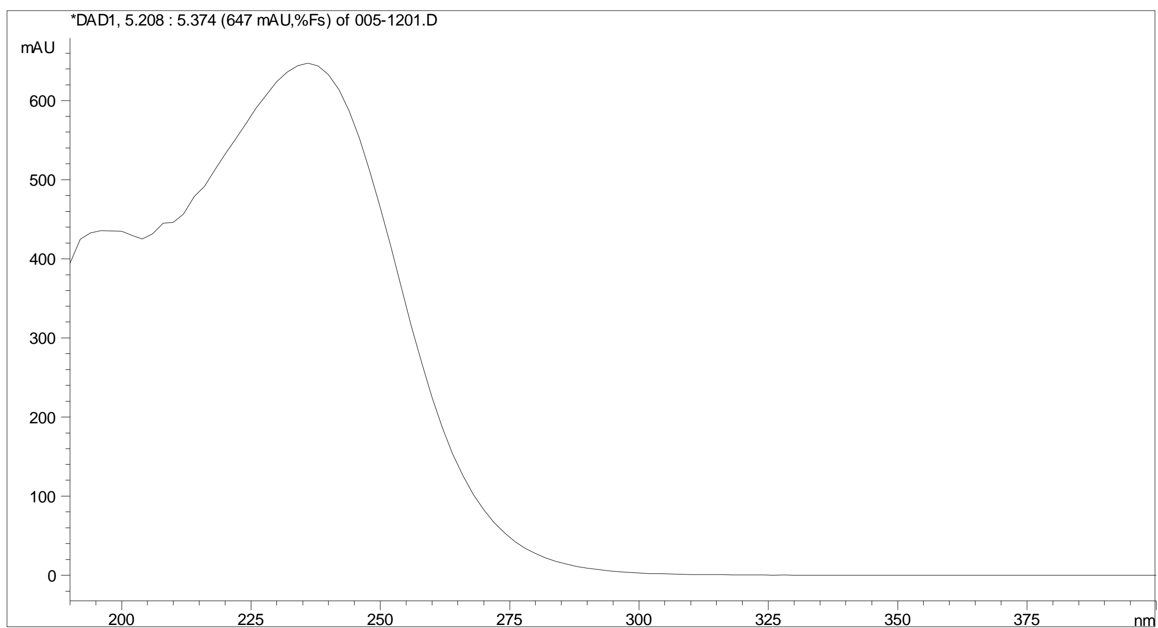

Figure S.14. BMX-2 (200 ppm) spectrum measured by LC-DAD.

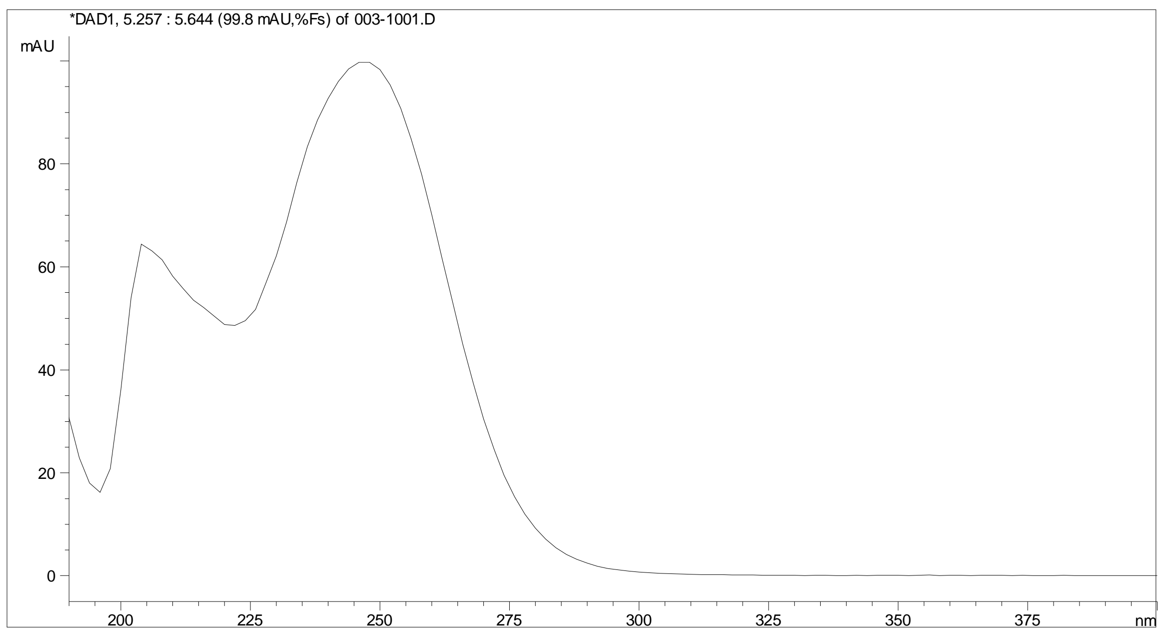

Figure S.15. BMX-3 (250 ppm) spectrum measured by LC-DAD.

SOP: ANALYSIS OF HALOGENATED FURANONES IN DRINKING WATER

Written August 15th 2015

AUTHOR: ____
Research Scientist | Laura Rascón Padilla | Date

APPROVED: ____
Primary Investigator | Dr. Gretchen Onstad | Date

A. PURPOSE AND SCOPE

This Standard Operating Procedure (SOP) describes the extraction and analysis of the five halogenated furanones (halofuranones): 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX), mucochloric acid (closed ring form), mucobromic acid (closed ring form), 3-chloro-4-(bromochloromethyl)-5-hydroxy-2(5H)-furanone (BMX-1), 3-bromo-4-(dibromomethyl)-5-hydroxy-2(5H)-furanone (BMX-3) in finished drinking water by solid phase extraction and liquid chromatography with mass spectroscopy detection (LC-MS/MS) analysis. Practical detection limits with a 1000:1 concentration factor ware 2 ng/L (BMX-3), 6 ng/L (BMX-1), 10 ng/L (MX), 7 ng/L (MBA), and 35 ng/L (MCA).

B. SUMMARY

This SOP describes a solid phase extraction and LC-MS/MS analysis of five halogenated furanone disinfection by-products (DBPs) in treated drinking water using solid phase extraction, separated by a Synergy Polar-Reversed Phase HPLC column, and detected by positive electrospray ionization MS/MS.

C. SAFETY AND SAMPLE HANDLING

Exposure to chemicals should be kept to a minimum. All procedures should be carried out under a functional fume hood. Safety Data Sheets should be accessible to all lab workers for proper chemical handling. Personal protective equipment including lab coat, gloves, and goggles must be worn at all times. Standard solutions are stored at -20 °C. Samples are stored at 4 °C. Mucobromic acid stocks stored for more than a month should be checked for degradation before using. All standard solutions and stocks should be stored in silinized amber glass vials to prevent degradation of analyte.

D. APPARATUS AND MATERIALS

- 1 L and 500 mL glass screw cap sample bottles with polytetrafluoroethylene (PFTE)-lined screw caps.
- Glass volumetric flasks with glass stoppers
- 20 mL graduated glass pipettes and bulbs
- Solvent proof tubing for SPE
- Phenomenex (Torrance, CA) Synergy Polar-RP HPLC column (250mm x 2mm, 4µm particle size)
- 47 mm glass microfiber filters, GF/F grade

- Sep-Pak Plus tC_{18} cartridge (tC_{18}) 400mg sorbent (Waters, Wexford, Ireland) P/N: WAT036810
- Oasis HLB Plus cartridge (HLB) 225mg of sorbent (Waters, Wexford, Ireland) P/N: 186000132
- Positive displacement pipettes with glass capillary tips (VWR Scientific)
- 2mL amber autosampler vials
- Agilent G6460 LC-MS/MS system (Palo Alto, CA)
- 0.2µm PTFE membrane, 13mm syringe filters (Pall Life Sciences)
- PTFE flow control valve liners for vacuum manifold Visiprep-DL (Supelco)
- Autosampler vial inserts, 250µL deactivated glass with polymer feet (Agilent)

E. REAGENTS AND CHEMICALS

- 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) (Toronto Research Chemicals Inc, Toronto, Ontario) P/N: C365665
- Mucochloric acid (closed ring form) (Sigma-Aldrich, St Louis, MO) P/N: M89803-100G
- Mucochloric acid (open ring form) (Toronto Research Chemicals Inc, Toronto, Ontario) P/N: M790800
- 3-Chloro-4-(bromochloromethyl)-5-hydroxy-2(5H)-furanone (BMX-1) (Toronto Research Chemicals Inc, Toronto, Ontario) P/N: B682395
- 3-Bromo-4-(dibromomethyl)-5-hydroxy-2(5H)-furanone (BMX-3) (Toronto Research Chemicals Inc, Toronto, Ontario) P/N: B683300
- Mucobromic acid (MBA) (Sigma-Aldrich, St Louis, MO) P/N: M89625-100G
- Acetone (Fisher Scientific Fair Lawn, NJ): , Optima grade
- Acetonitrile Fisher Scientific Fair Lawn, NJ): , Optima grade
- Methanol (Fisher Scientific Fair Lawn, NJ): , Optima grade
- Formic Acid (Fisher Scientific Fair Lawn, NJ): , Optima LC/MS grade
- Hydrochloric acid (36.5 – 38 %) (JT Baker, Phillipsburg, NJ) ACS Reagent grade
- Organic-free deionized water (18.2 MΩ) or also referred to as DIW (Barnstead Easypure, Dubuke, IA)
- L-Ascorbic acid in granular form (Fisher Scientific, Fair Lawn, NJ)
- Nitrogen gas (Praxair, 99.999% purity)

F. QC SAMPLES

- Primary stocks

  Mucochloric acid (10 mg/mL), mucocbromic acid (10 mg/mL), BMX-1 (4.25 mg/mL) and BMX-3 (2.5 mg/mL) are prepared by weight in methanol.

- Secondary Stocks
  MBA, MX, BMX-1, and BMX-3 are prepared at a concentration of 20 mg/L in methanol. MCA is prepared at a concentration of 10 mg/L in methanol.

- Tertiary stocks.
  Two mixtures, each containing MBA, MX, and BMX's, are prepared in methanol from secondary stocks at concentrations of 1 mg/L (1 µg/compound/mL) and 2 mg/L (2 µg/compound/mL).

- External calibration standards
  Using secondary and tertiary stock solutions, a total of six calibration standards are prepared in a mixture of 50/50 Acetonitrile/DIW (0.25% formic acid). Concentration range is 25-500 ng/mL. MCA is added as internal standard to each calibration solution at a concentration of 100 ng/mL using MCA secondary stock.

- Internal standard
  Fresh internal standard solution of MCA at 5 ng/mL is prepared each day in acetone from secondary stocks.

- Matrix spikes for standard addition curve
  500 mL aliquots of treated sample are spiked in duplicate at four different levels with MBA, MX, BMX-1, and BMX-3 (50, 75, 200, and 300 ng/L) using tertiary stocks. Treated samples are also spiked in triplicate with 100 ng/L of MBA, MX, and BMX's with tertiary stock.

- Blank samples
  500 mL of pH2 DIW (adjusted with HCl) is extracted in each sample batch

- Treated samples
  500 mL of a non-spiked treated sample is extracted in each sample batch.

H. SAMPLE COLLECTION AND PREPARATION

- Upon collection, 1L or 500 mL of treated sample water is quenched with L-ascorbic acid to a final concentration of 62 mg/L, and store at 4°C in a headspace free amber glass bottle with a PTFE-lined cap.
- To prepare all samples for extraction, filter through 47 mm GF/F grade glass filter under vacuum.
- Adjust pH to 2 with hydrochloric acid (HCl).
- 500 mL aliquots of sample (measured with volumetric flasks) are spiked at the aforementioned spike levels.

I. SOLID PHASE EXTRACTION

Conditioning solid phase extraction (SPE) cartridges

- Condition tC_{18} and with 15 mL of each acetone, methanol, and pH2 DIW (adjusted with HCl).
- Condition Oasis HLB cartridges with 5 mL of each ethyl acetone, methanol, and pH2 DIW (adjusted with HCl).

Solid Phase Extraction

- tC_{18} and oasis HLB cartridges are set up in train on Visiprep-DL vacuum manifold equipped with flow control liners. An SPE reservoir is attached with an adaptor to insert solvent proof tubing. Flow rate is ideally maintained at 10 mL/min.
- After sample pumping, pump 100 mL of pH2 DIW (adjusted with HCl) water through cartridges to ensure complete transfer of analytes to HLB cartridge.
- Dry HLB cartridge under vacuum for 15 min to remove any residual water.
- Elute analytes from HLB with 10 mL of acetone containing 5 ng/mL of MCA internal standard.
- Evaporate solvent almost to dryness (~50-100 µL) under gentle stream of nitrogen at 30 ℃.
- Reconstitute analytes in 500 µL of a mixture of 50/50 acetonitrile/DIW (0.25% formic acid). Initially, 400 µL of the solvent mixture is added to reconstitute the 50-100 µL of extract. If needed, additional solvent mixture is added to reach the 500 µL volume.
- Filter reconstituted extract through 0.2µm PTFE syringe filter.
- Transfer 150 µL of extract to autosampler vials equipped with glass inserts, and store at -20°C until analysis.

J. LC-MS/MS ANALYSIS

- Load a method into software with the following parameters:
  - Injection volume: 10 µL
  - Mobile phase flow rate: 300 µL/min
  - Column type: Synergy Polar-RP (250mm x 2mm, 4µm particle size)
  - Linear gradient: 28/72 ACN/DIW (0.25% FA) to 45/75 ACN/DIW (0.25% FA) in 20 minutes.
  - Multiple reaction monitoring (MRM) acquisition method with the following, transitions, acquisition time windows, fragmentor voltages and collision energies:

Table 1. MRM transition parameters

Compound	MRM Quantitation Transitions		MRM Confirmation Transitions		Acquisition Time	Fragmentor	Collision Energy
	Precursor ion (*m/z*)	Product ion (*m/z*)	Precursor ion (*m/z*)	Product ion (*m/z*)	min (SD)	(V)	(V)
MCA	169 $[MH]^+$	151 $[MH-H_2O]^+$	171 $[MH+2]^+$	152.9 $[MH+2-H_2O]^+$	5-7.2	50	5
MBA	258.9 $[MH+2]^+$	240.9 $[MH+2-H_2O]^+$	256.9 $[MH]^+$	238.9 $[MH-H_2O]^+$	7.2-10	55	10
MX	217 $[MH]^+$	199 $[MH-H_2O]^+$	219 $[MH+2]^+$	201 $[MH+2-H_2O]^+$	10-11.2	60	5
BMX-1	263 $[MH+2]^+$	244.7 $[MH+2-H_2O]^+$	261 $[MH]^+$	242.9 $[MH-H_2O]^+$	11.2-12.5	70	5
BMX-3	350.8 $[MH+2]^+$	332.9 $[MH+2-H_2O]^+$	352.8 $[MH+4]^+$	334.9 $[MH+4-H_2O]^+$	12.5-14	100	5

- Positive electrospray ionization (ESI)
- Capillary voltage: 4000 V
- Gas Flow: 11 mL/min
- Gas Temperature: 350 ºC
- Nebulizer pressure: 30 psi

- Sequence order and incorporation of QC samples:
  1. Reagent blank: It is recommended that a reagent blank is run first in full scan mode and subsequently in MRM mode at the beginning and end of each sequence.
  2. External calibrants: In duplicate or triplicate, and ascending concentration order. A reagent blanks should be run after 5-10 calibrant injections.
  3. Sample batch:
     - A reagent blank is run before and after each sample batch
     - Method blank: Run after reagent blank and before treated sample and matrix spiked samples
     - Matrix spiked samples: Run after treated sample
     - Three samples per batch are run in duplicate or triplicate

K. DATA ANALYSIS

- Extract MRM transition chromatograms (EICs)
- Integrate analyte quantitative transitions peak areas
- For relative response, $\frac{Peak\ Area\ (Analyte)}{Peak\ Area\ (MCA)}$
- Generate external and standard addition curve with relative response as a function of concentration

L. REFERENCES

Kubwabo, C., Stewart, B., Gauthier S.A., Gauthier, B. R. Improved derivatization technique for gas chromatography–mass spectrometry determination of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone in drinking water. *Analytica Chimica Acta* (20