Evaluation of Wastewater Drug Trends in Sacramento County by Liquid Chromatography Time-of-Flight Mass Spectrometry

By

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Abstract

Illicit drug use remains a significant global public health concern. To monitor drug trends, crime laboratories and public health agencies often rely on data derived from case work submitted by local authorities. Although effective, this approach limits an agency's ability to rapidly identify new drug trends as they emerge because there is an inherent delay in the use of a drug and its detection in toxicological samples. This is further complicated by the introduction of synthetic drugs known as novel psychoactive substances (NPS). These drugs are designed to mimic the effects of controlled substances, but their health risks are often unknown. Additionally, because these drugs are novel, crime laboratories and public health agencies struggle to develop methods to rapidly identify them. To address this detection gap, this study utilizes a novel method for NPS detection by analyzing wastewater samples using liquid chromatography time-of-flight mass spectrometry (LC-TOF/MS). Wastewater samples were collected at the entrance to the Sacramento County Wastewater Treatment Plant and analyzed at the Sacramento County District Attorney's Crime Laboratory to assess real-time drug use trends across Sacramento County. Substantial quantities of common drugs of abuse such as methamphetamine, fentanyl, and the primary metabolite of cocaine (benzoylecgonine) were detected in the wastewater, consistent with known local use data based upon blood and urine toxicology testing. Additionally, this method successfully identified all NPS evaluated reflecting the robustness of this method in identifying novel drugs when introduced into a community. Finally, this method of wastewater analysis offers a model for detecting drugs of abuse in the community before they are detected in clinical or forensic cases. This will enable faster responses from health agencies and crime labs and provide a powerful tool against constantly evolving drug use patterns.

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Introduction

Illicit drug use remains a persistent cause for concern worldwide due to the risk it poses to human health and its link to crime [1]. In addition to legacy drugs of abuse, a proliferation of new designer drugs known as novel psychoactive substances (NPS) has led to a highly dynamic illicit drug landscape [2]. NPS–synthetic or plant-based compounds– mimic the pharmacological effects of illegal drugs but are not yet regulated by government agencies [2]. Limited information is available regarding the health risks and potential for abuse associated with these drugs [3,4]. Additionally, changes in the drug market significantly outpace government and health agencies' ability to adapt to the health risks presented by NPS before they become ingrained in a community [5]. Global and national agencies such as the United Nations Office on Drugs and Crime (UNODC) Early Warning Advisory (EWA) and the United States Drug Enforcement Administration (DEA) monitor and report emerging NPS [2]. The Center for Forensic Science Research and Education (CFSRE) releases a quarterly report of national NPS trends by analyzing toxicology specimens and drug materials submitted from various agencies throughout the United States [6]. Despite evident governmental interest, a local network for illicit drug and NPS monitoring does not exist.

Traditional drug monitoring techniques such as self-reporting and crime laboratory identification often result in underreporting of drug use in a community. This can lead to a delay in the recognition of a new drug by law enforcement agencies [7]. For example, self-reporting of drug use is often hindered by stigma and limited awareness of what substances are consumed. Yet, epidemiological studies suggest that for traditional drugs such as methamphetamine, cocaine, and cannabis, self-reported illicit drug use aligns with analyzed biological samples [8]. However, these studies are limited in access to at-risk populations such as homeless individuals,

military personnel, and hospital patients [9]. This results in data that is not representative of drug trends across an entire community. Furthermore, use data is limited to the case work received by the laboratory. Crime lab methodologies often analyze a defined panel of common illicit drugs but typically lack the procedures or funding necessary to quickly develop methods to identify NPS. The combination of under-reporting of NPS by crime laboratories and biased self-reporting underscores the critical need for a robust local monitoring system capable of tracking drug use trends [10]. Moreover, these limitations contribute to a critical detection gap that slows the ability of local health agencies to properly respond.

To address this detection gap, a small number of local health departments outside of the United States have developed methods of analysis involving wastewater-based epidemiology (WBE) to monitor drug consumption trends [11,12]. It has been shown that drugs and their metabolites excreted into the sewage system and wastewater samples can provide a current "snapshot" of a community's drug use [13-15]. WBE analysis offers a comprehensive, real-time approach to drug detection. Zuccato et al. [13] first used WBE to assess drug trends in 2005. Their study demonstrated that illicit drugs and their metabolites were detectable as contaminants in untreated wastewater [13]. A similar study was conducted in the United States in 2009 by analyzing 24-hour composite wastewater samples to compare rural and urban trends of methamphetamine, cocaine, and methylenedioxymethamphetamine (MDMA) use [16]. Additional studies analyzed wastewater samples to determine specific drug use trends in prison facilities [1] and identify NPS in local communities [17].

In these initial studies researchers utilized solid phase extraction followed by liquid chromatography coupled with tandem mass spectrometry to quantify drug concentrations. These studies used this instrumentation to relate measured drug concentration in wastewater to a dose

[18]. More recently, solid phase extraction methods have been coupled with liquid chromatography time-of-flight mass spectrometry (LC-TOF/MS). One study, using LC-TOF/MS, quantified known psychoactive drugs through wastewater analysis and successfully identified NPS in pooled urine samples taken from three different festival locations [19]. This study found LC-TOF/MS to be a useful tool for wastewater analysis because it uses a non-targeted screening approach that can detect a wide array of known and unknown substances. Furthermore, acquired LC-TOF/MS data can be retrospectively analyzed–reevaluated for new drugs–which is crucial for the identification of novel substances.

To build on the existing body of research, this study introduces a novel technique for analyzing drugs in wastewater using LC-TOF/MS. The study's objective was to assess potential drug trends in Sacramento County and identify NPS entering the community. This work presents a new method for extracting drugs from wastewater samples for LC-TOF/MS analysis that eliminates the need for solid-phase extraction. Initially, a targeted screening was performed using methodology previously developed by the Sacramento County District Attorney's Crime Laboratory to identify traditional drugs of abuse. Subsequently, all acquired sample data was reprocessed through a broader suspect screening using a drug library to investigate the presence of NPS known to be prevalent in the United States during the sampling period. The ability to retrospectively analyze previously acquired data through this suspect screening technique makes this method a powerful tool capable of efficiently identifying NPS [19]. This method provides a framework for an early detection system so crime laboratories and local law enforcement can adapt their testing and enforcement efforts to the constantly evolving drug landscape.

Materials and Methods

Sample Collection and Preparation

Samples were provided by the Sacramento County Wastewater Treatment Plant (Regional San). Sewer water influent samples (untreated wastewater) were collected at the entrance to the sewer treatment plant as composite samples. A composite sample is a mixture of multiple individual samples collected over a 24-hour period to create a representative sample. Composite samples are collected by an autosampler that periodically draws a portion of the influent wastewater into a reservoir. After 24 hours, composite samples were collected from the water treatment plant's influent autosampler by Regional San personnel. Samples were immediately filtered to remove solid materials. A total of 67 samples were collected at an interval of three days per week. Sampling was conducted from 2/27/2024 to 7/30/2024 to evaluate drug trends over an extended period.

All laboratory analyses were conducted at the Sacramento County District Attorney's Crime Laboratory. Multiple extraction schemes were evaluated to determine the optimal method for extracting drugs from the wastewater. For all extraction schemes, high and low positive controls, and a method blank were analyzed with each extraction type to measure the efficiency of each extraction method. The positive controls were created from a laboratory-prepared drug standard (TOF standard) containing 123 certified reference materials (Cerilliant/Lipomed). The method blank was created with LCMS grade water. The drugs contained in the stock standard are listed in **Table 1.**

Table 1. List of 123 parent drugs and metabolites in the stock standard with internal standard (d7-7-aminoflunitrazepam) with expected neutral mass (Da)

7-aminoflunitrazepam	283.11	Dextromethorphan	271.19	Lidocaine	234.17	Oxazepam	286.05
8-aminoclonazolam	323.09	Diazepam	284.07	Lorazepam	320.07	Oxycodone	315.15
Acetaminophen	151.06	Dihydrocodeine	301.17	LSD	323.19	Oxymorphone	301.13
Adinazolam	351.13	Diphenhydramine	255.16	MDA	179.09	Paroxetine	329.14
Alprazolam	308.08	Doxepin	279.16	MDMA	193.11	Pentylone^	235.12
Alprazolam, alpha-hydroxy	324.07	Doxylamine	270.17	MDMB-4en-PINACA	357.20	Phencyclidine (PCP)	243.19
Amitriptyline	277.18	Duloxetine	297.12	Meperidine	247.16	Pheniramine	240.16
Amphetamine	135.10	Ecgonine methyl ester	199.12	Meprobamate	218.13	Phentermine	149.12
Aripiprazole	447.15	EDDP	277.18	Methadone	309.21	Pregabalin	159.13
Benzoylecgonine	289.13	Ephedrine/ Pseudoephedrine	165.12	Methamphetamine	149.12	Promethazine	284.13
Bromazolam^	352.03	Etizolam	342.07	Methylphenidate	233.14	Propoxyphene	339.22
Brompheniramine	318.07	Eutylone	235.12	Metonitazene^	382.20	Protonitazene^	410.23
Brorphine	399.09	Fentanyl	336.22	Midazolam	325.08	Quetiapine	383.17
Buprenorphine	467.30	Flualprazolam	326.07	Mirtazapine	265.18	Sertraline	305.07
Bupropion	239.10	Flubromazepam	331.99	Mitragynine	398.22	Strychnine	334.17
Caffeine	194.08	Flubromazolam	370.02	Morphine	285.14	Tapentadol	221.18
Carbamazepine	236.09	Flunitrazepam	313.08	Morphine, 6-monacetyl	327.15	Temazepam	300.07
Carfentanil^	394.23	Fluorofentanyl	354.21	N,N-dimethylpentylone ^	249.14	Tizanidine	253.02
Carisoprodol	260.17	Fluoxetine	309.13	Naloxone	327.15	Topiramate	339.09
Chlorpheniramine	274.12	Gabapentin	171.13	Nicotine	162.12	Tramadol	263.19
Citalopram/ Escitalopram	324.16	Hydrocodone	299.15	Norbuprenorphine	413.26	Tramadol, N-desmethyl	249.17
Clonazepam	315.04	Hydromorphone	285.14	Nordiazepam	270.06	Tramadol, O-desmethyl	149.17
Clonazepam, 7-amino	285.06	Hydroxybupropion	255.10	Norfentanyl	232.16	Trazodone	371.15
Clonazolam	353.07	Hydroxyzine	374.18	Norfluoxetine	295.12	Triazolam	342.04
Cocaethylene	317.16	Imipramine	280.19	Norhydrocodone	285.14	Trimipramine	294.20
Cocaine	303.15	Isotonitazene	410.23	Norketamine	223.08	Valpromide	143.13
Codeine	299.15	Kavain	230.09	Normeperidine	233.14	Venlafaxine	277.20
Cyclobenzaprine	275.17	Ketamine	237.09	Norsertraline	291.06	Venlafaxine, O-desmethyl	263.19
D7-7-aminoflunitrazepam*	290.16	Lacosamide	250.13	Nortriptyline	263.17	Xylazine	220.10
Desalkylflurazepam	288.05	Lamotrigine	255.01	Olanzapine	312.14	Zolpidem	307.17
Desipramine	266.18	Levetiracetam	170.10				

* internal standard
^ evaluated NPS

Crash Extraction (Experiments 1-3)

Initial testing of wastewater samples was performed using a crash extraction utilized by the Sacramento County District Attorney's Crime Lab for blood drug screening. This technique precipitates proteins out of the blood matrix to isolate drugs in the sample for analysis by LC-TOF/MS. This method was tested to determine if it was also suitable for wastewater extractions. For Experiment 1, 25 µL of TOF standard was added to 475 µL of water (Fisher Optima LC/MS) to make a stock standard. This was used to make the high (100 µL neat) and low (2x dilution) positive control. The method blank was created with 100 µL of water. One hundred µL of selected wastewater samples were pipetted into a microcentrifuge tube. Next, 100 µL of cold acetonitrile containing internal standard (d7-7-aminoflunitrazepam) was added to each tube. The samples were centrifuged for five minutes at 14,000 rpm, and a portion of the acetonitrile was transferred into a liquid chromatography autosampler vial for analysis. A similar crash extraction (Experiment 2) was performed with 200 µL.

Next, the crash extraction was conducted with the same initial volume (200 µL) but an increased volume of acetonitrile (225 µL) to improve crash efficiency (Experiment 3). These are the proportions of sample to acetonitrile used by the Sacramento County District Attorney's Crime Laboratory for its blood crash extraction. Despite improvements in the efficiency of the crash extraction, it was determined that to further improve drug recovery and further identify currently undetected compounds, a sample reconstitution step should be added to the extraction.

Sample Evaporation and Reconstitution (Experiments 4-10)

The next method tested introduced an evaporation and reconstitution step. The goal of evaporating the sample to dryness and reconstituting in a variety of solvent ratios was to improve drug responses and identify drugs previously undetected. Like previous extractions, a high

control, low control, and blank were extracted with wastewater samples. Again, 200 µL of each sample was transferred into labelled microcentrifuge tubes with 225 µL of internal standard containing acetonitrile (Experiment 4). For this experiment, the internal standard solution was diluted in half to better align the internal standard response with drug responses. The mixture was centrifuged then transferred into larger test tubes and evaporated to dryness under nitrogen with a Biotage TurboVap evaporator (30 min, 50 ºC). Next, all dried samples were reconstituted with 100 µL of varied H_2O:ACN ratios (75:25, 80:20, 90:10) to determine which reconstitution solvent was most effective. The resulting reconstitutions were transferred into liquid chromatography autosampler vials for analysis by LC-TOF/MS. A subsequent experiment (Experiment 5) was performed with 400 µL of sample and 600 µL of acetonitrile with a 90:10 H_2O:ACN reconstitution.

Initial experimentation was conducted using a Waters G2 XS LC-TOF/MS. During the experimentation period, the Sacramento County District Attorney's Crime Laboratory acquired a newer version of the G2 XS instrument, a Waters Xevo G3 LC-TOF/MS. This instrument offers higher sensitivity compared to its predecessor. Thus, subsequent experimentation was conducted on the new instrument. Using the new instrument, Experiment 6 was conducted to confirm the 90:10 reconstitution method with the original 200 µL sample to 225 µL acetonitrile scheme to conserve sample volume. The results of this retest were like the original 90:10 experiment (Experiment 4).

Next, an experiment was conducted to (1) compare analyte responses between acetonitrile and methanol as reconstitution solvents, and (2) evaluate how increasing sample volume increases analyte responses (Experiment 7). Three samples were extracted with varied reconstitution solvent and initial volumes. Sample initial volumes and reconstitution solvent

mixtures are listed in **Table 2**. Subsequent experiments were conducted with increased sample volumes (Experiments 8-10: 500 µL, 1 mL, and 2 mL, respectively). Due to limited sample volume, initial sample volumes were not tested in amounts greater than 2 mL. The drug responses for all 2 mL samples were largest, and this sample volume was used as the final extraction scheme.

Table 2. Summary of the reconstitution solvents and initial volumes for Experiment 7.

Sample	Initial Volume	Reconstitution Solvent
1	200 µL	100 µL 90:10 H_2O:ACN
2	200 µL	100 µL 80:20 H_2O:MeOH
3	500 µL	100 µL 80:20 H_2O:MeOH

Hydrochloric Acid Keeper Evaluation (Experiment 11)

Additionally, an experiment was performed to evaluate whether the recovery of certain drugs could be improved by the addition of a keeper solution at the evaporation stage. A keeper solution is a solution added to prevent the loss of an analyte during an extraction. For this experiment, addition of 100 µL of 1% (v/v) concentrated hydrochloric acid in methanol solution greatly improved recoveries for drugs such as amphetamine and methamphetamine; results can be seen in **Figure 1**.

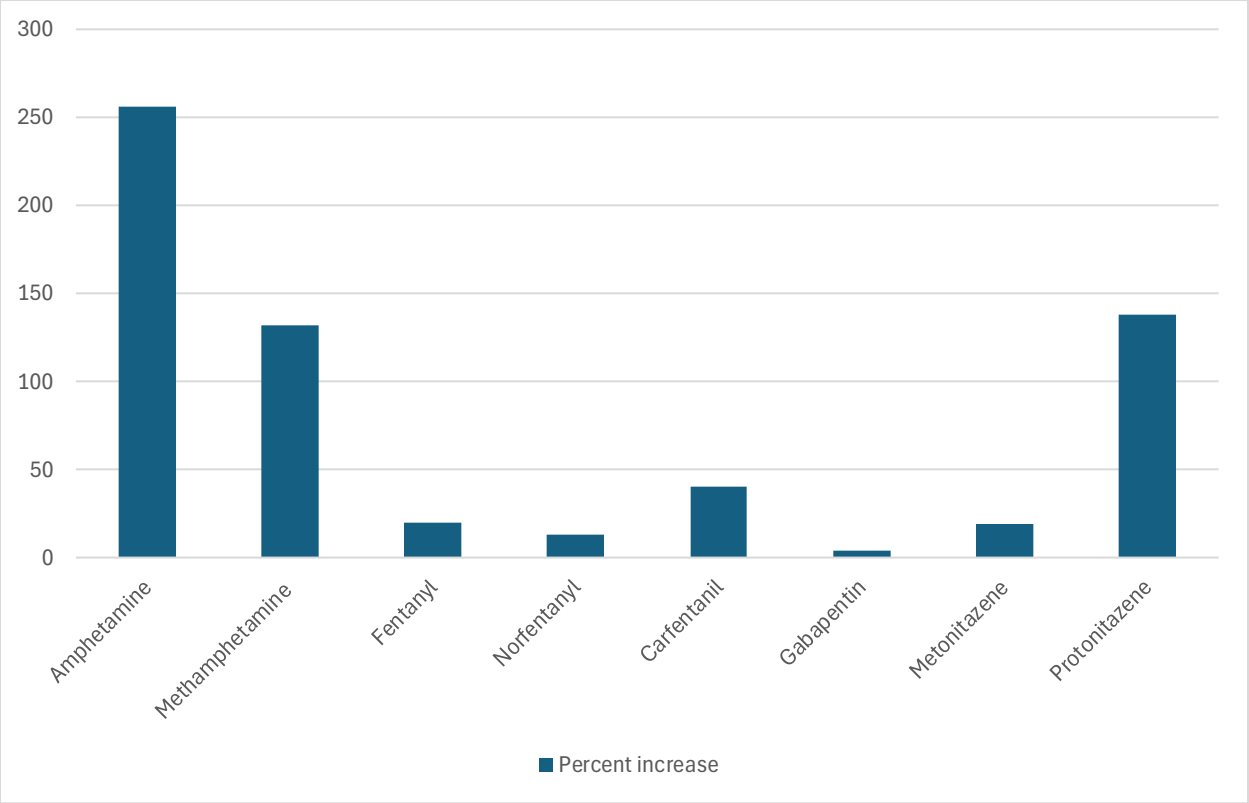

Figure 1. Percent increase in drug response after addition of 100 µL of HCl keeper solution.

Final Extraction Method

Based on prior experimentation a volume of 2 mL was used as it provided the highest drug recoveries. To create the high positive control, 25 µL of stock standard was diluted to 2 mL in water. A two times dilution of the high control was used to create the low control. A method blank was created with 2 mL of water. To 15 mL disposable centrifuge tubes, 2 mL of wastewater, 225 µL of internal standard (0.216 µM d7-7-aminoflunitrazepam in ACN) and 100 µL of HCl keeper were added. Samples were vortexed one minute then evaporated to dryness using a nitrogen evaporator (50 °C, 60 min). To all samples, 100 µL of reconstitution solvent (90:10 H_2O:ACN) was added and vortexed for 1 minute. Extracts were transferred to autoinjector vials for LC-TOF/MS analysis. A summary of all extraction methods is detailed in **Figure 2**.

Instrumentation

Samples were analyzed with a Waters Xevo G3 LC-TOF/MS in positive sensitivity mode and were ionized by electrospray ionization (ESI). The capillary and sample voltages were set to 0.80 kV and 25 V, respectively. The injection volume was 2 µL, the initial sample temperature was 10°C, and the column temperature was set at 50°C. Samples were analyzed for 15 minutes using a gradient solvent system (initial 87:13, MPA:MPB) ramping from an aqueous to organic solvent concentration. A summary of sample analysis initial conditions can be found in **Table 3**.

Drugs were identified by their retention time, parent mass, detected fragment ions, and by comparison to drug standard spectra. Drug comparisons were conducted using the extracted ion chromatograms (XIC), and both the low and high energy mass spectra. The low energy spectra are created using a lower collision energy that yields fewer fragments, while the high energy spectra utilizes a higher collision energy that fragments molecules more extensively. In addition to the in-house reference library, all samples were processed against the Waters Toxicology Library [POS waters-connect_UNIFI_v1]. This is a positive ionization library containing retention times, parent masses, and in some cases, expected fragment ions, for over 1500 compounds, including a wide range of drugs of abuse, NPS, and their metabolites.

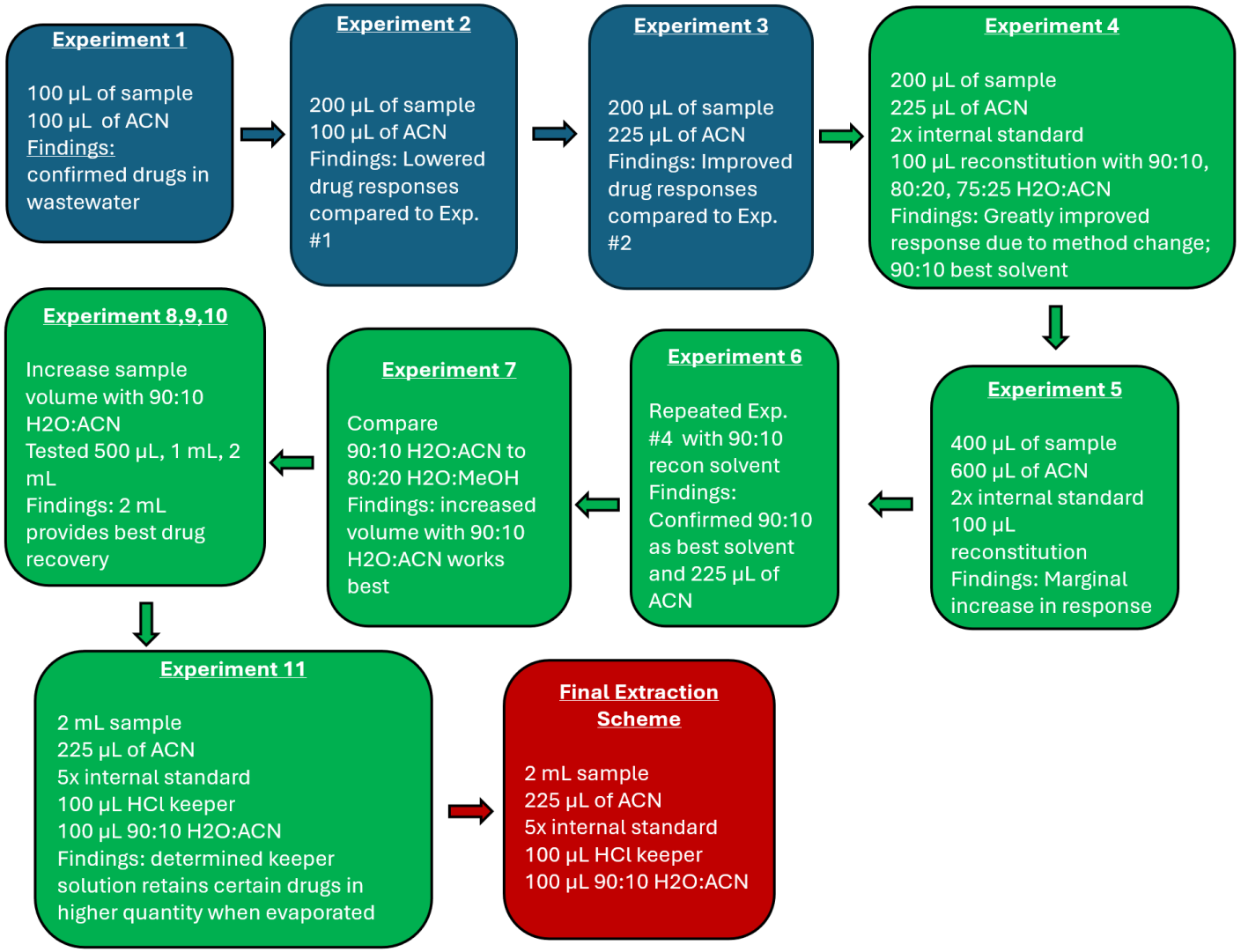

Figure 2. Method development and final extraction scheme used for sample analysis.

Table 3. Summary of materials and instrumentation used for analysis.

Reagents	TOF Standard	Created using 123 certified reference materials (Cerilliant/Lipomed)
	Internal Standard	0.216 µM d7-7-aminoflunitrazepam in ACN (Fisher)
	Mobile Phase A (MPA)	10 mM ammonium formate with 0.1% formic acid in 4L water
	Mobile Phase B (MPB)	0.1% formic acid in 4L ACN
	Reconstitution Solvent	90:10 (H_2O:ACN)
	Keeper solution	1% v/v concentrated HCl in methanol
Instrumentation	Waters Xevo G3 LC-TOF/MS	
	Column	Waters Aquity UPLC HSS C18 1.8 µm (2.1 x 150 mm) + Pre-Column (2.1 x 5 mm)
	Flow rate	0.400 mL/min
	Injection Volume	2 µL
	Initial solvents (gradient)	87:13 (MPA:MPB)
	Sample temperature	10 °C
	Column temperature	50 °C
	Analysis time	15 min
	Capillary voltage	0.80 kV
	Sample voltage	25 V
	Low Spectra Fragmentation energy	6 V
	High Spectra Fragmentation Energy	10-40 V
	Mass Range Acquired	50-950 m/z

Results

Overview

The purpose of this study was to evaluate the feasibility of wastewater analysis as an early warning system in identifying common and novel drugs of abuse in Sacramento County. A total of 67 wastewater samples were analyzed by LC-TOF/MS. Target drug analytes were identified by retention time, identified fragment ions and mass error (difference between theoretical and identified mass of an analyte). Additionally, a comparative analysis of the extracted ion chromatograms (XIC), and both the low and high energy mass spectra was conducted between the high control and selected wastewater samples. The retention times, expected and observed fragments, mass error, and common high and low fragment peaks for selected drugs are listed in **Table 4**. The comparative XIC and mass spectra are shown in **Appendix A**. A single wastewater sample for each comparison was used as a representative example for all analytes, as it reflects the overall sample set.

A series of experiments was conducted to develop a method that best identified analytes of interest in wastewater samples. Initial experimentation (Experiments 1-3) proved that a crash extraction could effectively isolate drugs present in the wastewater for analysis by LC-TOF/MS. In Experiment 1, the response of many drugs was low compared to known concentrations in the high and low controls. A similar extraction (Experiment 2) was conducted to improve drug responses by increasing initial volumes to 200 µL. This experiment resulted in significantly lower response for all drugs, potentially due to an inadequate ratio of acetonitrile (100 µL) to sample volume needed to facilitate the crash extraction. Experiment 3 utilized a 200 µL initial volume with increased acetonitrile. This resulted in increased recoveries for all targeted analytes

and the internal standard. Notably, fentanyl, previously undetected in any samples, was detected. The fentanyl responses in the known standard were greatly improved from previous experiments.

To improve upon the crash extraction, a sample evaporation and reconstitution extraction was tested for all additional experiments (Experiments 4-11). Compared to the results of the crash extractions, the sample evaporation and reconstitution method greatly improved drug responses. For these additional experiments, a two-time dilution of the internal standard was used. Despite this dilution, the chromatographic response of the internal standard (D7-7-aminoflunitrazepam) was almost double. Further experimentation (Experiments 4-7) determined that a reconstitution of 90:10 H_2O:ACN yielded the highest responses across targeted analytes. The starting mobile phase conditions for positive ion analysis utilized by the Sacramento County District Attorney's Crime Laboratory is 87:13 H_2O:ACN. The 90:10 reconstitution ratio is closest to these starting conditions and may account for improved responses. Experiments 8-10 confirmed that increasing the initial volume of samples led to a direct increase in analyte response. Experiment 11 showed that the addition of a keeper solution at the evaporation step increased analyte recoveries for drugs such as amphetamine and methamphetamine. Based on these results the final sample extraction method was developed using a 2 mL initial volume and 100 µL of 90:10 H_2O:ACN.

All samples were evaluated for the presence of 123 common drugs of abuse and NPS. Many of the analytes evaluated and identified in the wastewater are prevalent drugs of abuse in Sacramento County. The method successfully identified a large array of drugs of abuse in the wastewater. Benzoylecgonine, fentanyl, norfentanyl, methamphetamine, and gabapentin were identified in all analyzed samples and trends of use were evaluated by plotting relative responses of these drugs over time. Relative responses were calculated by normalizing measured drug

response to internal standard response and plant flow data. When a drug and its metabolite were both identified in a sample, the metabolite response was typically greater than that of the parent drug. Methamphetamine was a notable exception to this trend. Retention times of identified drugs in the wastewater samples closely matched those observed in the high control. Additionally, mass spectral data showed strong agreement between control and sample data, supporting the reliability of analyte identification. Moreover, all NPS evaluated in this study were identified in the positive control samples but not identified in detectible quantities in any wastewater samples.

Table 4. Chromatographic and spectral data for high positive control and wastewater samples*.

Drug	Ionized Mass (Da)	Exp. RT	Obs. RT in Control	Obs. RT in Samples	Exp. Frag.	Obs. Frag. in Control	Obs. Fragments in Samples	Mass Error in Control	Mass Error in Samples	Expected Fragment Peaks (m/z)	Low Energy Peaks (m/z)†	High Energy Peaks (m/z)†
Benzoylecgonine	290.14	3.00	3.06	3.06	3	3	3	-0.8	-1.1	82.06 105.03 168.10	290.14	105.03 168.10
Cocaine	304.15	4.55	4.53	4.54	4	4	4	-0.3	0.3	82.07 150.09 168.10	304.15	304.15
Fentanyl	337.23	6.37	6.28	6.3	3	2	3	0.5	-1.8	105.07 188.14 216.07	337.23	105.07 337.23
Norfentanyl	233.16	3.35	3.34	3.34	1	1	1	-1.7	-3.0	84.1	233.16	-
Methamphetamine	150.13	2.62	2.58	2.59	2	2	2	-4.2	-4.3	91.05 *119.09*	150.13	91.05 119.09
Phentermine‡	150.13	2.62	2.58	2.59	2	1	1	-4.2	-	91.05 *133.10*	-	-
Amphetamine	136.11	2.32	2.28	2.30	2	2	2	-3.2	-3.2	91.05 119.09	119.09 136.11	91.05 119.09
Gabapentin	172.12	1.88	1.88	1.89	2	2	2	-2.0	-3.2	137.10 154.13	148.11 154.12 172.13	137.09 154.12
Bromazolam	353.04	8.96	9.06	-	2	0	-	0.2	-	158.98 275.04	-	-
Carfentanil	395.23	7.25	7.09	7.07	5	5	-	0.7	-	194.12 246.15 279.19 335.21	-	-
Metonitazene	383.21	5.80	5.74	-	2	2	-	0.7	-	100.11 121.06	-	-
Protonitazene	411.24	8.20	8.08	-	1	1	-	0.6	-	100.1	-	-
Pentylone	236.12	3.82	3.80	3.98	1	1	-	-1.7	-	188.1	-	-
N, N-dimethylpentylone	250.14	4.09	4.04	-	2	2	-	-1.2	-	149.02 205.09	-	-

* Samples were analyzed in three batches with three uniquely extracted high controls. Results of only one high control have been reported.
† NPS fragments are only from high control spectra.
‡ Phentermine data is only included to show the difference in expected fragments related to methamphetamine.

Internal Standard Response

The internal standard (d7-7-aminoflunitrazepam) was added to all samples to identify any matrix effect or instrument variation during sample analysis. Samples were analyzed in order of date collected. Over the analysis period, internal standard responses gradually declined. However, mass accuracies across a sample run remained unchanged. To evaluate if this was an increasing matrix effect or an instrument drift occurring during the instrument run the instrument was recalibrated. All samples were then reinjected in reverse data order. The trend of diminishing internal standard response remained, indicating instrument drift. Original internal standard responses showing this drift over time are shown in **Figure 3**. Data points on 3/31/24, 5/9/24, 6/23/24, and 7/9/24 were removed due to low internal standard recoveries. These samples were not reextracted because of sample volume constraints.

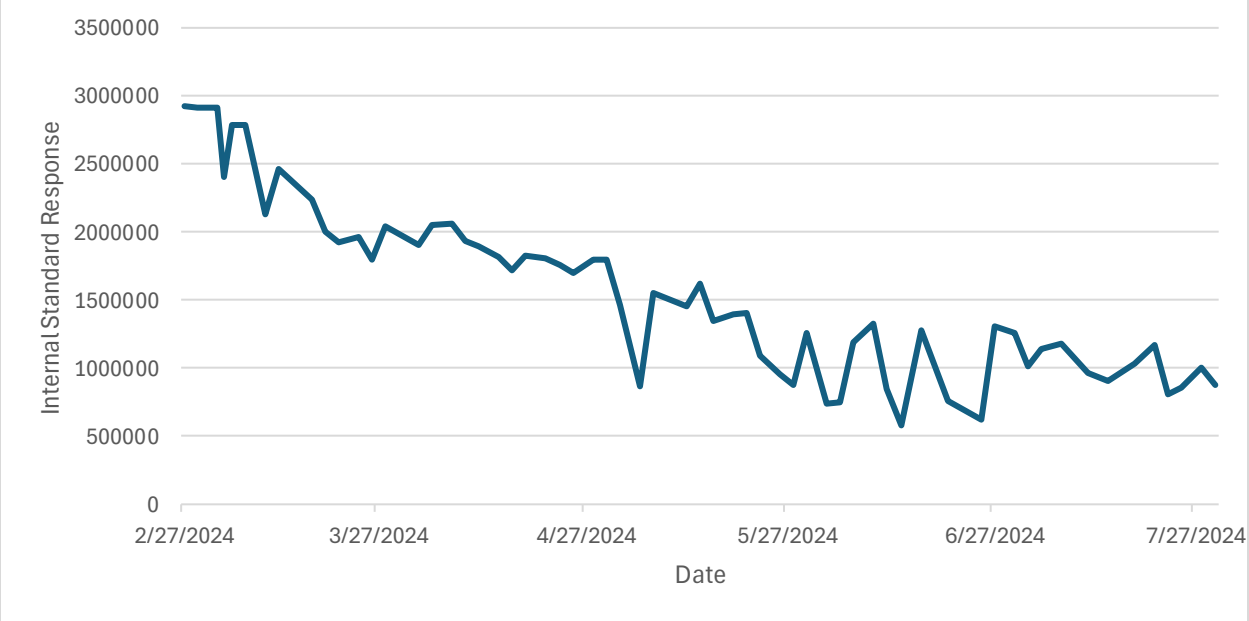

Figure 3. Internal standard responses detailing instrument drift over time.

Adjusting for Matrix Variability

Samples were collected as composite influent samples over a four-month period. Due to environmental factors, such as rain, the flow of wastewater moving through the water treatment plant is variable. Plant flow data during the sampling period is listed in **Figure 4**. The average flow rate of the plant during the sampling period was 156.3 million gallons per day (MGD). To account for variations in plant flow rate internal standard responses were normalized with the flow rate of the plant. Once adjusted for plant flow, drug responses were normalized to the internal standard response for that sample and date. The normalized internal standard responses are displayed in **Figure 5**.

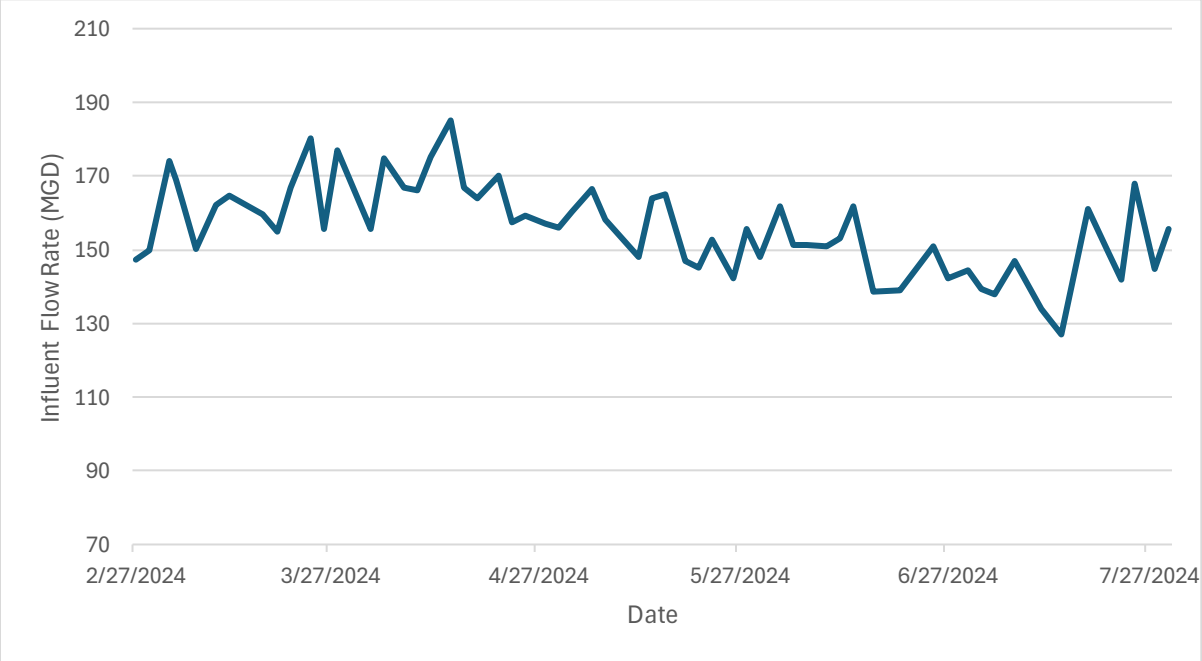

Figure 4. Net influent flow rate of Regional San 2/27/24 to 7/30/24.

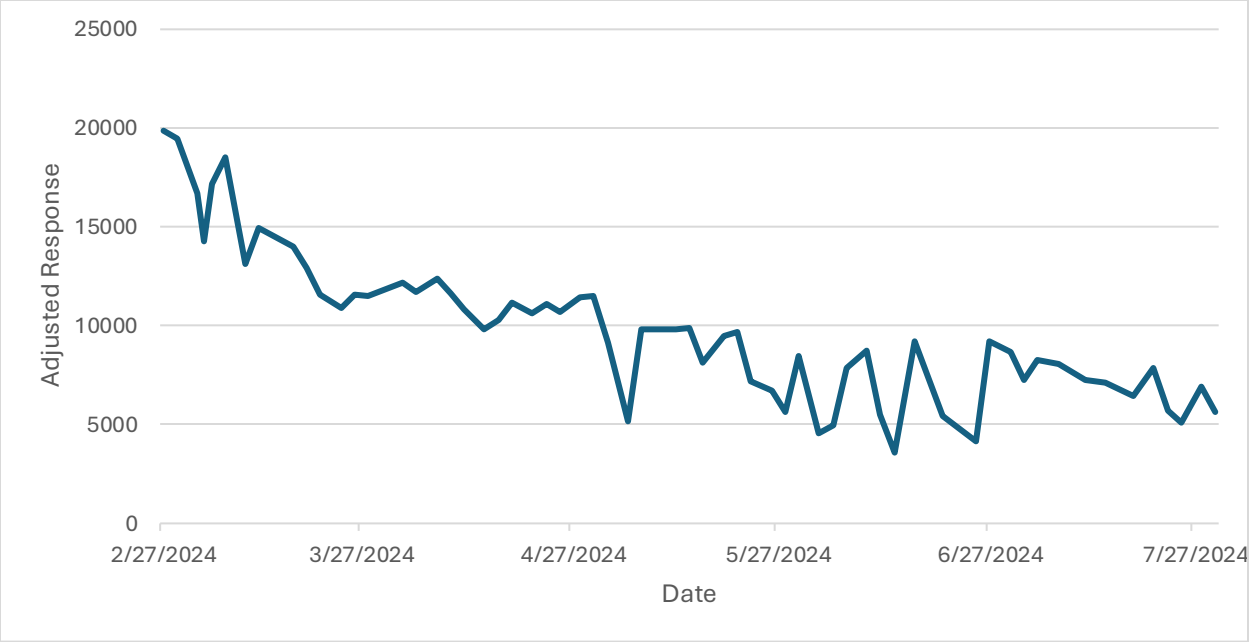

Figure 5. Internal standard response over time normalized to plant flow.

Drug Presence in the Wastewater of Sacramento County

In general, drugs identified in the wastewater were consistent with commonly identified drugs in the toxicology case work at the Sacramento County District Attorney's Crime Laboratory. The top 20 drugs confirmed by the laboratory in 2024 and during the sampling period are listed in **Table 5**. Alcohol and tetrahydrocannabinols (THC) were not evaluated in this study and have not been included. Drugs such as benzoylecgonine, fentanyl, and methamphetamine were confirmed in high frequencies in both the wastewater analysis and toxicological data. Norfentanyl and gabapentin were both identified with high frequency in the wastewater but are not currently routinely confirmed by the Crime Lab. Prescription drugs such as diphenhydramine, methadone, citalopram, and lidocaine were also identified at a high rate in the wastewater and toxicology data. Benzodiazepines such as alprazolam, diazepam and its

metabolite nordiazepam, lorazepam, midazolam, and clonazepam were all identified in the blood toxicological data, but not the wastewater.

Table 5. Top 20* drugs confirmed in Sacramento County blood samples in 2024 and the sampling period compared to number of samples that drug was identified in the wastewater.

Drug	Confirmations 2024	Confirmations 2/29/24- 7/30/24	Wastewater Identifications
methamphetamine	272	113	67
benzoylecgonine	264	114	67
caffeine	216 (2,285)†	87 (990)†	67
amphetamine	169	74	10‡
fentanyl	112	51	67
cocaine	103	51	12‡
gabapentin	68†	23†	67
alprazolam	50	25	0
nordiazepam	38	23	0
lorazepam	31	11	0
diphenhydramine	28	13	67
hydrocodone	23	11	66‡
diazepam	19	13	0
methadone	18	6	67
topiramate	18	8	0
citalopram	17	8	67
lidocaine	16	6	67
fluoxetine	15	6	0
midazolam	15	8	0
clonazepam	13	7	0

* Alcohol and THC are the top 2 identified drugs in Sacramento County, but were not evaluated in this study.
† Indications of a drug in both blood and urine samples, but not confirmed.
‡ Identified in trace amounts.

Cocaine and Benzoylecgonine

Cocaine was not detected in significant amounts in any of the samples tested. Conversely, benzoylecgonine was identified in all samples analyzed. Quantities of benzoylecgonine were relatively constant. Benzoylecgonine responses are listed in **Figure 6**. The benzoylecgonine XIC and the mass spectra in the high control compared to wastewater sample on 6/30 are listed as an example of the presence of benzoylecgonine in the wastewater in **Figures A1-A4.**

Fentanyl and Norfentanyl

Both fentanyl and its primary metabolite norfentanyl were identified in almost all tested samples. Like benzoylecgonine to cocaine, norfentanyl was identified with higher responses than fentanyl in every sample tested. Relative responses also remained consistent during the sampling period for both drugs. Fentanyl and norfentanyl responses in the high control are listed in **Figure 7**. Fentanyl and norfentanyl XIC and the mass spectra data can be found in **Figures A9-A16**

Methamphetamine

Methamphetamine was also identified in all samples. Unlike with benzoylecgonine and norfentanyl, its metabolite amphetamine was not identified in significant amounts in any samples. Methamphetamine responses steadily decreased over the sampling period like internal standard responses. Methamphetamine responses can be seen in **Figure 8**. Additionally, methamphetamine and phentermine have similar retention times. Furthermore, the presence of both methamphetamine and phentermine in the high control make the mass spectra indistinguishable from each other. However, the absence of phentermine's 133.06 m/z peak in the wastewater spectra suggests the presence of methamphetamine in the wastewater rather than phentermine. The XIC and the mass spectra for methamphetamine/phentermine, and amphetamine can be found in **Figures A17-A22.**

Gabapentin

Gabapentin was identified in all samples tested. However, no major metabolites were identified. Gabapentin responses remained mostly constant during the sampling interval and did not experience the same steady decline in drug response over time that methamphetamine did. Gabapentin responses can be found in **Figure 9**. The gabapentin XIC and the mass spectra in the

high control compared to the wastewater sample on 6/16 are listed as an example of the presence of gabapentin in wastewater in **Figures A25-A28.**

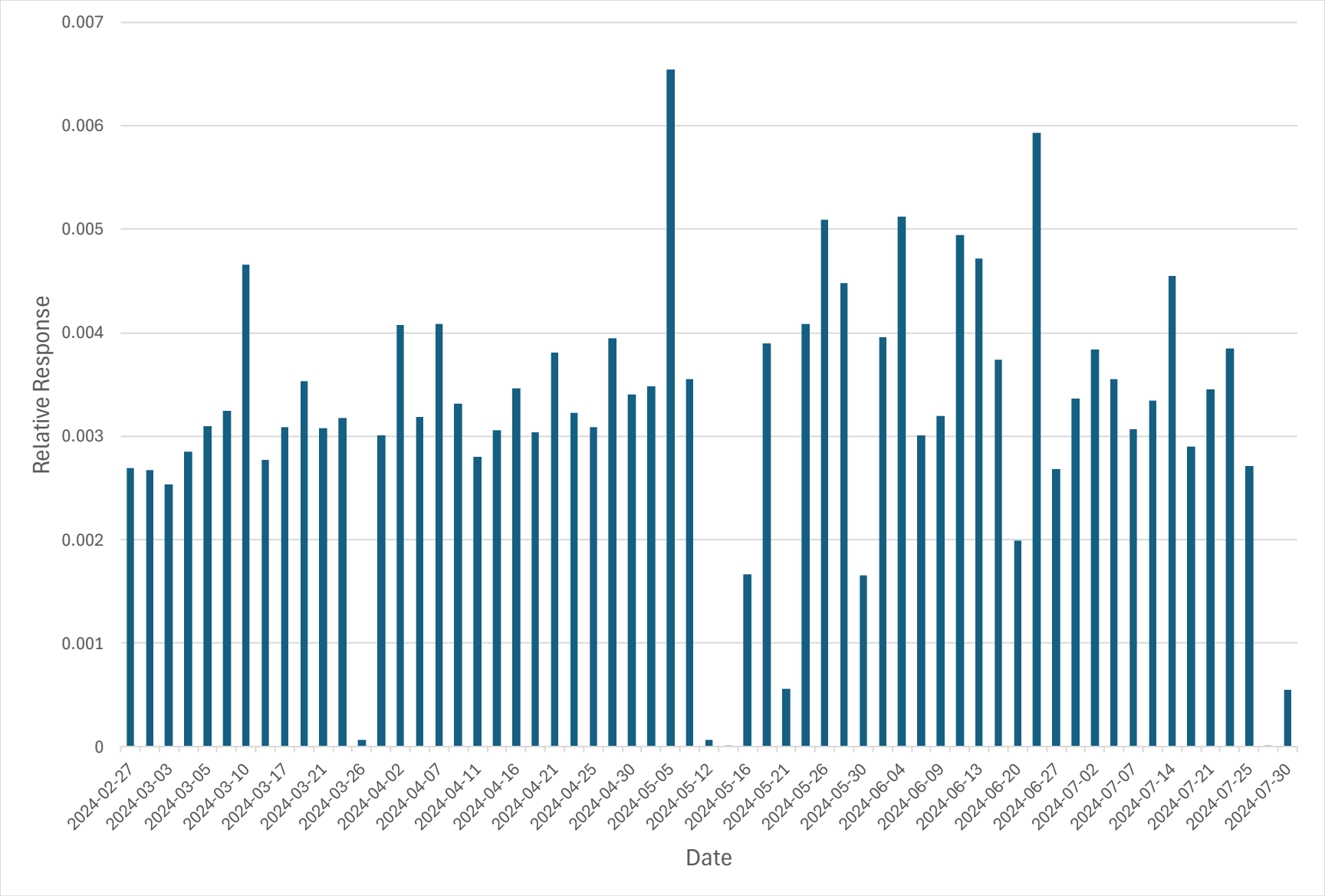

Figure 6. Benzoylecgonine relative response over time.

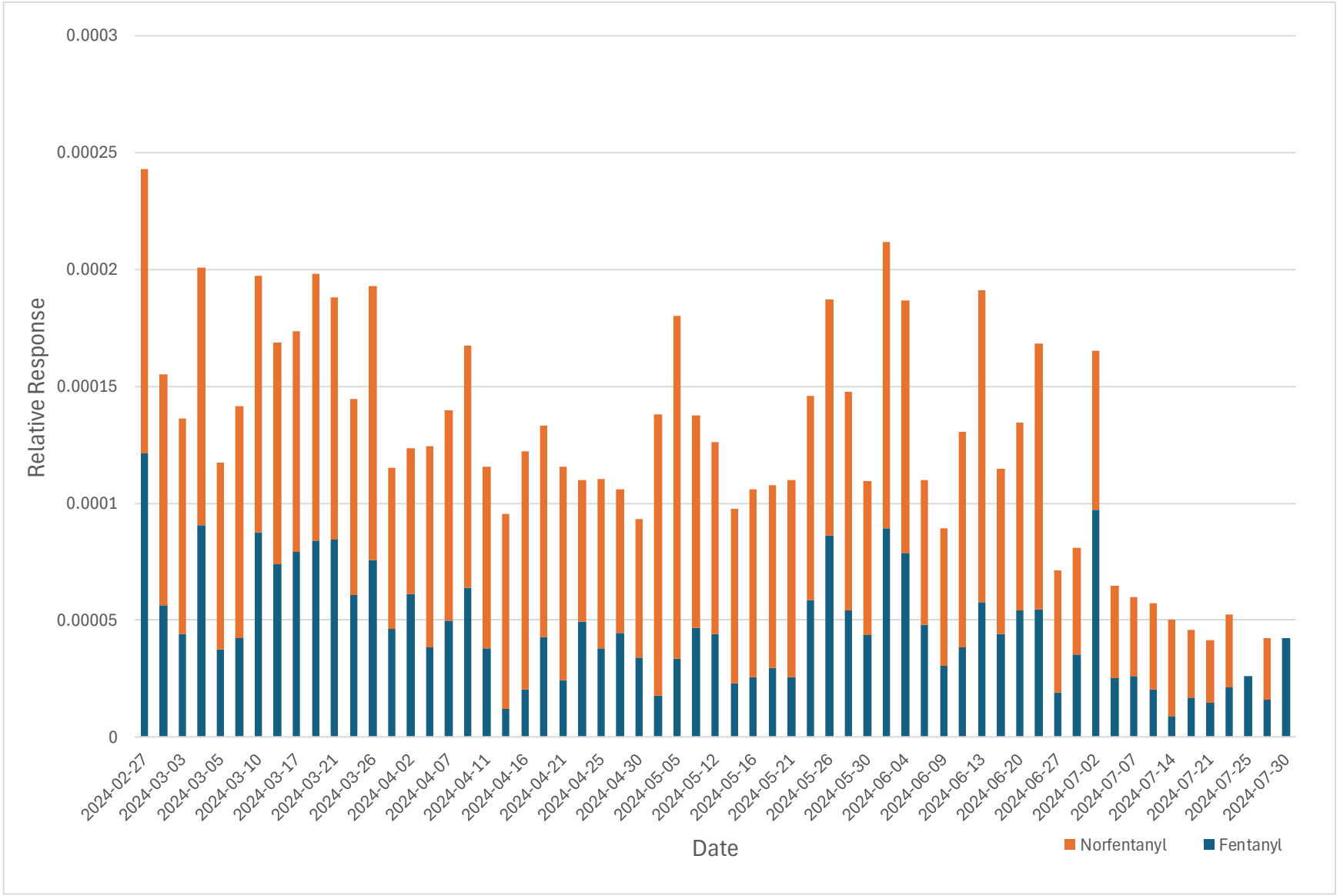

Figure 7. Fentanyl and norfentanyl relative responses over time.

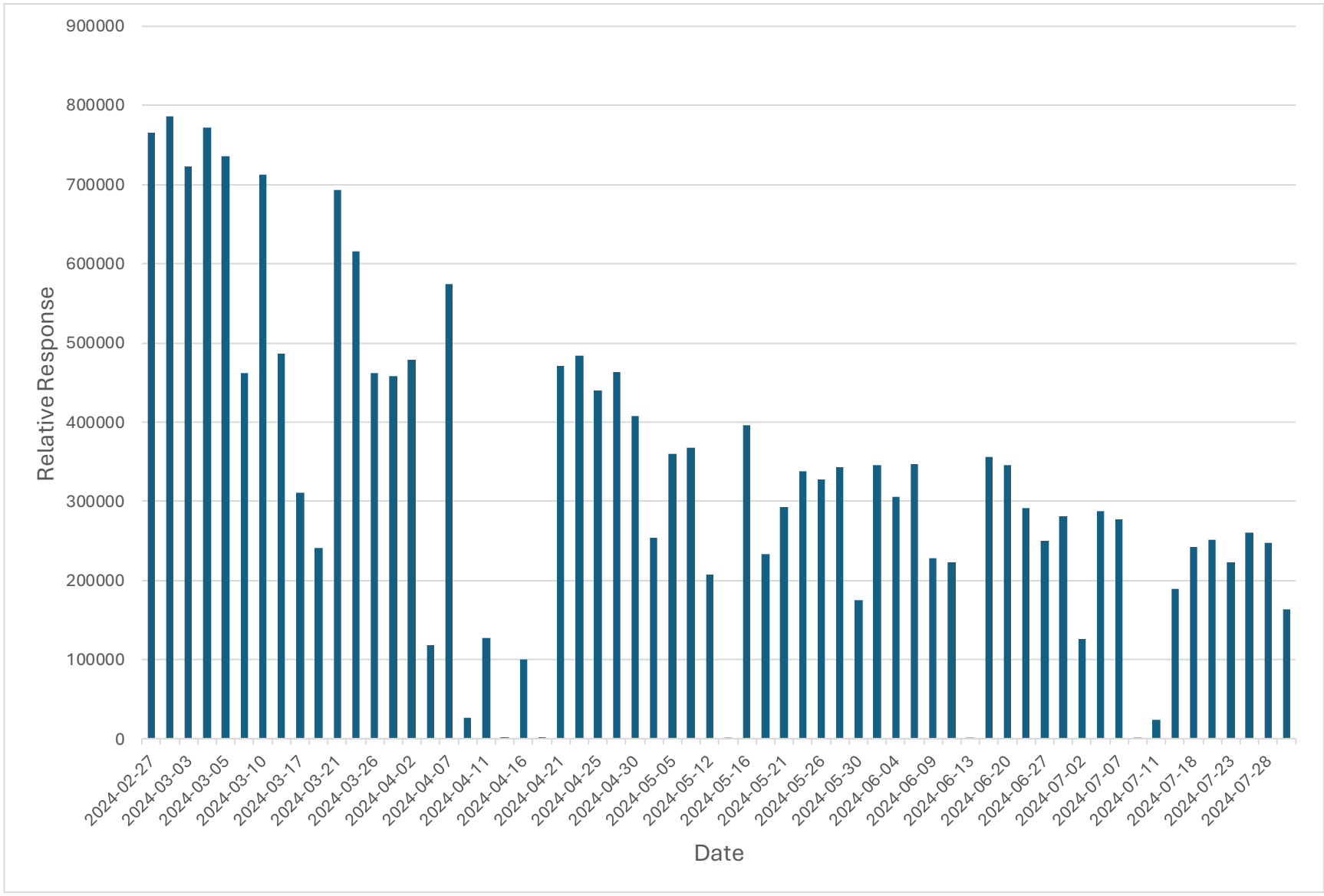

Figure 8. Methamphetamine relative response over time.

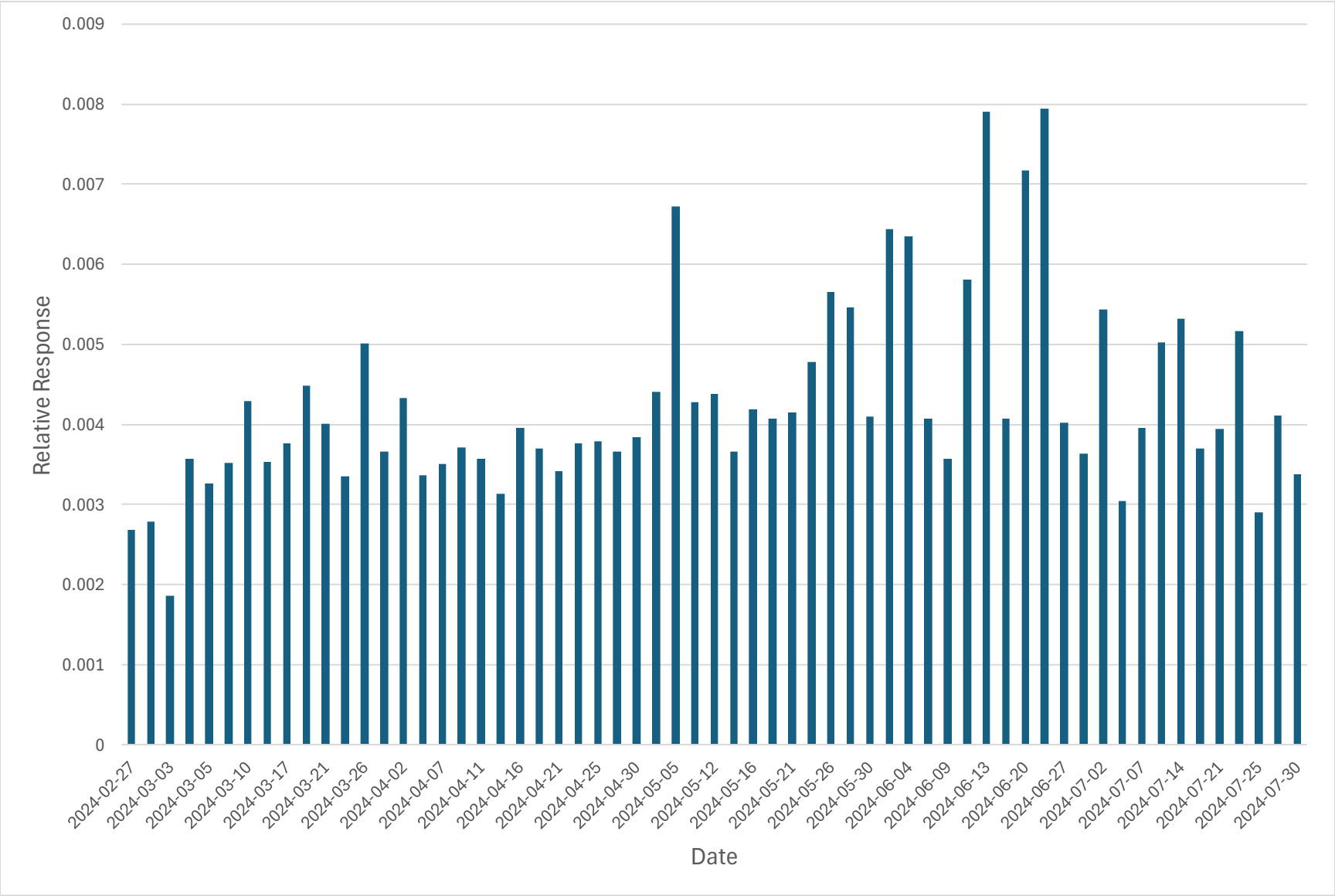

Figure 9. Gabapentin relative response over time.

Novel and Emerging Drugs in Sacramento County:

In addition to evaluating common drugs of abuse, NPS data from CFSRE was used to target specific NPS that may be present in the wastewater of Sacramento County. Quarterly, CFSRE releases a report with emerging NPS in the United States based on toxicological and seized drug data. The specific NPS evaluated in this study are listed in **Table 6**. These drugs were chosen as specific targets due to their known prevalence in the United States during the sampling period. Sample data was also reprocessed against the Waters Suspect Screen library which contains over 1,700 prescription, emerging, and commonly abused drugs. Similar to the in-house library used for initial data acquisition, analytes were identified by retention time, mass error, and measured expected fragments.

Table 6. NPS evaluated based on CFSRE emerging NPS reports during sampling period, and the number of potential sample identifications for each NPS.

NPS	Drug Class
bromazolam	Benzodiazepine
carfentanil	Opioid
metonitazene	Opioid
protonitazene	Opioid
pentylone	Stimulant
N, N-dimethylpentylone	Stimulant

All six targeted NPS were either not identified or identified in trace quantities by the Waters data analysis software. A drug was deemed at a trace level if the data analysis software identified that specific drug, but further investigation showed either: poor chromatographic resolution, low response, large mass error (>10 ppm), or a lack of expected fragment ions. However, all six NPS evaluated were successfully identified in the high and low controls with proper retention time, mass error and expected fragments. Bromazolam, protonitazene, and N, N-dimethylpentylone were not identified in any of the samples tested. Carfentanil, metonitazene,

and pentylone were identified in trace amounts in multiple samples, however, further investigation into mass spectral data did not provide significant confidence in a positive identification due to large mass error and a lack of identified fragments. The XIC and mass spectral data for all six identified NPS in the high control are found in **Appendix B.**

Discussion

Entrenched Illicit Drugs

Wastewater is a viable matrix for the identification of drugs [12-16]. This study shows the ability of this method to identify drug trends at the local level and thus can reasonably be applied to NPS monitoring at the community level. The use of certified reference materials in the TOF standard, added to the positive controls, provides confidence that the listed spectra in the high control can be compared to the spectra in the wastewater samples. The XIC and mass spectra comparative analysis for benzoylecgonine, fentanyl, norfentanyl, methamphetamine, and gabapentin confirm their presence in the wastewater. Furthermore, the detection of these drugs align with known toxicological data in Sacramento County and suggests widespread use in the community. The correlation between wastewater and toxicological data verifies the reliability of the method and its local relevance. Moreover, it reaffirms this method's potential for detecting NPS in the community.

Toxicology Unit Workflow

The Sacramento County District Attorney's Crime Laboratory receives blood and urine toxicological specimens with offenses such as driving under the influence (DUI) and sexual assault. The lab also analyzes post-mortem blood and tissues samples provided by the Sacramento County Coroner's Office. Most samples received are analyzed for alcohol, then screened by LC-TOF/MS to identify any drugs that may be in the sample. Identified drugs are then confirmed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Due to restraints such as sample volume and current methodologies of the lab, not all drugs identified during TOF screening are confirmed. For example, in 2024, caffeine was identified by LC-TOF/MS screening in 2,285 case samples but was only confirmed 216 times. This is because the

presence of caffeine provides little investigative information about impairment for an offense such as DUI. Additionally, gabapentin was identified by TOF screening 68 times but not confirmed in any samples. This is because the laboratory has not yet developed a method for gabapentin confirmation. Despite variations in toxicological confirmation data the results in **Table 5**, suggest that benzoylecgonine (cocaine), fentanyl, and methamphetamine have widespread use in the community.

Prevalence of Benzoylecgonine vs. Cocaine

Cocaine is a potent central nervous system (CNS) stimulant administered by smoking, insufflation, and intravenous injection [20]. It was identified at a higher rate in the toxicology samples (51 cases) during the sampling period yet was minimally detectable in the wastewater. This is likely because cocaine's presence in the blood is relatively short lived ($t_{1/2} = 0.8 \pm 0.2$ hrs) as it is rapidly converted to its primary urinary metabolite benzoylecgonine ($t_{1/2} = 6$ hrs) [20]. Conversely, benzoylecgonine was identified in all wastewater samples. The rapid metabolism of cocaine to benzoylecgonine likely accounts for the lack of unconverted cocaine detected in the wastewater. Lastly, benzoylecgonine is a metabolite unique to cocaine therefore it can be inferred that the high wastewater prevalence of benzoylecgonine is from illicitly consumed cocaine that was metabolized and then excreted into the wastewater.

Fentanyl vs. Norfentanyl

Unlike cocaine, which is rapidly metabolized, unmetabolized fentanyl was detected in all analyzed samples. Fentanyl is a synthetic opioid that has become increasingly prevalent in the United States since its introduction into the illicit market beginning around 2017. During the COVID-19 pandemic fentanyl use greatly increased, and in 2021 accounted for 60% of all seized narcotic analgesics [21]. Fentanyl is metabolized into a variety of metabolites, but its

predominant metabolite, norfentanyl, was also detected in all samples tested. Fentanyl was also detected in the toxicological samples at a high rate (51 cases). The Sacramento County District Attorney's Crime Laboratory does not currently confirm norfentanyl; however, it was tentatively identified in 64 toxicology cases during the sampling period. The presence of norfentanyl in higher amounts further reflects that excreted metabolites are also detectable in higher amounts. The presence of both fentanyl and its metabolite also align with national trends of fentanyl abuse.

Methamphetamine, Amphetamine, and Phentermine

Methamphetamine, like cocaine, is a CNS stimulant that can be snorted, smoked, or injected intravenously. It is metabolized in the body and converted into active metabolite amphetamine. Methamphetamine has legitimate clinical use for the treatment of narcolepsy and attention deficit hyperactivity disorder (ADHD), but due to its high potential for abuse, it is infrequently prescribed [20]. Most methamphetamine is consumed illicitly to increase alertness and provide feelings of euphoria [22]. Like methamphetamine, amphetamine can also be prescribed for the treatment of ADHD (Adderall) [19]. Methamphetamine was identified in all wastewater samples, yet amphetamine was not identified in any samples. This diverges from identified amphetamine in the toxicology data (74 cases). This may be because only 6-7% of smoked or injected methamphetamine is excreted in the urine as amphetamine [23, 24]. Likely, amphetamine recoveries in the wastewater were not detectable because of their low abundance in the urine when excreted. Additionally, amphetamine is not a unique metabolite of methamphetamine and cannot be used as a conclusive indicator of methamphetamine. Considering this, the best indicator of methamphetamine use is the trend established in the wastewater from unconverted methamphetamine.

The identification of methamphetamine, however, is complicated by its structural isomer phentermine. Both analytes have a protonated mass of 150.12 m/z and were identified in the XIC at 2.58 minutes in the high control. However, due to small differences in their structures, they can be differentiated by their mass spectral fragmentation patterns. Methamphetamine and phentermine share an expected fragment at 91.05 m/z, but a fragment at 133.10 m/z is far more prominent in phentermine. This fragment is likely due to the loss of one of phentermine's methyl groups or a primary amine. In the high control the methamphetamine and phentermine spectra appear identical because both analytes are in the standard and were identified at the same retention time. Conversely, when analyzing the spectra for the wastewater samples the 133.10 m/z phentermine peak is absent, and both methamphetamine fragments are present (91.05, 119.08 m/z). Given the unique spectra in the wastewater samples and known trends of methamphetamine use in Sacramento County, it is reasonably assumed that the methamphetamine/phentermine identifications in the wastewater are due to methamphetamine use.

Widespread use of Gabapentin

Gabapentin is a prescription medication often prescribed as an oral dose for the treatment of epileptic disorders, chronic pain, and alcohol use disorder [20]. However, illicitly, it is self-administered to reach euphoric highs [25-27]. Recently, abuse of gabapentin has risen with increased prescription rates [27]. The presence of gabapentin in high amounts suggests high use in Sacramento County. Like methamphetamine, no metabolites of gabapentin were detected. It is excreted in the urine virtually unchanged by metabolism (76 to 81% unmetabolized) [20, 26]. This metabolic profile likely accounts for the large amount of parent drug identified in the wastewater. Due to the lack of metabolite detected it is difficult to assess how gabapentin is

being introduced into the wastewater supply. It is possible that gabapentin is introduced into the sewer system from dumping of unused prescription medications. However, the presence of gabapentin was consistent over the sampling period. This consistency suggests that the gabapentin detected is likely from a community of users excreting consumed drugs over time. Further study of gabapentin's metabolites in the wastewater needs to be conducted to better evaluate how gabapentin is being introduced into the wastewater supply.

Lack of Benzodiazepines

Benzodiazepines are a widely prescribed class of CNS depressants used for the treatment of anxiety, insomnia, epilepsy, or alcohol withdrawal [28]. Based on the toxicological data from the laboratory, benzodiazepine use was expected to be widely detected in the wastewater. However, no benzodiazepines were identified in significant quantities in any of the wastewater samples. This discrepancy could be due to how benzodiazepines are excreted in the urine. Benzodiazepines are metabolized in the liver then glucuronidated and excreted by the kidneys [29]. For example, diazepam is metabolized in the liver to temazepam and nordiazepam then oxazepam, all are then glucuronidated by the kidneys and excreted [30]. This method sought to identify parent benzodiazepines and non-glucuronidated metabolite forms. Given the metabolism of benzodiazepines, there is likely minimal parent drug reaching the wastewater. Further investigation into the glucuronidated forms of these drugs was beyond the scope of this research.

Variations in Data

Both benzoylecgonine and methamphetamine recoveries were less consistent across the sampling period compared to other drugs. These drugs may be more susceptible to varied matrix conditions compared to drugs like gabapentin and norfentanyl. Additionally, drugs such as methamphetamine and benzoylecgonine are more stable in their hydrochloride salt form. This

was why the HCl keeper solution was added to every sample. Inter-sample variability may suppress how easily methamphetamine and benzoylecgonine convert to their HCl salt forms and may account for poor recoveries on some testing dates. On the dates where methamphetamine or benzoylecgonine recoveries were low there was not a correspondingly low internal standard recovery, suggesting that the missing drug response is due to the chemistry of the drug rather than global matrix interference that reduces all analyte recoveries.

In addition to inter-sample variations, internal standard recoveries steadily declined over time. The final extraction and analysis were performed in three batches which consisted of all sample dates. One batch of samples was tested each day for three days (in order of date collected). One hypothesis for this gradual decline in internal standard recovery is that variations in the chemistry of wastewater over time caused this decline. Due to the variable nature of wastewater flowing into the wastewater treatment plant, it is possible that as winter and spring rain ends the composition of influent flowing into the plant becomes more organic. This may result in a more pronounced matrix effect over time. An additional reason for this trend may be attributed to a decline in detector performance as samples were analyzed. It is unclear what may cause this decline in sensitivity across an analysis, but temperature fluctuation, a buildup of charge around the detector, and system cleanliness deteriorating across a sample run may affect detector performance.

To test this theory the instrument was recalibrated after all three batches were analyzed. After recalibration, a small set of previously analyzed samples were reinjected to determine if internal standard responses would increase. Recalibration greatly increased internal standard responses despite reinjecting old extracts. To further test this, all previously injected samples were reinjected post calibration in reverse date order with standards bracketing the wastewater

samples. Despite the reverse in order, a similar drift in the internal standard recoveries occurred proving that internal standard recoveries decreased due to instrument performance rather than sample conditions.

NPS Evaluation

There were six NPS specifically evaluated in this study. In addition to identifying drug trends in Sacramento County, this study sought to identify any NPS in the community not currently seen in casework at the Sacramento County District Attorney's Crime Laboratory. Of the six NPS specifically evaluated all were either identified in trace amounts or not identified. However, all six NPS were successfully identified in the control samples demonstrating the ability of this method to identify these drugs in the wastewater if present in significant quantities. Considering this, the absence of NPS in the wastewater suggests either the NPS evaluated were not present in the wastewater during sampling or the NPS were not present in detectable amounts.

This potential detection gap may be influenced by broader drug trends. Drug use across the country is highly variable and depends on several factors including time of year, accessibility, social norms, and community accessibility to treatment services [3]. These factors highlight that while certain NPS may be common in one part of the nation it does not mean that all communities will experience the same drug trends. This variability is why a local monitoring system is critical for early NPS identification. Conversely, if NPS are present, but not yet detectable by this method, it underscores the need to consistently monitor these drugs for increases over time. Despite the lack of NPS detection in the wastewater samples, all six NPS evaluated were detectable in both the high and low controls. This confirms this method as a robust approach to identifying NPS in the wastewater at sufficiently high concentrations. To

consistently monitor NPS, however, more research needs to be performed to determine their limits of detection.

Lack of Bromazolam

Bromazolam is a synthetic benzodiazepine with increased CNS depressant properties compared to conventional benzodiazepines [32]. Bromazolam was originally synthesized 40 years ago but was never approved for medical use [33]. Despite this, recently it has gained popularity illicitly. One recent study in San Francisco reported 44 bromazolam related deaths in the year 2023. This study also found that bromazolam use was often combined with opioids such as fentanyl and stimulants such as methamphetamine [32]. Due to the proximity of San Francisco and Sacramento County it is possible that drug use trends may be similar due to geographical proximity. Bromazolam is not currently confirmed by the Sacramento County District Attorney's Crime Laboratory; however, indications of bromazolam were found in five DUI related cases in 2024. Due to this lack of confirmation data, bromazolam use is likely under reported in the Sacramento County. The number of deaths in a nearby community paired with indications of bromazolam use reflect the public health risk this drug presents.

Despite toxicological data in Sacramento and a nearby community, bromazolam was not detected in the wastewater. Like other benzodiazepines, bromazolam is glucuronidated during metabolism [33]. This likely accounts for the lack of identification of bromazolam in the wastewater samples as the glucuronidated form of bromazolam was not tested. As was previously stated, all NPS in the positive controls were identified in the analysis reflecting this methods ability to detect bromazolam in the wastewater. However, to properly evaluate the presence of bromazolam the glucuronidated form should be added to the stock standard and analysis library. Conversely, a hydrolysis step could be added to cleave the glucuronidated

metabolite and convert bromazolam back to its unmetabolized form. This further shows a limitation in this method for the identification of benzodiazepines.

Trace Levels of Carfentanil

One of the NPS that was identified in multiple samples was carfentanil. However, all carfentanil identifications were deemed false, due to large mass error, lack of diagnostic fragments identified, and low response. Carfentanil was originally developed to tranquilize large animals such as elephants and other large mammals but recently has entered the illicit NPS market with increased prevalence [34]. It is a synthetic opioid that is approximately 100 times more potent than fentanyl [35]. The high potency of carfentanil presents an analytical challenge because less drug is required to reach an intended effect. Thus, the detection of carfentanil, even in trace amounts, is potentially significant. Despite the lack of carfentanil in the wastewater, the laboratory has intermittently detected carfentanil in solid dose formulations. Recently, the laboratories Controlled Substances Unit has identified indications of carfentanil mixed with other fentanyl samples. Tentative identifications have steadily increased, but like the results of the wastewater samples, low concentrations of carfentanil make it difficult to confirm in such samples. Despite this, carfentanil was not identified in any toxicology samples in 2024. Given the known presence of carfentanil in Sacramento County, the ability of this method to detect carfentanil provides an incredibly useful tool for local agencies to better adapt to the constantly evolving drug landscape.

Limitations

One limitation of this study in identifying NPS is the dilute nature of the sample received. Identifying trace amounts of a drug becomes increasingly difficult the more dilute a sample is. This holds especially true for NPS because consumption of NPS has not reached levels

comparable to classic illicit drugs such as methamphetamine [16]. This paired with limited volume for this study is likely a large contributor to why some NPS were not identified or were identified in trace amounts. For drugs such as methamphetamine, use is widespread and deeply ingrained in the population, so it's abundance in the wastewater is expected. For drugs that are not yet widespread it is difficult to identify meaningful quantities. Considering this, there is still an inherent benefit to consistent monitoring for these drugs because as a novel drug becomes more widespread its presence in the wastewater will increase. Any variation in drug trends in the wastewater is still useful to local agencies because it dynamically reflects drug use in a population. This data allows for earlier action to mitigate health risks from new drugs.

Conclusion

The aims of this study were to evaluate sewage wastewater for illicit drugs, to determine the feasibility of LC-TOF/MS wastewater analysis for the identification of NPS at the local level and assess trends in drug use. The method proved to be effective at confirming known trends in drugs of abuse such as cocaine, methamphetamine, and fentanyl. It also successfully identified a significant trend in gabapentin use in the local community that is present in the toxicological screening data but not yet being confirmed in toxicological casework in Sacramento County. Though this method showed its usefulness in confirming drugs already deeply rooted in the community it did not successful identify emerging NPS in the wastewater samples collected. Notably, the method successfully identified reference NPS in the control samples, despite the absence of NPS in the wastewater. This is likely because NPS use in the community is still a relatively small percentage of the total drug load in the wastewater resulting in low, or undetectable NPS levels by this method. However, should NPS use increase, their concentrations in wastewater will increase and become detectable by this method allowing evaluation of trends of use. This presents a significant surveillance tool for local agencies in the constant battle to keep pace with highly dynamic drug use trends.

Further study of the method needs to be conducted to address the lack of benzodiazepines identified. Due to the metabolism of these drugs by glucuronidation, identification of benzodiazepines was negligible. To better assess the presence of these drugs a method needs to be developed to either identify benzodiazepines in their metabolite form, or a hydrolysis step added to cleave the glucuronide formed by metabolism. This may help to identify designer benzodiazepines such as bromazolam or future iterations of the drug. Moreover, to improve the method, a larger sample volume should be collected and extracted. In doing so, low or currently

non-detectable drug levels may be found. It is likely some trace level drugs and NPS were undetectable using the small sample volumes available for this study. However, despite these limitations this provides a useful tool for local health agencies, police departments, and crime laboratories as a local monitoring system. It provides a non-invasive real time summary of drug trends at the local level which can provide interested groups with information to adjust to the changing drug landscape.

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Appendix A

Benzoylecgonine

Figure A1. Benzoylecgonine XIC in high control.

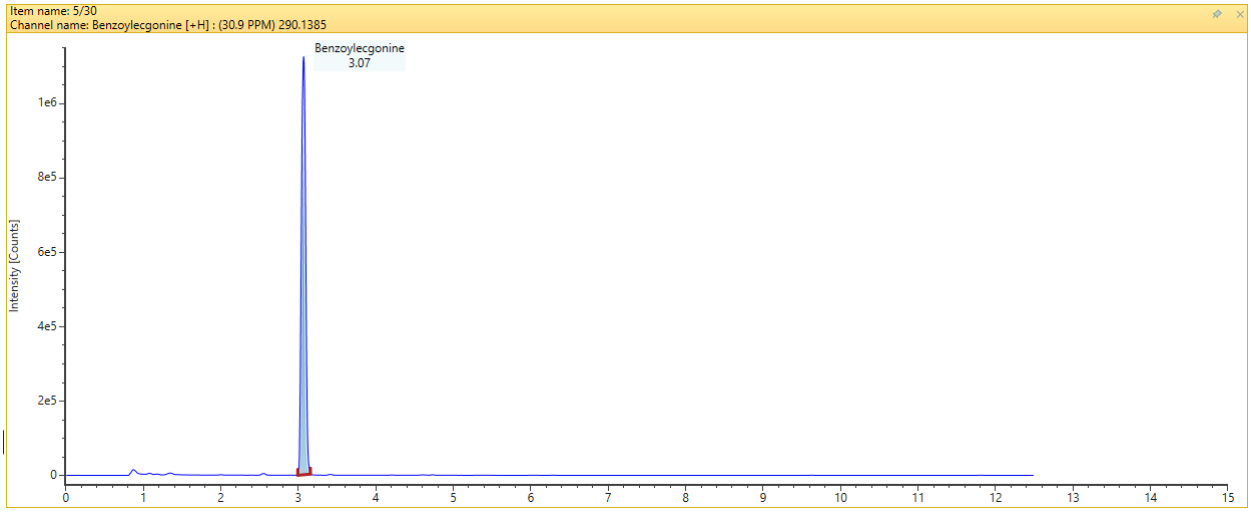

Figure A2. Benzoylecgonine XIC in 5/30 wastewater sample.

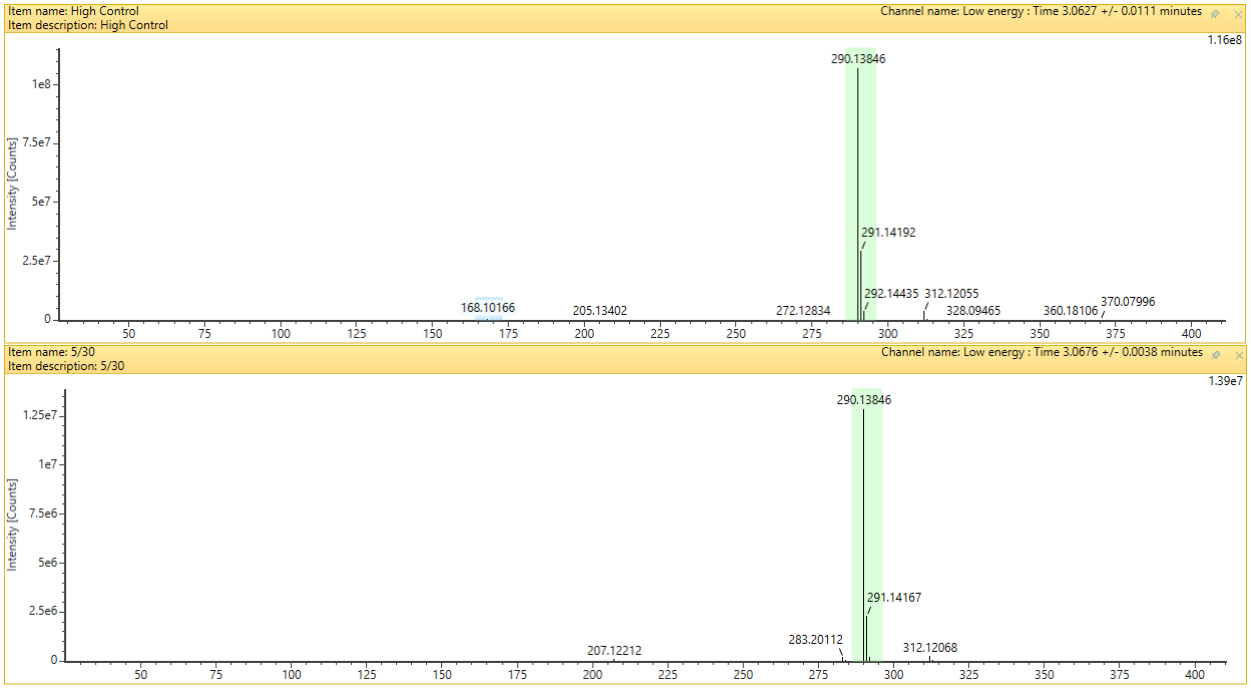

Figure A3. Low energy mass spectra for high control and sample 5/30 at approximately 3.06 minutes.

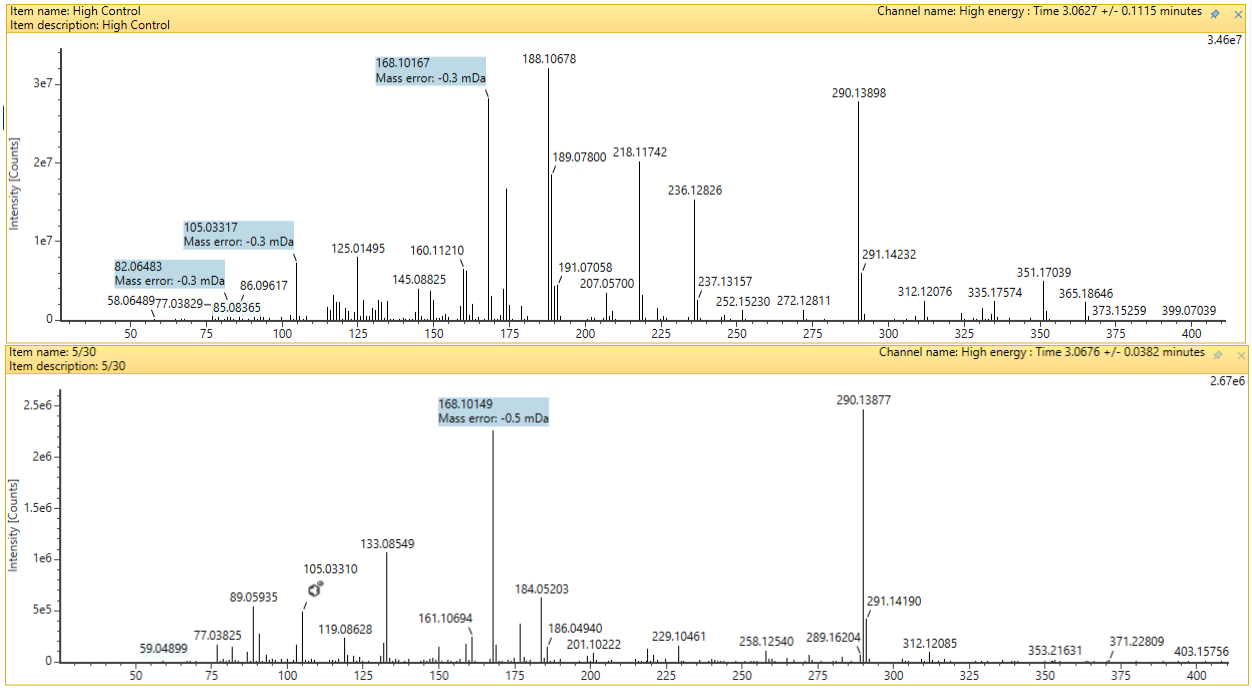

Figure A4. High energy mass spectra for high control and sample 5/30 at approximately 3.06 minutes.

Cocaine

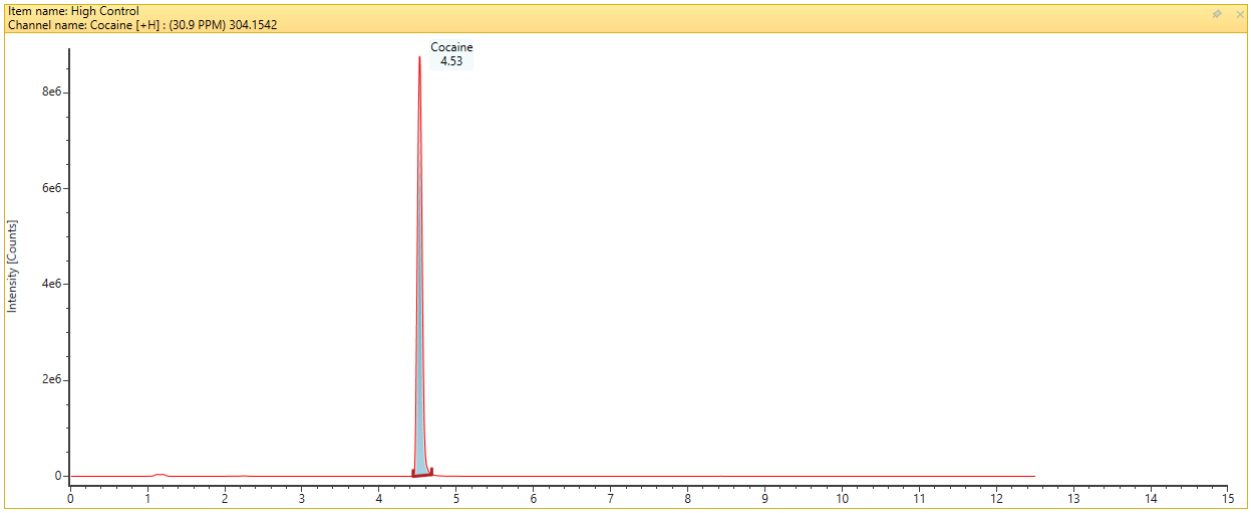

Figure A5. Cocaine XIC in high control.

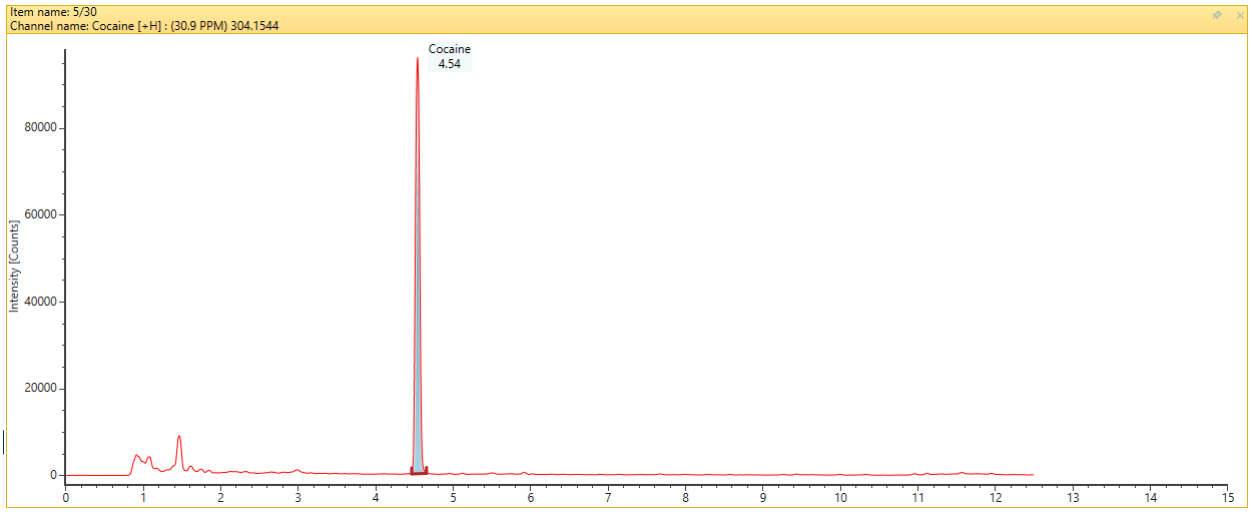

Figure A6. Cocaine XIC in 5/30 wastewater sample.

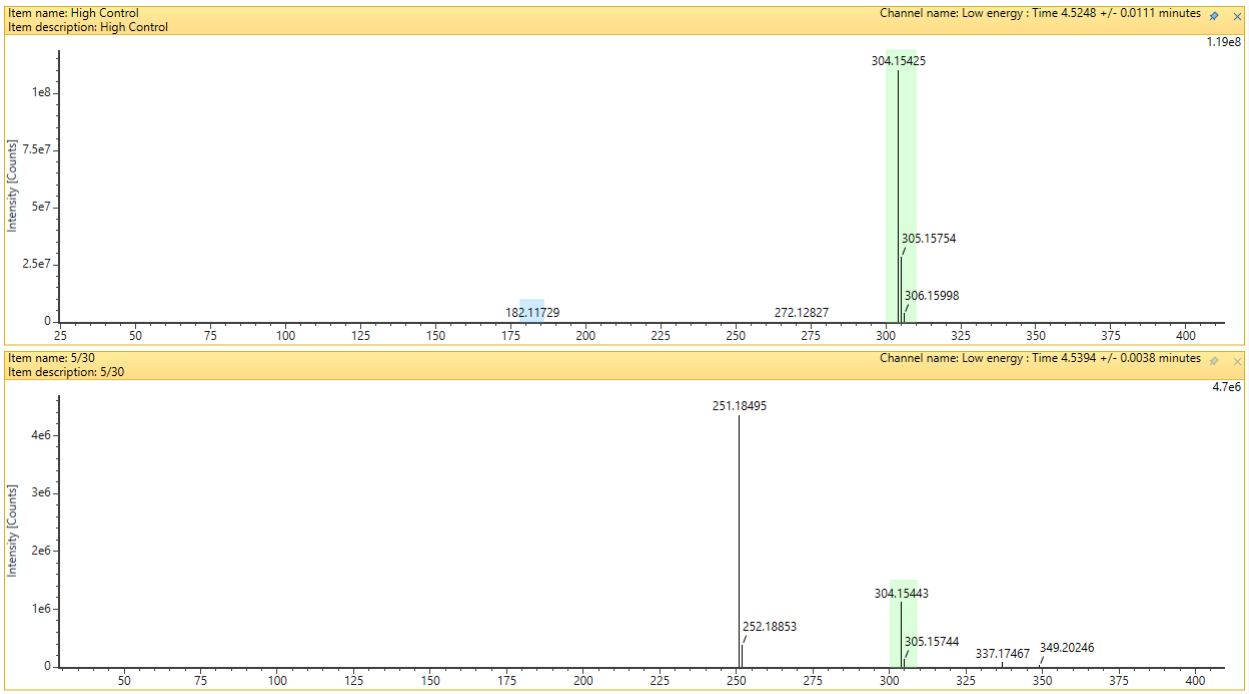

Figure A7. Low energy mass spectra for high control and sample 5/30 at approximately 4.52 minutes.

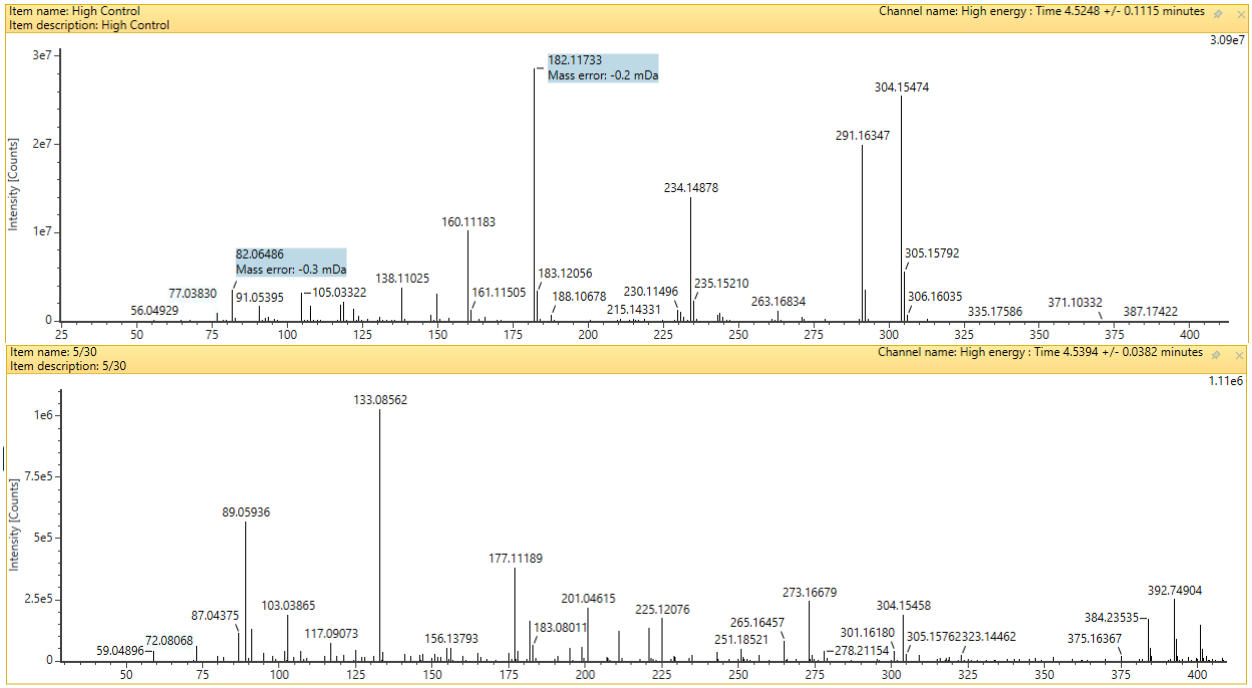

Figure A8. High energy mass spectra for high control and sample 5/30 at approximately 4.52 minutes.

Fentanyl

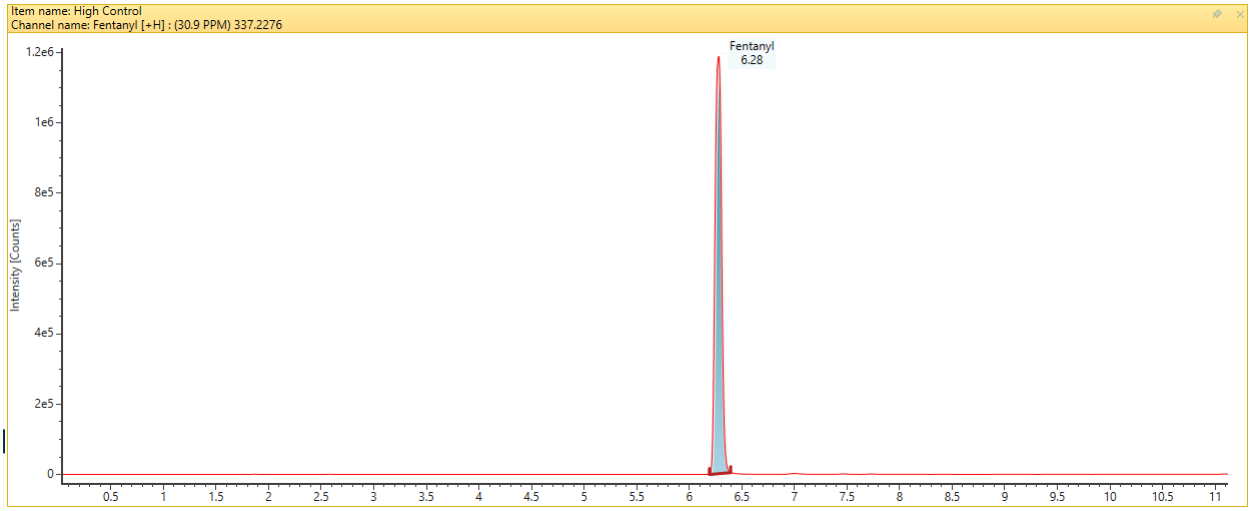

Figure A9. Fentanyl XIC in high control.

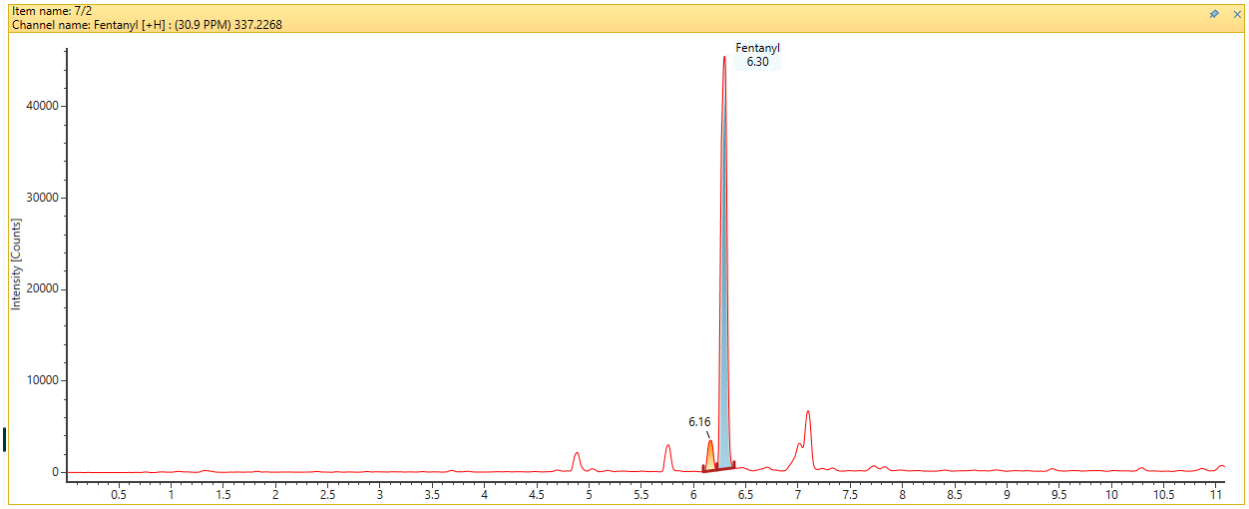

Figure A10. Fentanyl XIC in 7/2 wastewater sample.

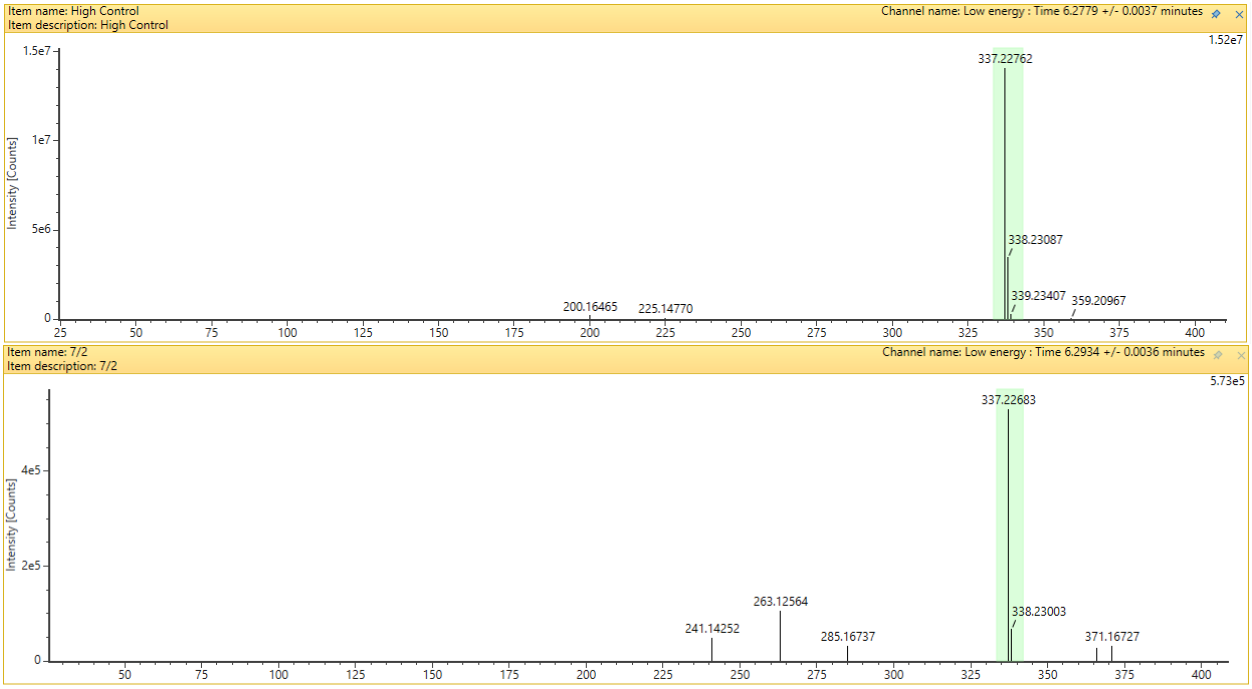

Figure A11. Low energy mass spectra for high control and sample 7/2 at approximately 6.27 minutes.

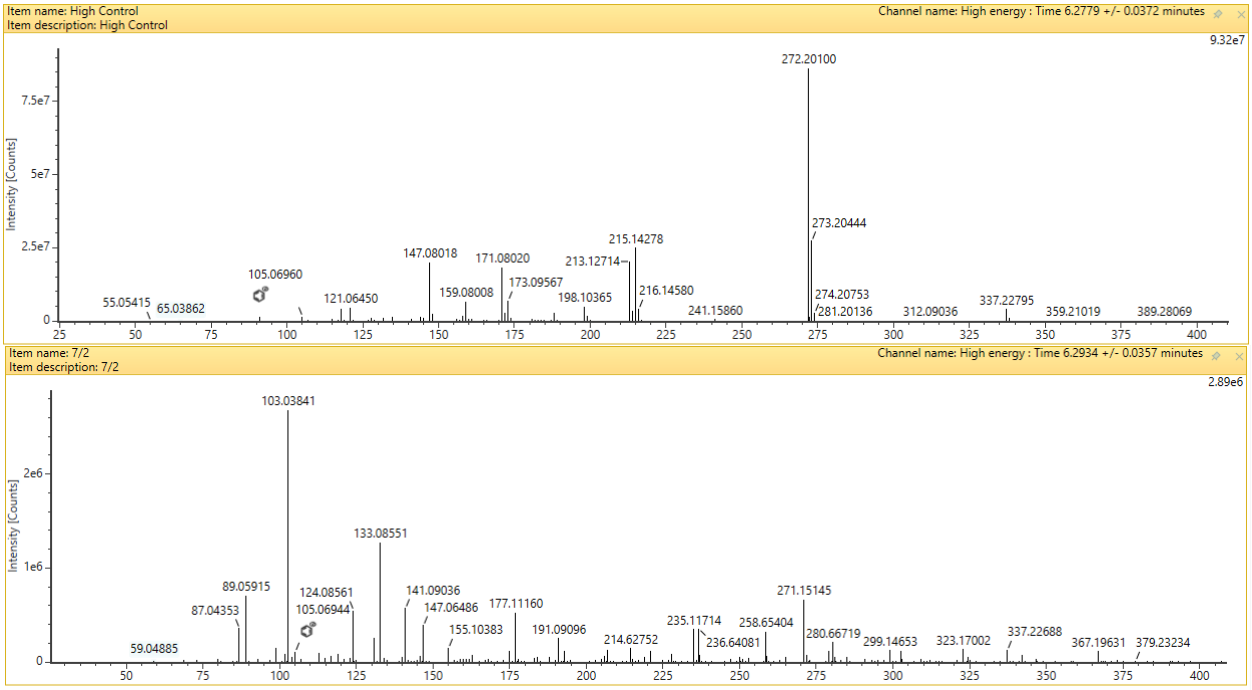

Figure A12. High energy mass spectra for high control and sample 7/2 at approximately 6.27 minutes.

Norfentanyl

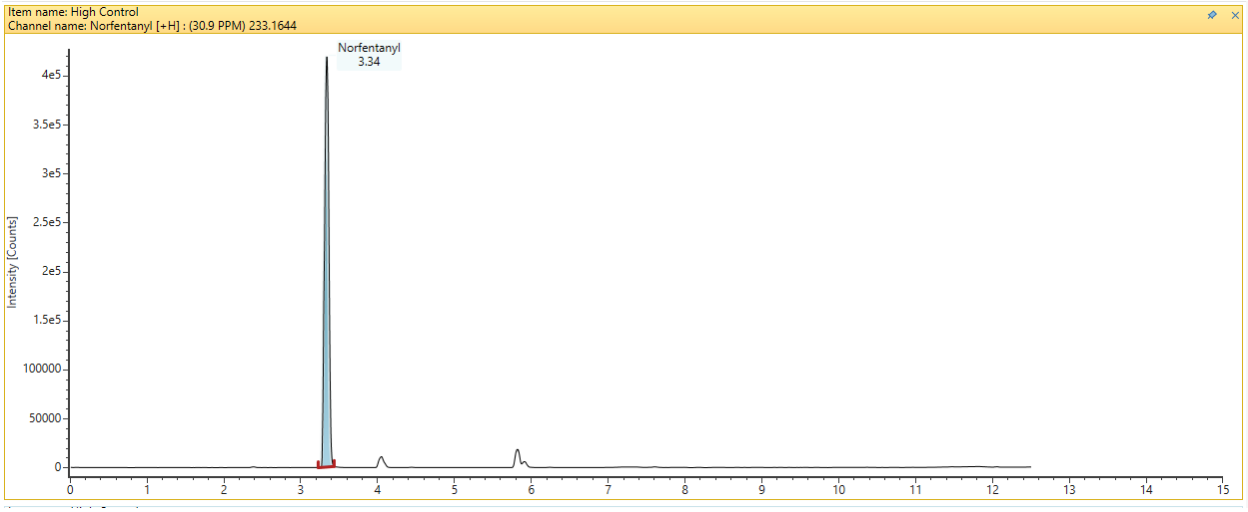

Figure A13. Norfentanyl XIC in high control.

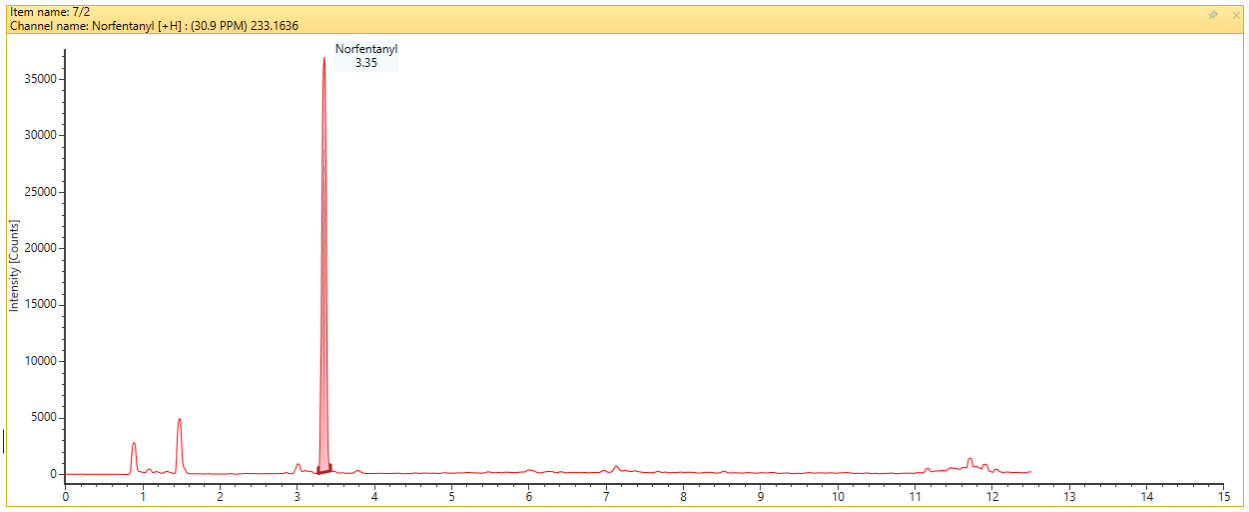

Figure A14. Norfentanyl XIC in 7/2 wastewater sample.

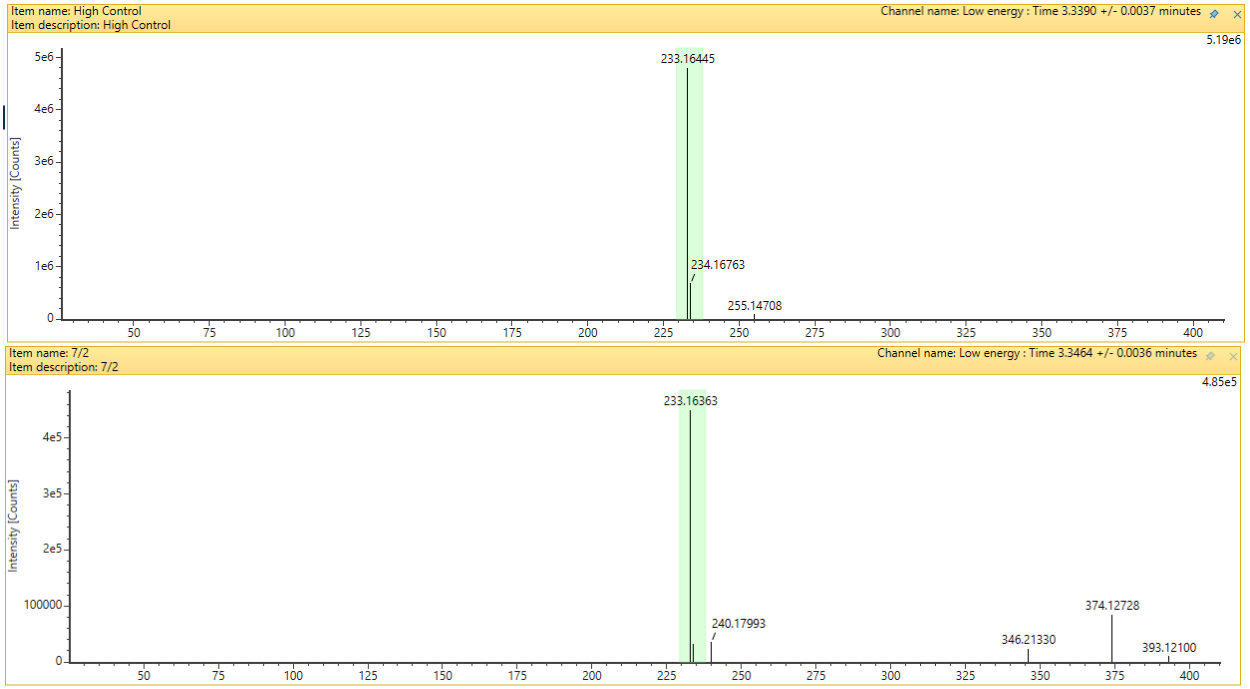

Figure A15. Low energy mass spectra for high control and sample 7/2 at approximately 3.34 minutes.

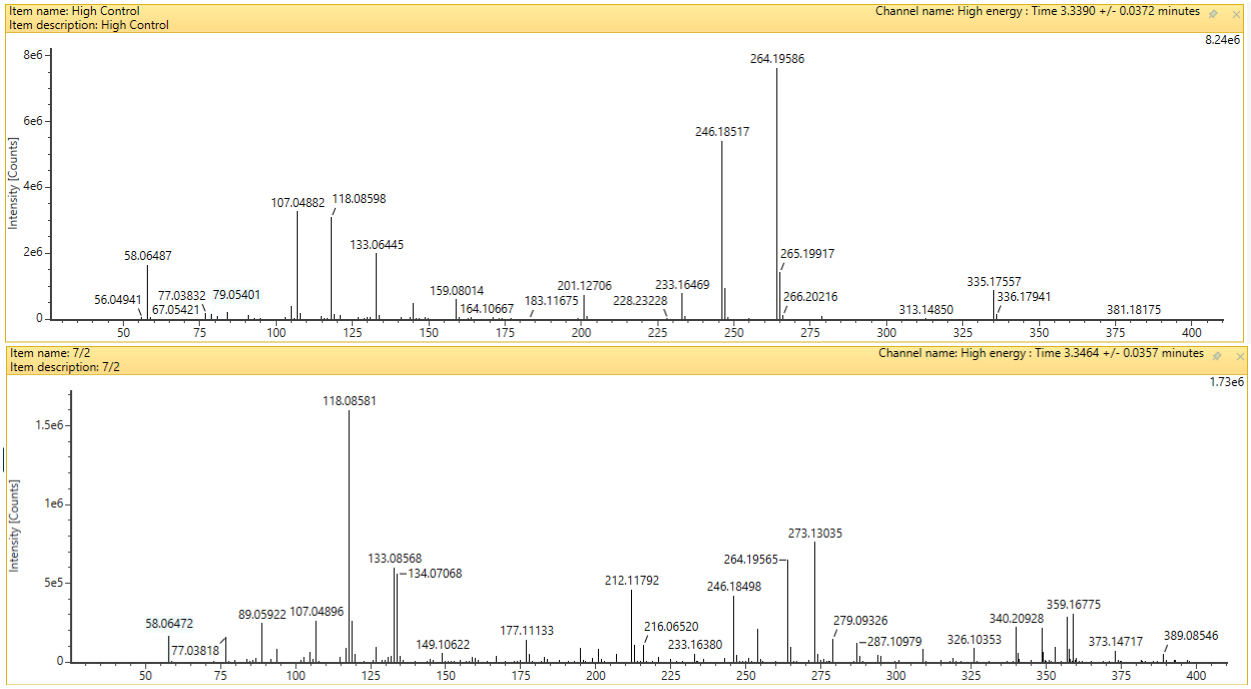

Figure A16. High energy mass spectra for high control and sample 7/2 at approximately 3.34 minutes.

Methamphetamine/Phentermine

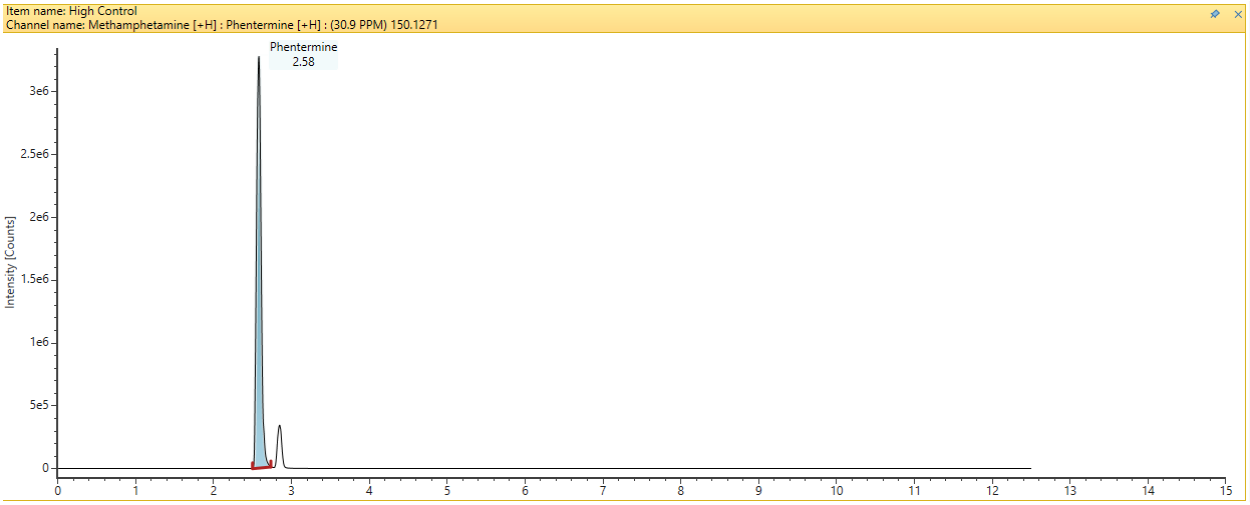

Figure A17. Methamphetamine/Phentermine XIC in high control.

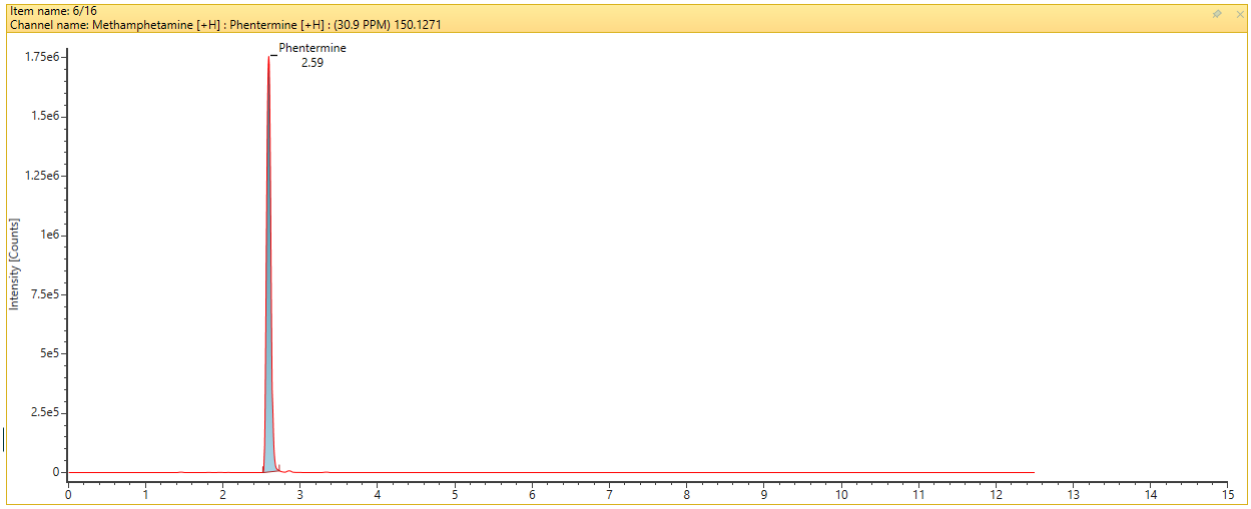

Figure A18. Methamphetamine/Phentermine XIC in 6/16 wastewater sample.

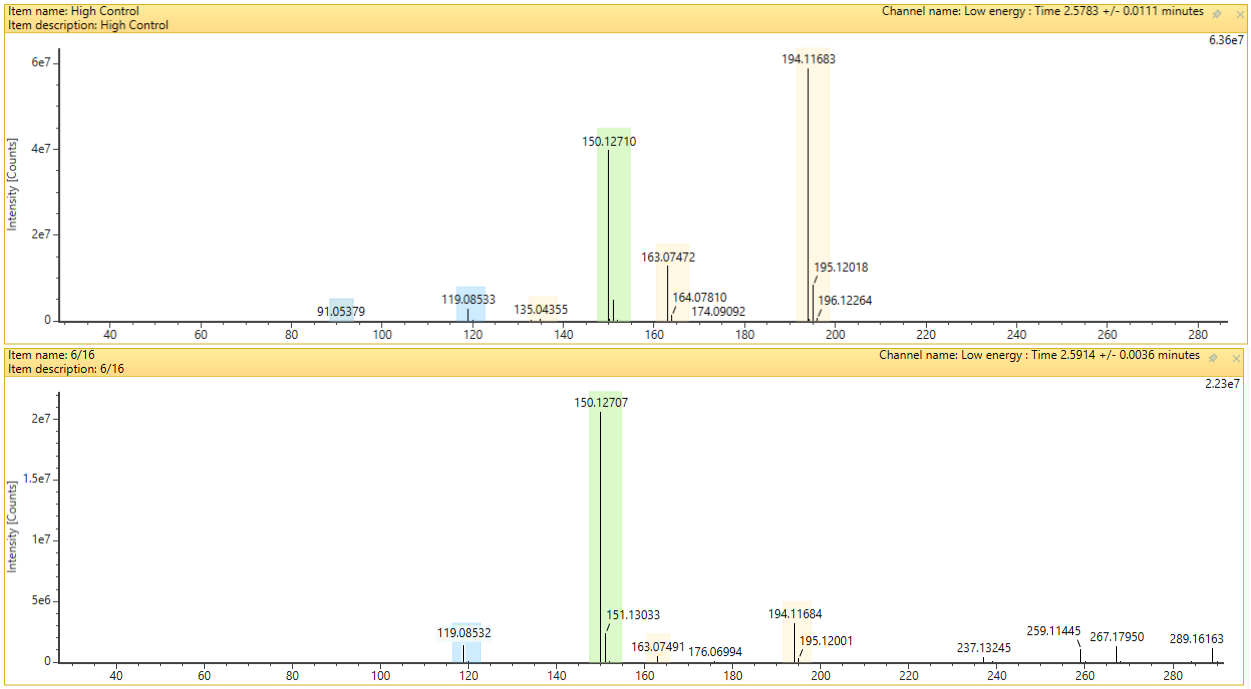

Figure A19. Low energy mass spectra for high control and sample 6/16 at approximately 2.58 minutes.

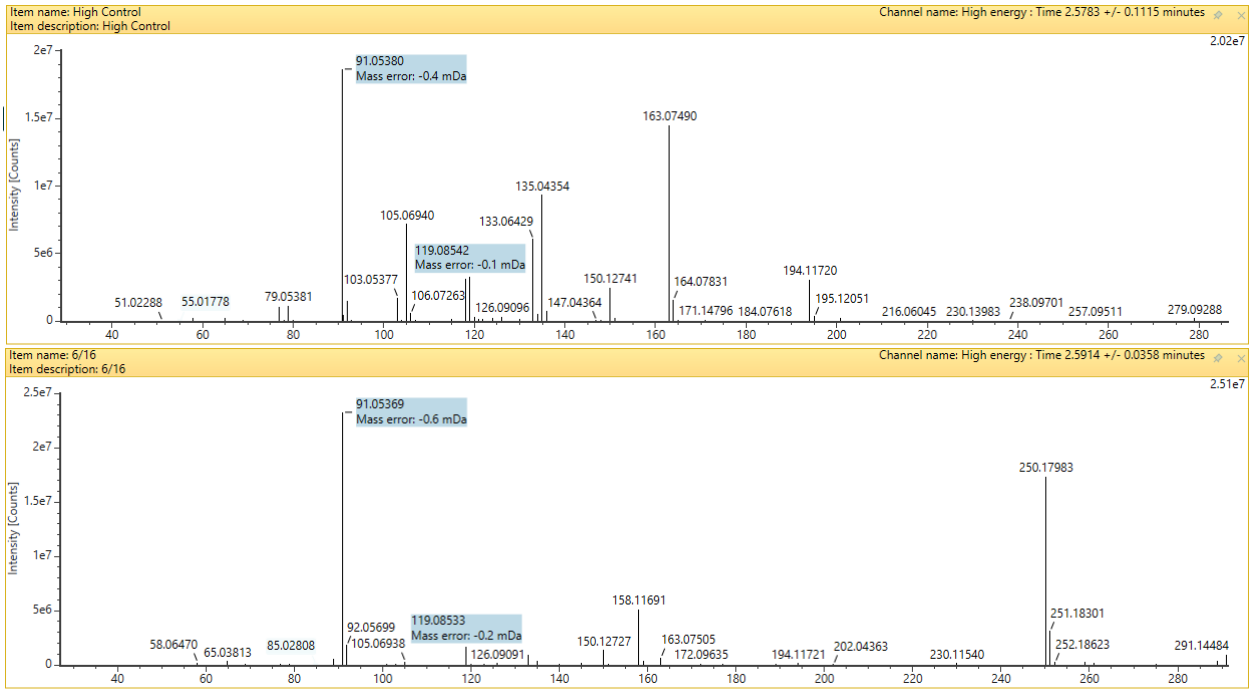

Figure A20. High energy mass spectra for high control and sample 6/16 at approximately 2.58 minutes.

Amphetamine

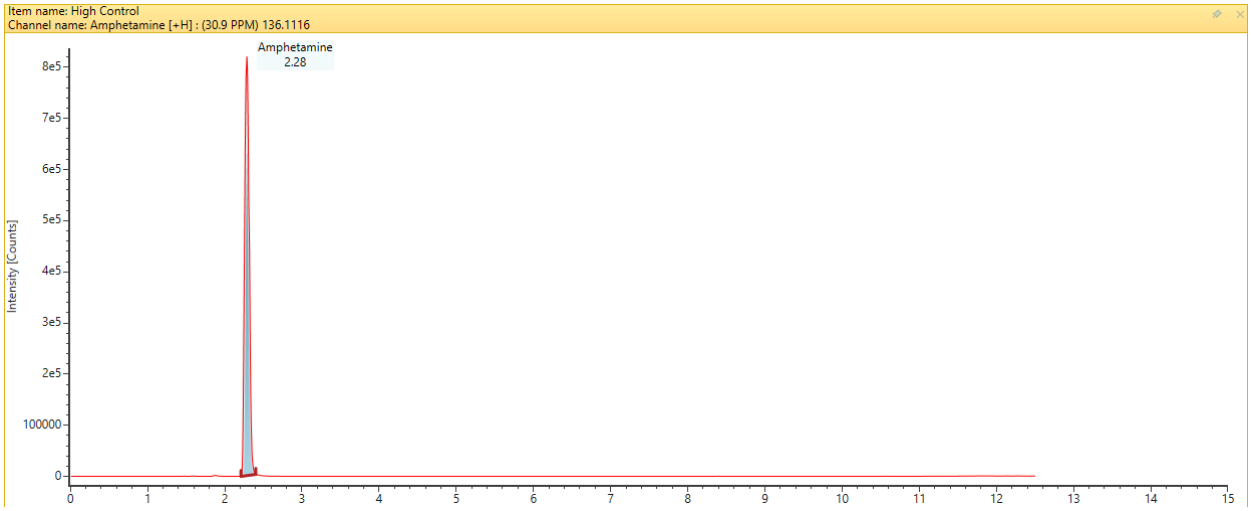

Figure A21. Amphetamine XIC in high control.

Figure A22. Amphetamine XIC in 6/16 wastewater sample.

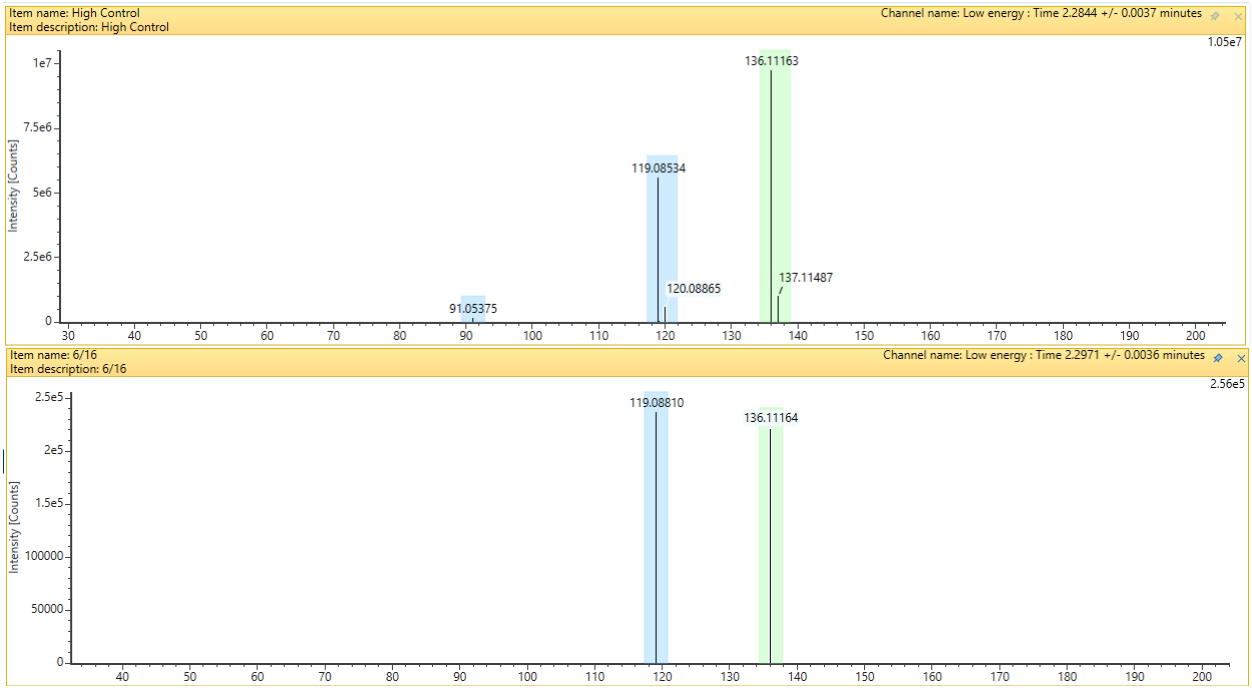

Figure A23. Low energy mass spectra for high control and sample 6/16 at approximately 2.28 minutes.

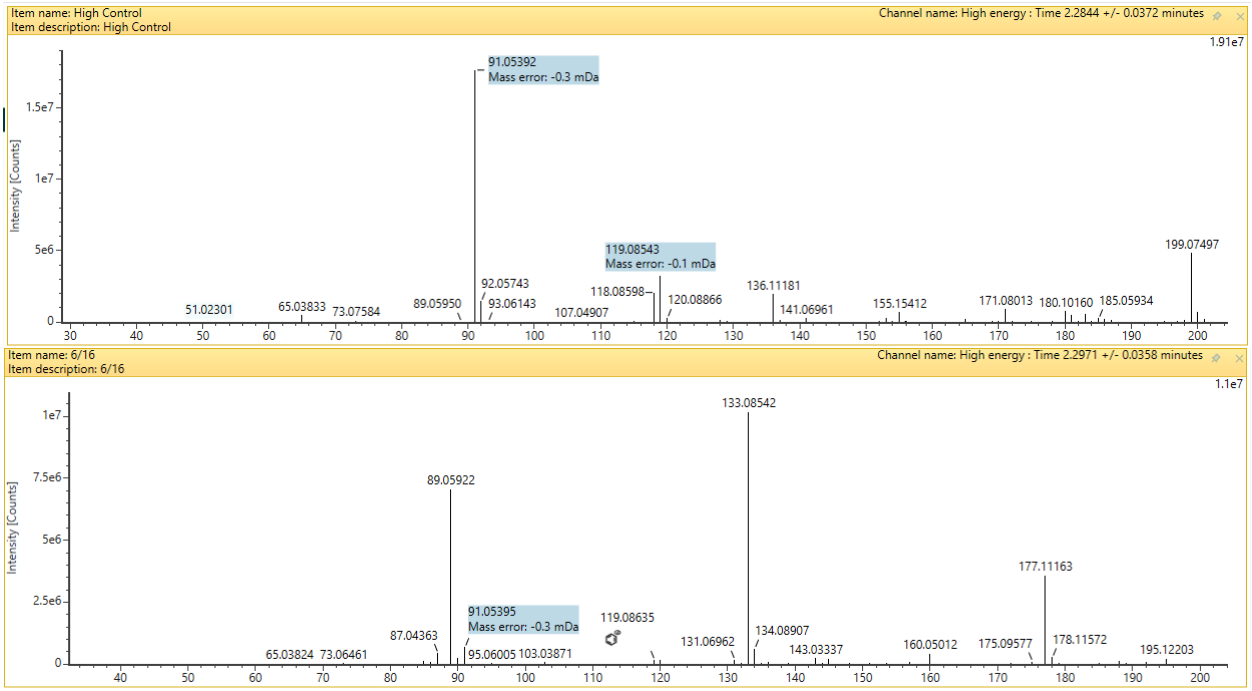

Figure A24. High energy mass spectra for high control and sample 6/16 at approximately 2.28 minutes.

Gabapentin

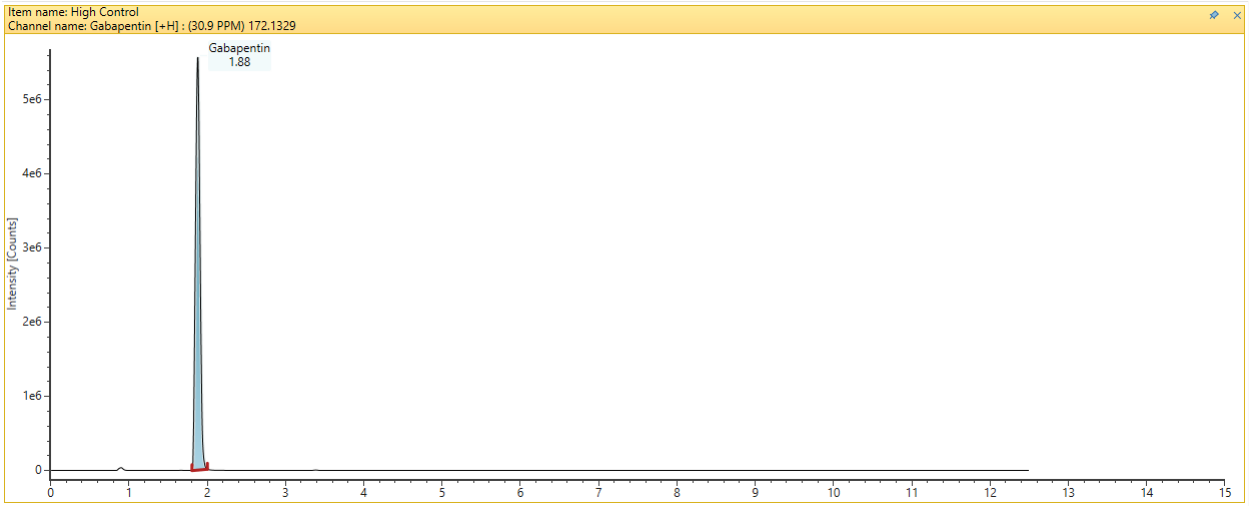

Figure A25. Gabapentin XIC in high control.

Figure A26. Gabapentin XIC in 6/16 wastewater sample.

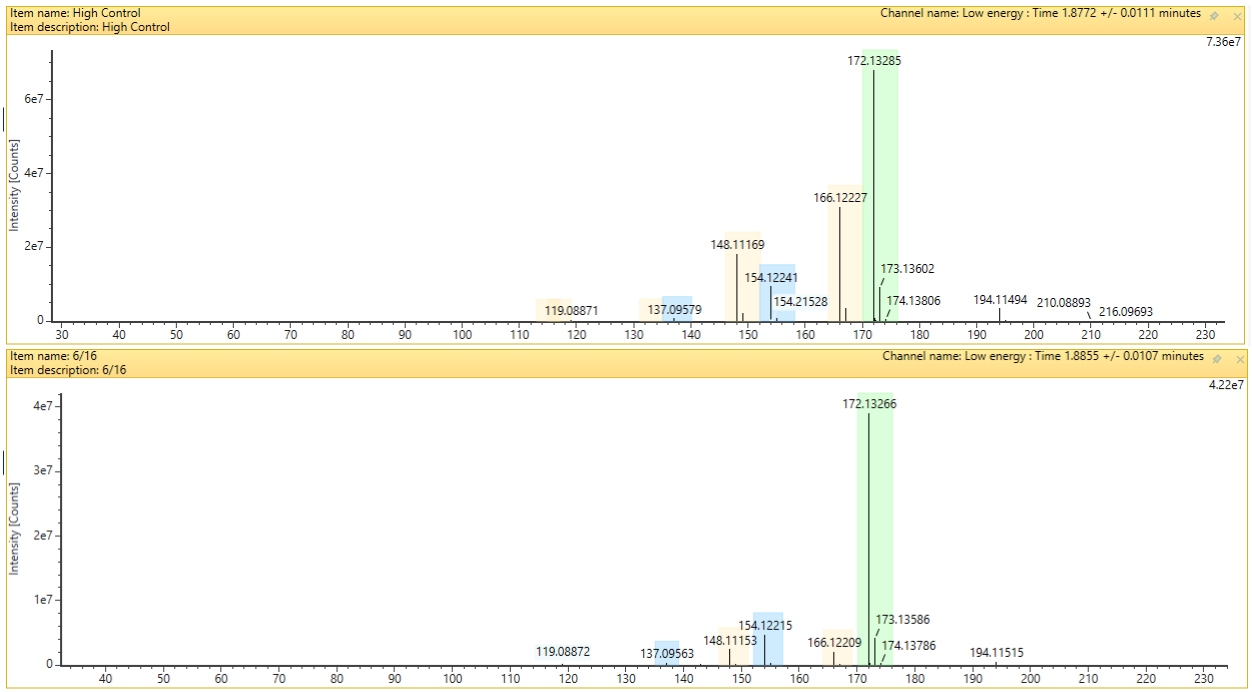

Figure A27. Low energy mass spectra for high control and sample 6/16 at approximately 1.88 minutes.

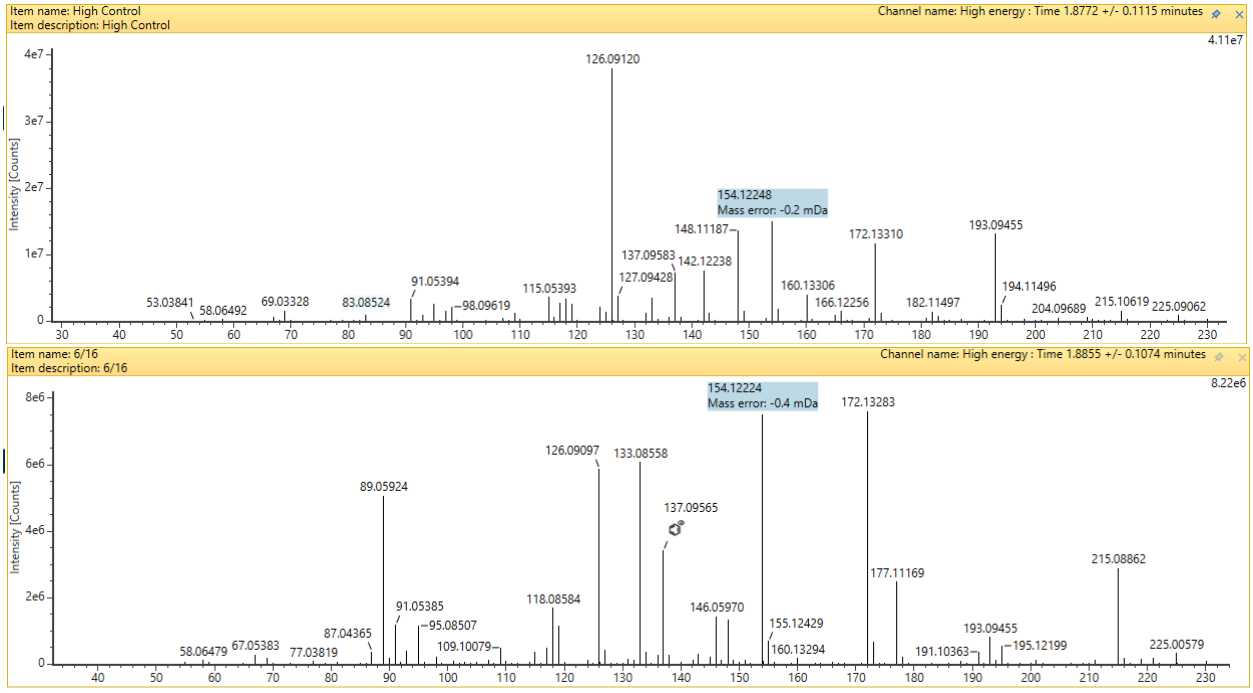

Figure A28. High energy mass spectra for high control and sample 6/16 at approximately 1.88 minutes.

Appendix B

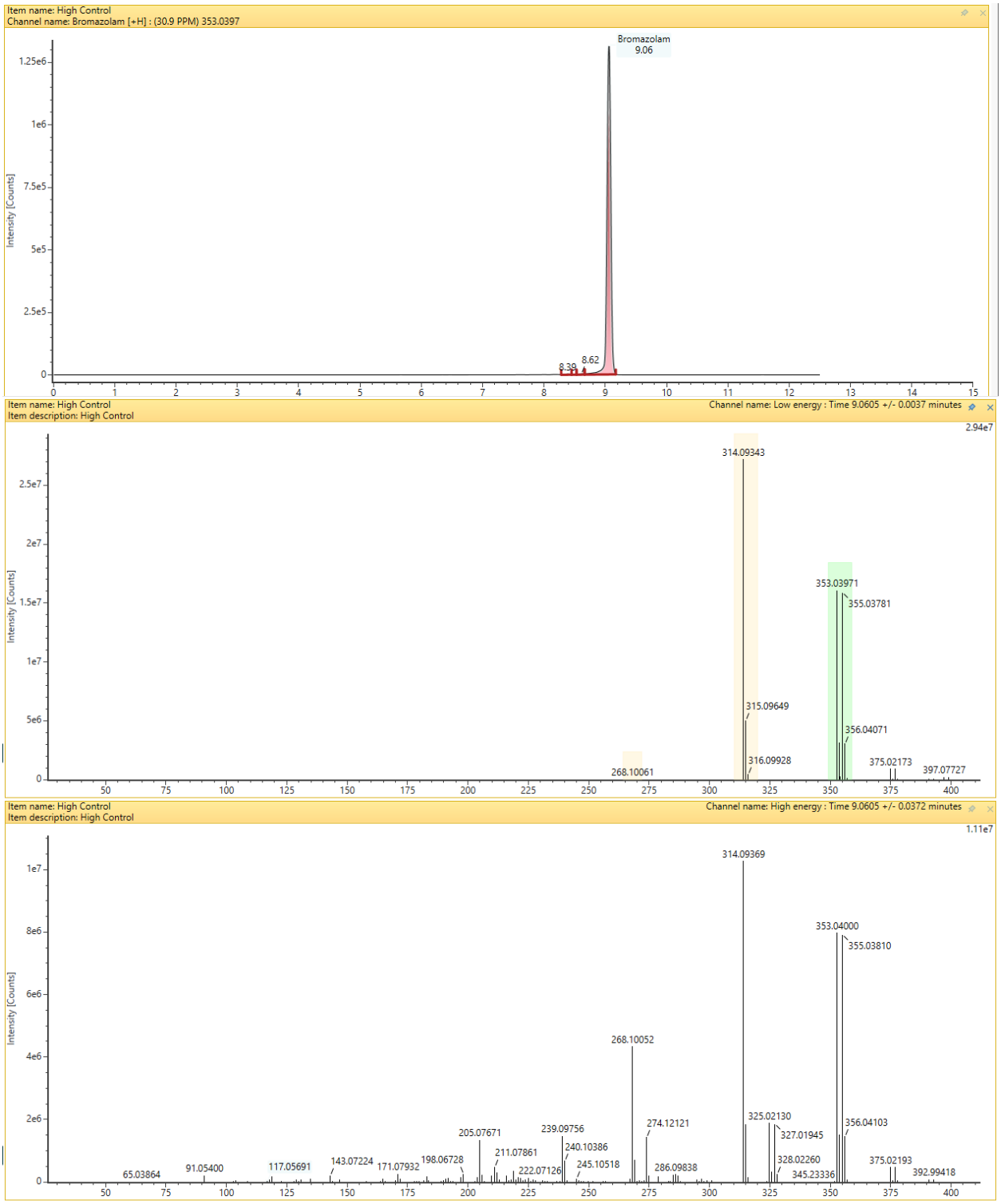

Figure B1. XIC, low and high mass spectra for bromazolam at 9.06 minutes in the high control.

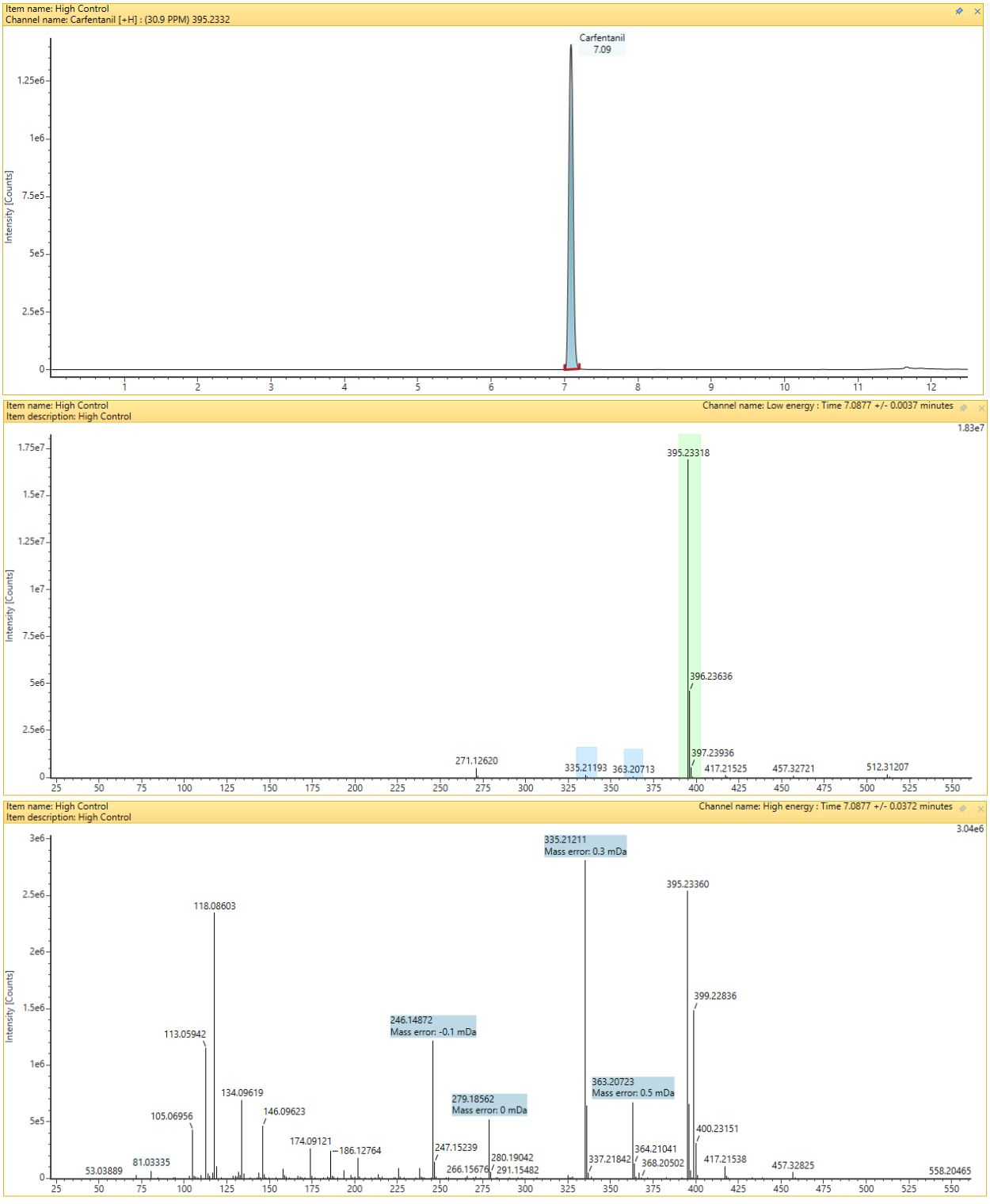

Figure B2. XIC, low and high mass spectra for carfentanyl at 7.08 minutes in the high control.

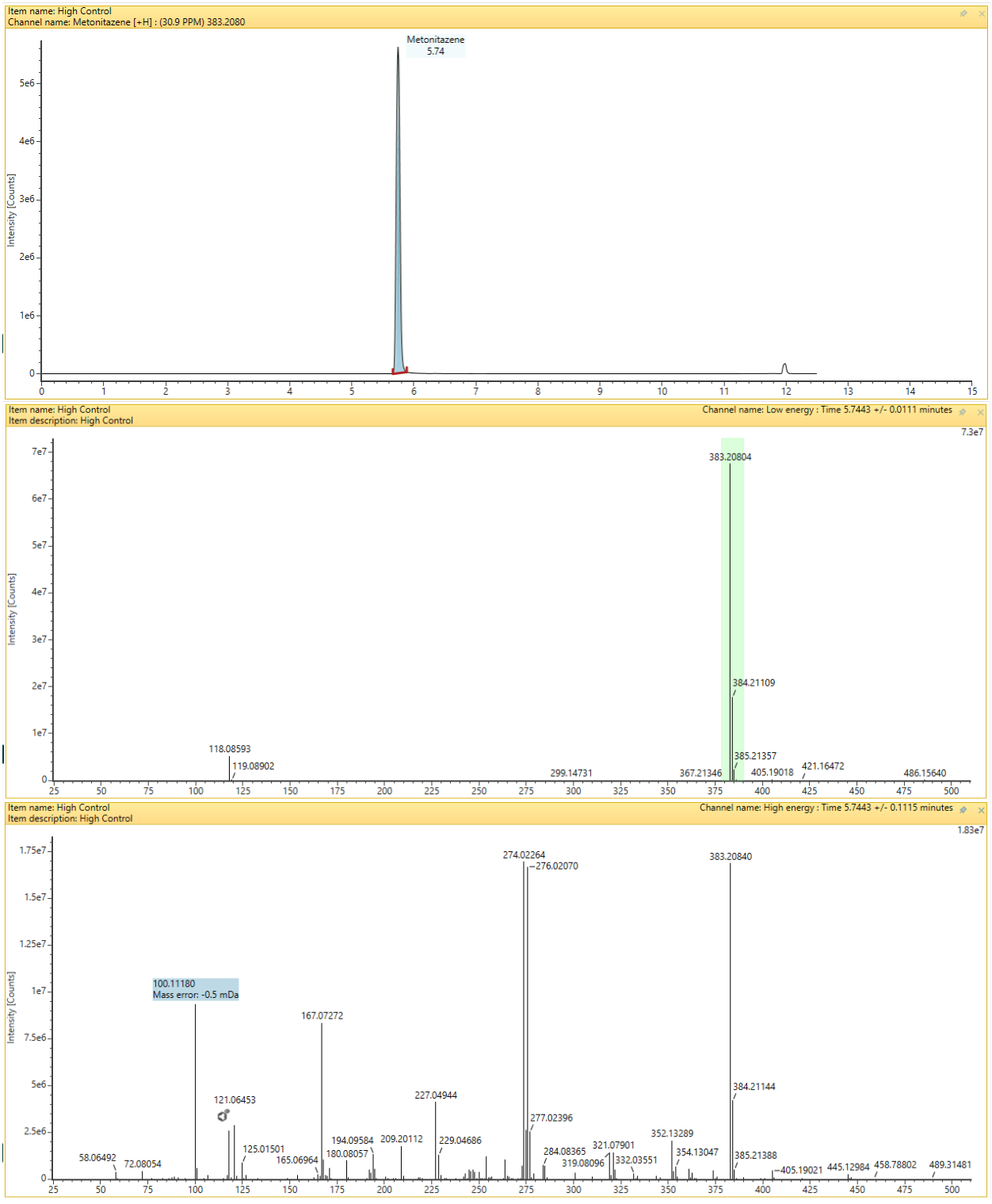

Figure B3. XIC, low and high mass spectra for metonitazene at 5.74 minutes in the high control.

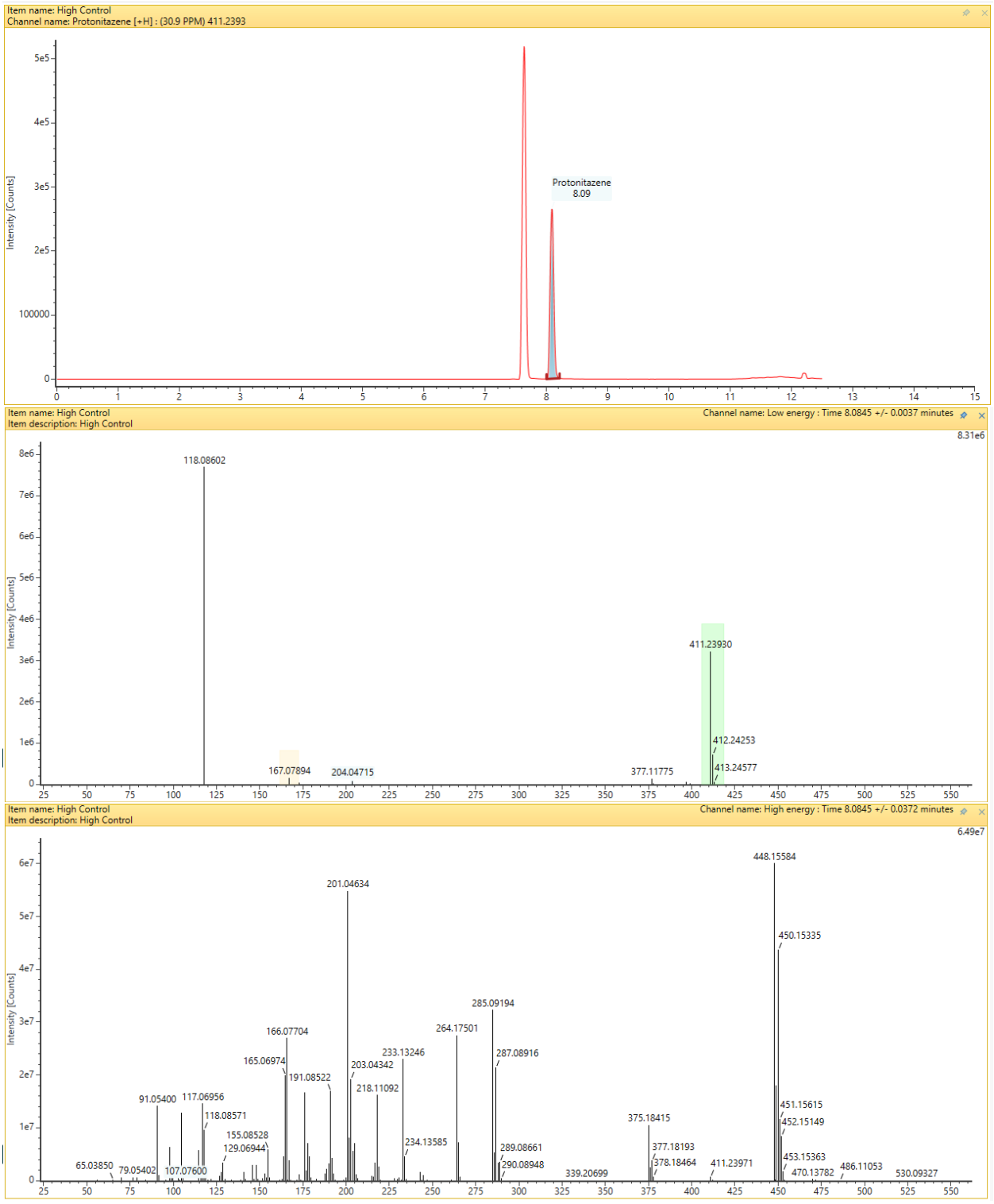

Figure B4. XIC, low and high mass spectra for protonitazene at 8.08 minutes in the high control.

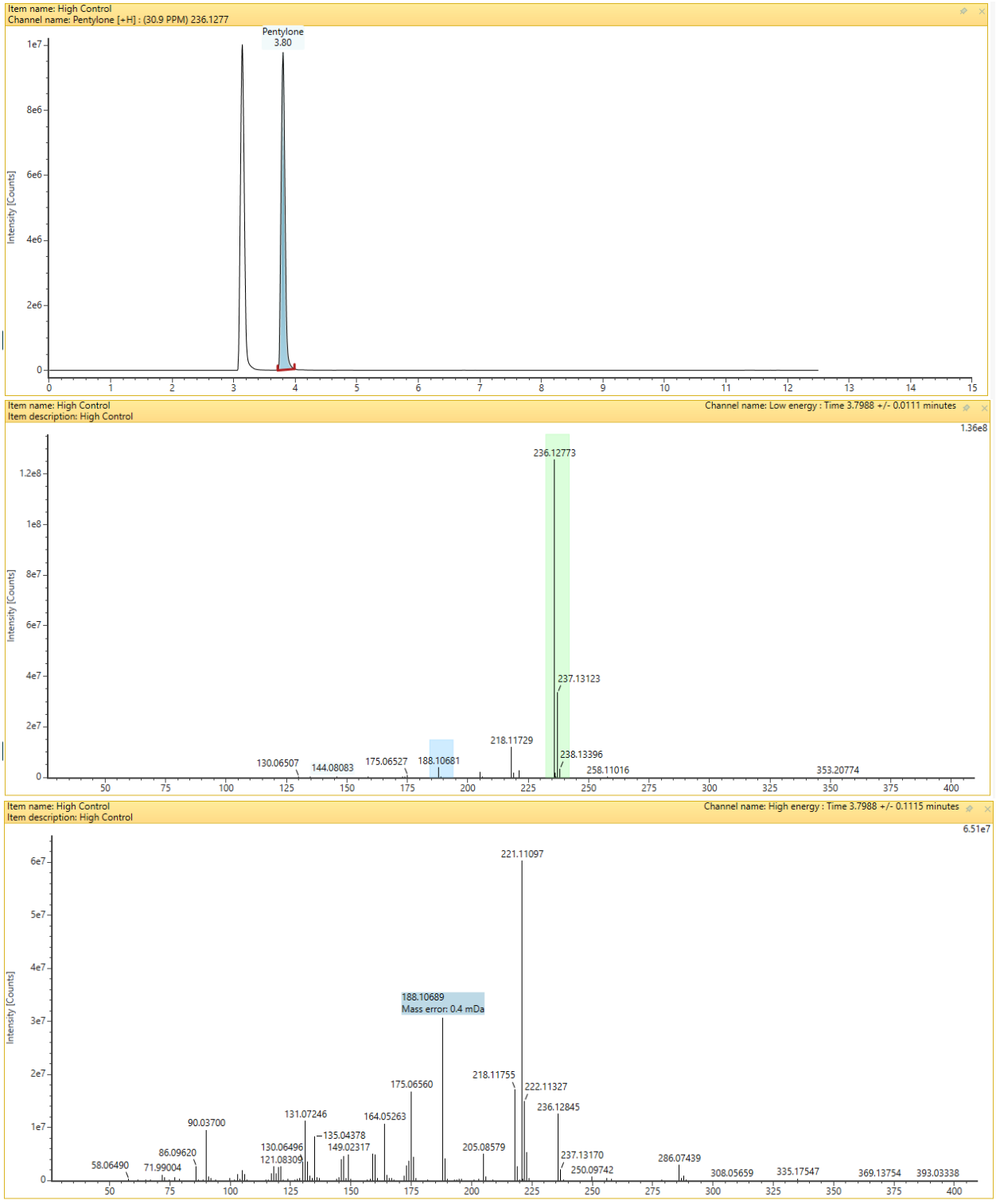

Figure B5. XIC, low and high mass spectra for pentylone at 3.80 minutes in the high control.

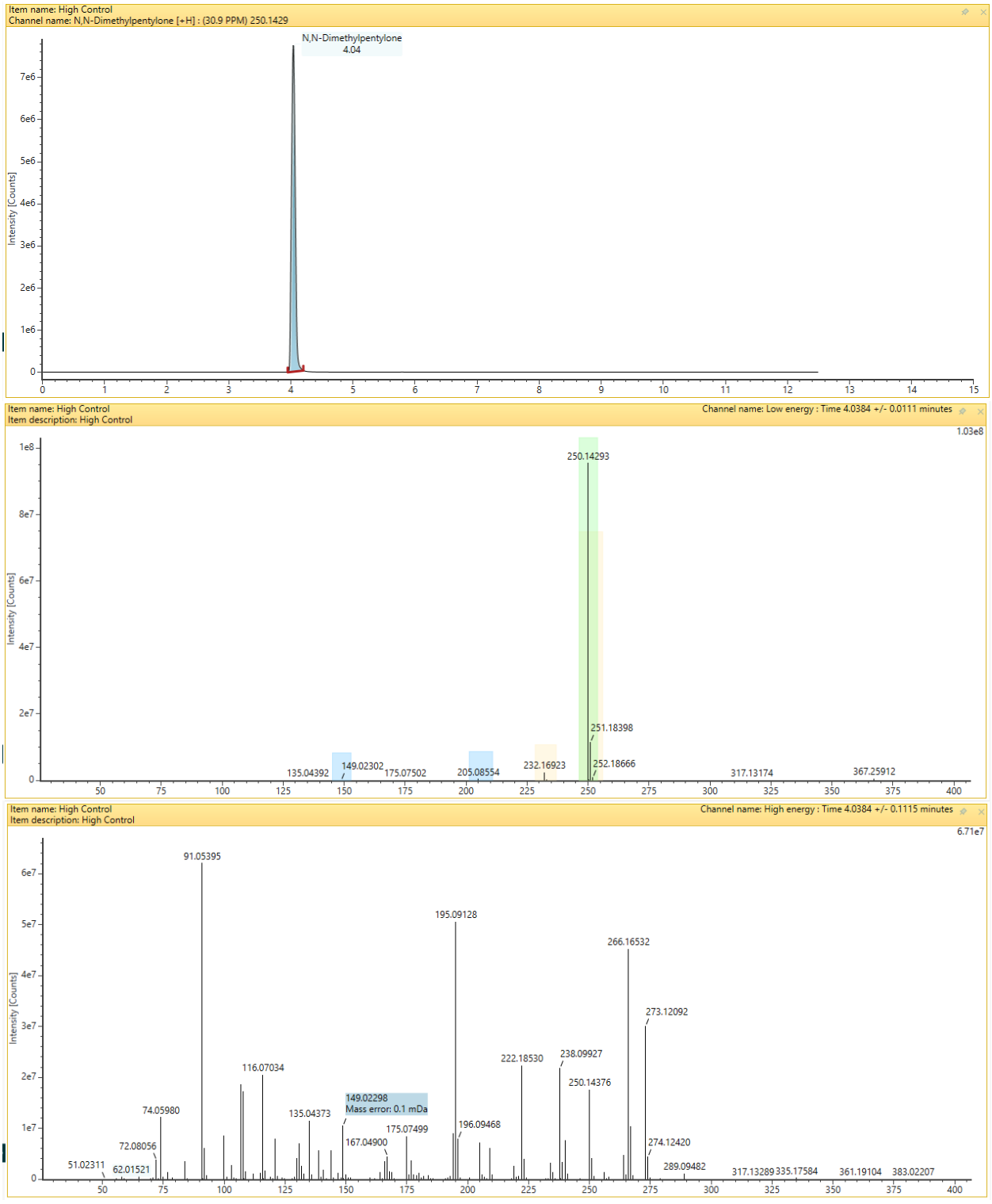

Figure B6. XIC, low and high mass spectra for N,N - dimethylpentylone at 4.04 minutes in the high control.