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# ORGANIC WASTE COMPOUNDS IN BUTTE AREA SURFACE WATERS

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By

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## Abstract

As human dependence on pharmaceuticals and household products containing a broad variety of organic compounds increases, so does the discharge of residual components of these compounds into surface and groundwaters. Organic wastewater chemicals (OWCs) result when human or animal discharge appears in the environment through a variety of waste disposal mechanisms. Historically environmental standards for organic wastewater chemicals have not been a concern when compared to biological hazards, metal contamination, acid/base hazards and radioactive hazards. At present, the U.S. Environmental Protection Agency (EPA) does not have standards for organic wastewater chemicals for surface waters; it is imperative that research be conducted regionally and locally so that national standards can be established to address new environmental hazards as organics become more applicable to everyday use.

Organic wastewater chemicals represent an expansive range of compounds that includes hormones, pharmaceuticals, industrial chemicals and biocides. At low concentrations these chemicals have been linked to a variety of physiological problems, including breast and testicular cancers. This project sampled for organic wastewater chemicals in five field sites along Silver Bow Creek in year 2014 and year 2015. These sites span from Silver Bow Creek in the city of Butte, Montana to the wastewater treatment plant, and 3 sites downstream of the plant, to the Warm Springs Ponds Operable Unit in Warms Springs, Montana. This project is part of a narrow study to determine the presence and quantity of three organic wastewater chemicals. The compounds of interest are 17-α-ethynyl estradiol, 17-β-estradiol, and N, N-diethyl-meta-toluamide (DEET). The waters were extracted and analyzed based on methodology developed by the U.S Geological Survey (USGS).

The study found DEET present at all 5 sites for both years, and the estradiol compounds were present in 45% of total sites tested. Concentrations of the compounds discovered in the samples were determined by their peak area and the calibration curves constructed by compound standards performed prior to sample analysis. These results may lead to further investigations of organic waste in area surface waters and influence future waste water treatment considerations for the city of Butte.


Key Words: Pharmaceuticals; Silver Bow Creek; Estradiol; Gas Chromatography Mass Spectroscopy; Surface Waters

**Dedication**

I would like to dedicate this work to Kenneth Jr, Evan, Noah and Maddox. (Who will probably never read this)

## Acknowledgements

| | |
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## Glossary of Terms & Acronyms

| Term | Definition |
|---|---|
| 17-β estradiol | The primary female sex hormone and a steroid. |
| α-ethynyl estradiol | Derivative of 17-β estradiol that can be found in oral contraceptives. |
| DEET | N, N-diethyl-meta-toluamide, used as insect repellent |
| [X] | Numbers in brackets denote concentration |
| ORP | Oxidation reduction potential (mV) |
| WSPOU | Warms Springs Ponds Operable Unit |
| DO | Dissolved oxygen |
| DIC | Dissolved inorganic carbon includes 3 forms ($CO_2$, $HCO_3^-$ and $CO_3^{2-}$) |
| GCMS | Gas chromatography mass spectroscopy |
| Alkalinity | Capacity of an aqueous solution to neutralize acid |
| EE | Diethyl Ether |
| Method Detection Limit (MDL) | The minimum concentration of a compound that can be determined by GCMS Analysis with a high confidence that the compound concentration is greater than zero |
| Solid Phase Extraction (SPE) Cartridges | An extraction apparatus that separates dissolved compounds from other compounds based on different physical characteristics or parameters, such as mass, pH, and conductivity |
| m/z | Mass to charge ratio used in chromatogram analysis. |
| ng/µL | Nanograms per microliter |
| SBC | Silver Bow Creek |

# 1. Introduction

## 1.1. Background

In response to an increase of organic waste compounds (OWCs) present in many surface waters, the U.S. Geological Survey National Water Quality Laboratory has developed a method for determining the presence of 67 compounds typically found in waste waters (Zaugg et al, 2002). The USGS method is designed to identify organic compounds that are normally associated with waste water produced by industrial and domestic practices. (Zaugg et al, 2002)

## 1.2. Study Objective

Multiple studies have determined that domestic-based organic chemicals are beginning to impact aquatic species as they are regularly exposed to these chemicals for extended periods of time (Hutchins, 2007, Zaugg et al, 2002, Routledge et al, 1998). To date the U.S. Environmental Protection Agency (EPA) does not impose surface water quality standards for pharmaceuticals that are suspected and or known to interfere with or disrupt endocrine systems in fish. These compounds are called endocrine disrupting compounds (EDCs) and several studies have confirmed that 17-β estradiol is linked to endocrine disruption in fish, and α-ethynyl is suspected to have similar effects on fish as well (Hutchins, 2007, Zaugg et al, 2002, Heiko et al, 2011, Shappell 2010). Further research is necessary to determine the presence as well as the quantity of these compounds in both surface waters and ground waters. Without this knowledge environmental standards cannot be effectively developed and implemented, and the waters may continue to go untreated for EDCs, as concentrations are increasing in surface waters and beginning to make their appearance up the food chain (Hutchins et al 2007).

This project focused on determining the presence and concentrations of three organic compounds at five surface water sites along Silver Bow Creek from Butte to the Warms Springs

Ponds Operable Unit (WSPOU). Two of the three compounds are EDCs: 17-β estradiol and α-ethynyl estradiol. N, N- diethyl-*meta*-toluamide (DEET), the third compound of interest, is the active ingredient in insect repellents. DEET has not specifically been linked to endocrine disruption in fish, but it is often persistent as a surface water contaminant; for this reason, it was also chosen for analysis in addition to the estradiol compounds. The chemical structures for the three compounds of interest are as follows.

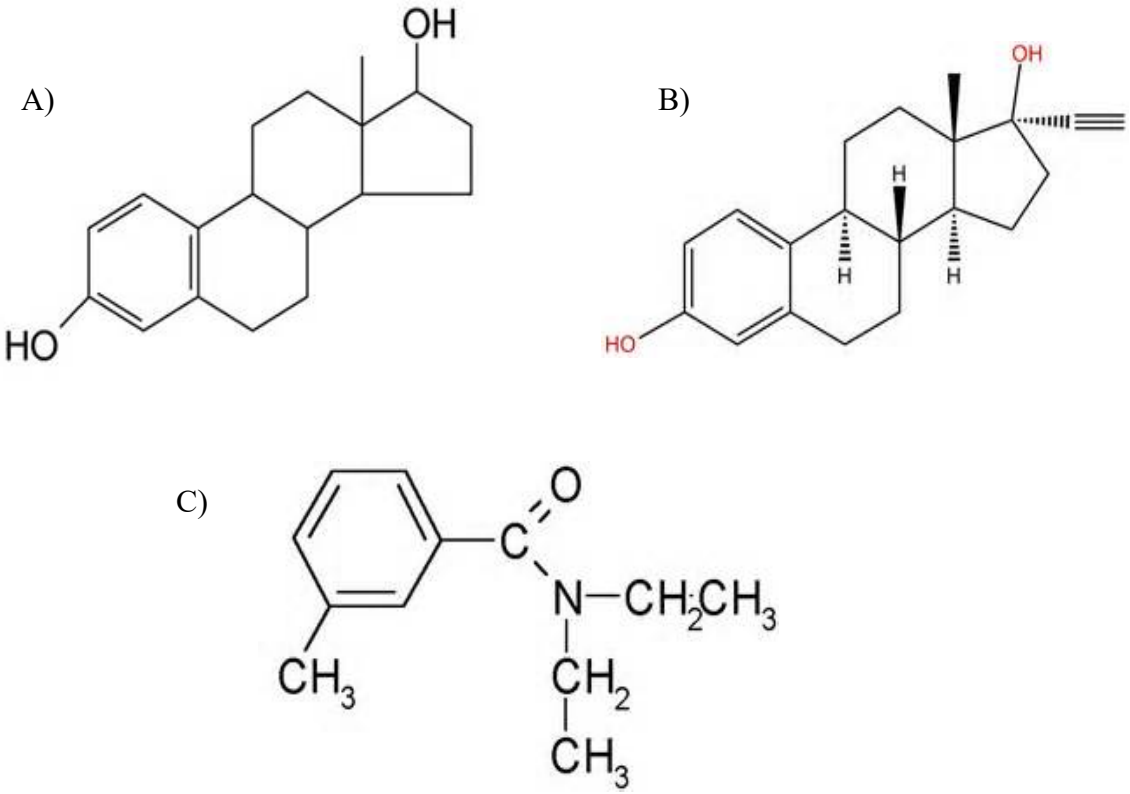


**Figure1. A) 17-β estradiol, B) α-ethynyl estradiol, C) N, N-diethyl-*meta*-toluamide (DEET)**

## 1.3. Outside Studies

Two previous studies conducted at Montana Tech identified amounts of OWCs in the Butte Summit Valley and Silver Bow Creek areas (J. Timmer, unpublished results). The first study used an Enzyme-Linked Immunosorbent Assay (ELISA) and found that every study site

contained sulfamethoxazole and 40% of samples contained 17β-estradiol, a compound of interest in this study. (J. Timmer, unpublished results).

A second qualitative study was conducted in 2013, and it analyzed surface waters from the same sampling sites involved in this study. The analysis was done using liquid chromatography-mass spectrometry (LCMS) based on EPA method 1694. (Reid et al, 2013). Due to instrument selectivity, the compounds of interest in Reid's study are different than the ones involved in this study. Reid did successfully determine a high presence of five pharmaceutical based compounds (Carbamazepine, Miconazole, Sulfamethoxazole, Thiabendazole, and Ciprofloxacin), and this observation prompted a more in-depth investigation of other OWC's in the area such as this one.

A third study reported in 2016 analyzed for OWCs collected from wastewater, surface water and ground water sites from various locations throughout of Gallatin County Montana (Icopini et al, 2013). This study analyzed water samples using ELSIA methods for several pharmaceutical compounds including 17-β estradiol. The Gallatin County study determined the presence of this compound between 2.25 ng/L (ppb) to 9.05 ng/L (ppb) in surface waters (Icopini et al, 2016).

## 1.4. Study Area

Copper and silver mining has been conducted in Butte, Montana, since the late 1800's. Prior to its diversion; Silver Bow Creek's (SBC) headwaters started at the Continental Divide north east of Butte, ran through the location of the present-day Berkeley Pit and continued to the northwest making its contribution to the head waters of the Clark Fork River (Helgen et al, 2007). As mining in and around Butte progressed, contamination from acid-mine drainage and spent-metal compounds began to have a significant impact in the area. Through numerous

studies, the effects of contamination typical of mine waste have been well documented (Helgen et al, 2007, Moore, Luoma, 1990, Quivik, 2001). Contamination was introduced into ground and surface waters by seepage from the Anaconda copper ore Smelter, Parrott Smelter and other mineral processing activates, Butte Pole and Treatment Plant (MPTP), and domestic waste practices. SBC now flows into the Yankee Doodle Tailing Ponds north of Butte and then re-starts at the base of the Butte Hill near the location of the Civic Center. From SBC's new origin it flows through the city of Butte past the waste water treatment plant west of the city, and further to the northwest through Warms Springs Pond Operable Unit (WSPOU) treatment (Quivik, 2001). The Warms Springs Pond Operable Unit was constructed as a series of settling basins for remediation and mine-water treatment prior to entering it into the Clark Fork River. The area of interest in this study starts in the city of Butte where SBC passes under Montana Street and extends to the northwest ultimately entering WSPOU (Helgen, et al 2007). After an extended residence time in WSPOU the waters eventually become the headwaters of the Clark Fork River.

Samples were collected at five different sites along Silver Bow Creek. The first site is located near Montana street. SBC-2 is 1.2 Km downstream from Butte's wastewater treatment plant. The third site Miles Crossing (Miles X) is about 13 Km downstream from the wastewater treatment plant and the fourth site SBC-6 is about 33 Km downstream from the wastewater treatment plant. The final site is the outflow into the Warms Springs Ponds Operable Unit (WSPOU). The water from SBC-6 has a 1 to 2-month retention time within the treatment ponds (Parker, 2013), prior to exiting to the headwaters of the Clark Fork River. The distances between sites is listed in Table 1 and Figure 2 is a map of the study area.

**Table 1 Distance between sites**

| Site | Distance |
|---|---|
| Site 1: Montana Street | 1.3 Km above Waste Water Treatment Plant (WWTP) |
| Site 2: SBC -2 | 1.2 Km downstream WWTP |
| Site 3: Miles Crossing (Mils X) | 13 Km downstream WWTP |
| Site 4: SBC- 6 | 33 Km downstream WWTP |
| Site 5: WSPOU | 37 Km downstream WWTP |

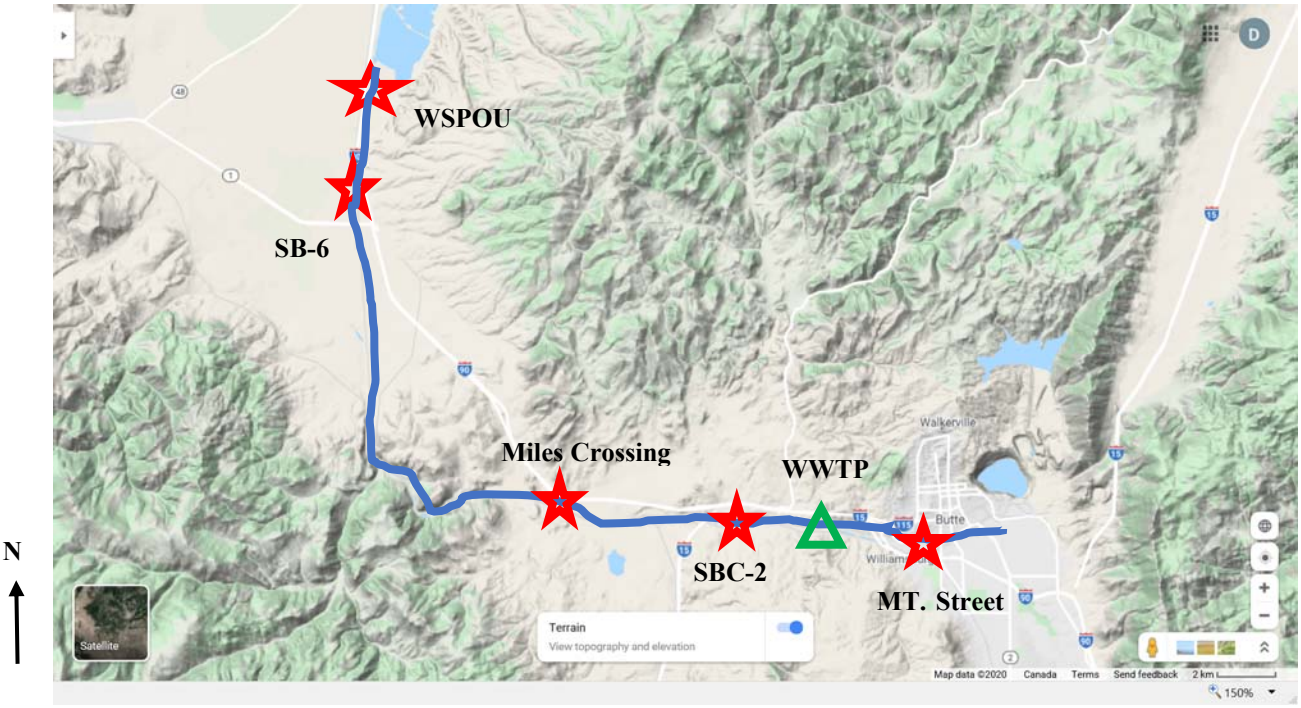


**Figure 2. Study area Silver Bow Creek**

## 2. Methods

The sampling and analysis method used in a study was based on a method developed by the U.S. Geological Survey (USGS). The water samples collected in the USGS study were filtered to remove suspended particulate matter, extracted by vacuum through disposable solid-

phase cartridges that contained polystyrene-divinylbenzene resin. The cartridges were then dried with nitrogen gas, and the sorbed compounds were eluted off the cartridges using a dichloromethane-diethyl: ether (4:1) solvent and analyzed by capillary-column gas chromatography/mass spectrometry.

Qualitative identification visually compared the sample compound spectra to the reference standard spectra and a confirmation of a reasonable match was used to qualitatively and quantitatively identify the compounds in the water samples. The retention time of the quantitation ion for the compound of interest should be within 0.1 minutes (± 6 seconds) of the expected retention time (as calculated from the relative retention time of calibration standards and the retention time of internal standard in the sample). (Zaugg et al, 2002)

The methods used in this study were adapted from the USGS study *Methods of analysis by the U.S. geological survey national water quality laboratory-determination of wastewater compounds by polystyrene-divinylbenzene solid-phase extraction and capillary-column gas Chromatography/Mass spectrometry* (Zaugg et al 2002). The paper was used as a guide for this study with modifications to account for the different equipment and instrumentation that was available at Montana Tech.

## 2.1. Field Methods & Sampling

In addition to collecting water samples, a Hydrolab MS-5 Datasonde was also used to collect additional field data at each site. The Hydrolab was calibrated independently prior to field analysis and submerged in water at each site during the time of water collection. The readings were taken after enough time had elapsed (≈ 3-5min) to allow equilibration of temperature, pH, conductivity, dissolved oxygen and oxidation-reduction potential.

The Hydrolab determined specific conductivity (SC, ±2 µS $cm^{-1}$), oxygen reduction potential (ORP vs. SHE, ± 0.5 mV), pH (SU, ± 0.1 pH units), and dissolved oxygen reported in two different units; percent saturation (%), and concentration (mg/L± 0.2 mg/L).

In addition to the Hydrolab readings, a small water sample was taken and filtered using a 0.2 µm polyethersulfone (PES) filter and sent to the Montana Bureau of Mines and Geology (MBMG) for carbon isotope analysis. Both carbon isotope analysis and alkalinity tests were only conducted during the 2014 sample event.

Alkalinity was measured in the field during the time of water collection using digital titration methods. Sulfuric acid was added by a digital titrator to a flask containing water collected from each stream site until the solution changed from blue to pink indicating a neutral endpoint. When the solution reaches a pH of 4.2 a color change occurs indicating all the alkaline compounds (bicarbonate, carbonate, and hydroxide) in the water are used up by neutralizing the acid compounds in the water. Alkalinity was tested in 2014 and applies to the acid neutralizing capacity of solutes in a water sample reported in mg/L $CaCO_3$. Alkalinity thus consists of the sum of titratable carbonate and noncarbonate chemical species in an unfiltered water sample (mg/L of $CaCO_3$) (Radtke et al, 98).

The dissolved inorganic carbon (DIC) in the 2014 water samples were analyzed using an Aurora 1030W TIC/TOC analyzer interfaced with a Picarro G2131-i carbon isotope analyzer at the Montana Bureau of Mines and Geology.

Water was collected at different points spanning the width of each stream, to ensure a representative sample was collected on August 29, 2014 and July 27, 2015. The water samples were collected using 2-liter amber glass bottles to avoid photochemical degradation as well as potential phthalate and preservation contamination. Once the samples reached the lab they were

stored in a refrigerator prior to filtering and extraction. Samples were extracted within the 14-day hold time as set by the USGS study (Zaugg et al, 2002) to recover the organic compounds of interest.

## 2.2. Sample Preparation

Upon returning from sample collection, the 2-L samples were divided into two cleaned 1-L glass bottles, one portion was used for the procedure relative to this experiment and the other portion was used for another research project outside the scope of this study. After separation, the 1-L samples were filtered through a 0.7µm nominal pore diameter glass fiber filter. The filters were used to remove suspended particulates from the water to avoid plugging up the solid phase extraction (SPE) cartridges. Nitrile gloves were donned during collection, filtration, extraction and analytical processes to avoid contamination. The water was pumped through the filters by a tubing system and a Geo-Tech peristaltic pump. The 2015 samples were not filtered as there did not appear to be a significant number of particulates due to stream flow.

After filtering, 60g of NaCl was added to the filtered samples to ensure preservation. Salting the samples improves non-polar compound recovery by increasing ionic strength (Zaugg et al 2002).

After filtration the quality-control samples and the environmental samples were acidified with 3mL of acetic acid: sodium acetate buffer. The acetic acid: sodium acetate buffer was made by diluting 30g of acetic acid and 15g of sodium acetate in 1L of reagent water. The acetic acid was added to sodium acetate until the pH of the solution was 4.3 as determined by a pH meter. The pH meter calibration was verified prior to taking the buffer reading. After acidification the samples were extracted at the Bureau of Mines analytical laboratory.

## 2.3. Quality Control

Quality control (QC) samples are introduced to determine analysis reliability as well as, identify any method and instrument contamination or carryover. After filtration and before sample extraction, a set of quality control samples were added to the batch of environmental samples and processed at the same time. A batch of samples included 5 environmental samples (one sample from each site), and a blank (both composed using 18MΩ-filtered water). D-caffeine was added to each sample at various volumes. D-caffeine is used as a surrogate spike because it is not found in nature and would not be identified in the water samples separate from actual addition (Zaugg et al, 2002). The sample preparation and extractions occurred at different dates and additional surrogate was added to the 2015 samples prior to the samples going through the extraction process.

Concentrated standards of DEET, 17-β estradiol, and α ethynyl estradiol were made by weighing 20 mg to the nearest 0.002g of the neat material and diluting with approximately 2.65g $\pm$ 0.05g of methylene chloride (dichloro-methane, DCM) to a concentration of approximately 10,000 µg/L (10mg/mL). Since DCM is a volatile solvent it was brought to mass as quickly as possible to get as close to 2.65g to avoid loss due to evaporation. The density of DCM (1.33g/mL) was used to convert the volume needed to the mass used in making the standards, as a 2mL volumetric flask was not available in the lab. A calibration curve was constructed after serial dilutions of the standards were analyzed.

Surrogate (D-caffeine) recovery from the environmental samples indicates a percentage of analyte lost during extraction, instrument drift, or matrix effects that may be encountered during the entire process (Harris, 2007). A calibration curve for D-caffeine was constructed in

the same way as the standard curves derived for the compounds of interest. The surrogate was made in the same manner as the intermediate method compound standards.

100µL of the 10,000 µg/L D-caffeine surrogate, was added to a 50-mL volumetric flask and brought to volume with methanol to a final concentration of 20ng/µL. The USGS study added 100µL of the 20ng/uL standard to a 1-L sample to obtain a surrogate spike solution of 2.0 ug/L. A surrogate concentration of 5.0 ng/µL is expected from a 0.40-mL extract if 100 percent of the surrogate is recovered through the sample preparation for the USGS method. (Zaugg et al 2002). The 2014 environmental sample areas fell within acceptable peak area range as determined by D-caffeine standard analysis and percent recover was calculated for these samples. Figure 3 displays an example of one of the chromatograms derived from the 2014 D-caffeine peak isolation. The top chromatogram shows the full spectrum, the middle is isolated by m/z for the quantitation ion and the bottom chromatogram is isolated by m/z for the confirmation ion. The area for the quantitation ion is used to determine the concentration of D-caffeine.

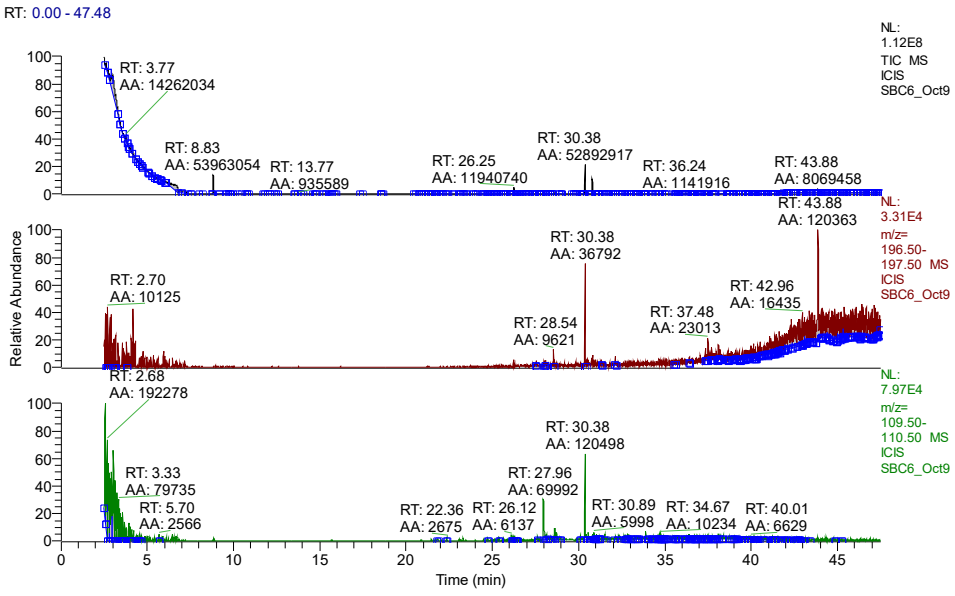


**Figure 3. Chromatograms for D-caffeine peak areas for the 2014 samples (RT = 30.38)**

The 2015 environmental samples were double spiked creating peak areas greater than 20% of the largest areas analyzed during D-caffeine standard analysis. The percent recovery for D-caffeine was not determined for the 2015 samples as the peak area count greatly exceeded the calibration curve as can be seen in Figure 4.

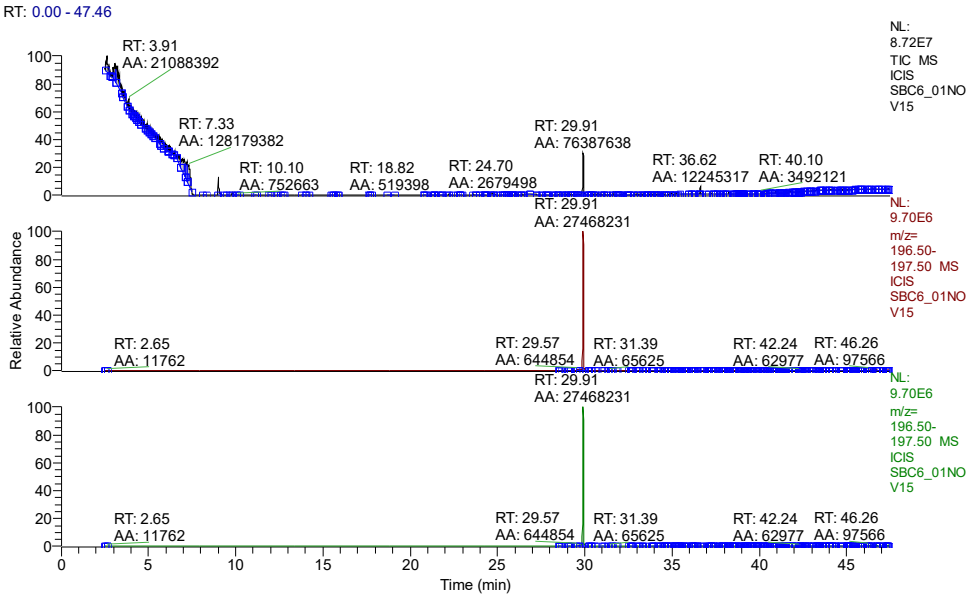


**Figure 4. Chromatogram for D-caffeine peak area 2015 samples (RT = 29.91)**

A blank sample was analyzed before the environmental samples and isolated for the compounds of interest by m/z ratio. A blank was analyzed between each sample and produced no carryover from the compounds of interest between runs. Figure 5 displays a chromatogram isolated by mass to charge for the quantitation and confirmation ion ratios for 17-β estradiol. Peaks with retention times close to the retention times determined from standard analysis ($RT_{DEET} \approx 26.25$min, $RT_{17\text{-}\beta\ estradiol} \approx 37.52$min, $RT_{\alpha\text{-}ethynyl\ estradiol} \approx 38.05$), did not appear in the blank.

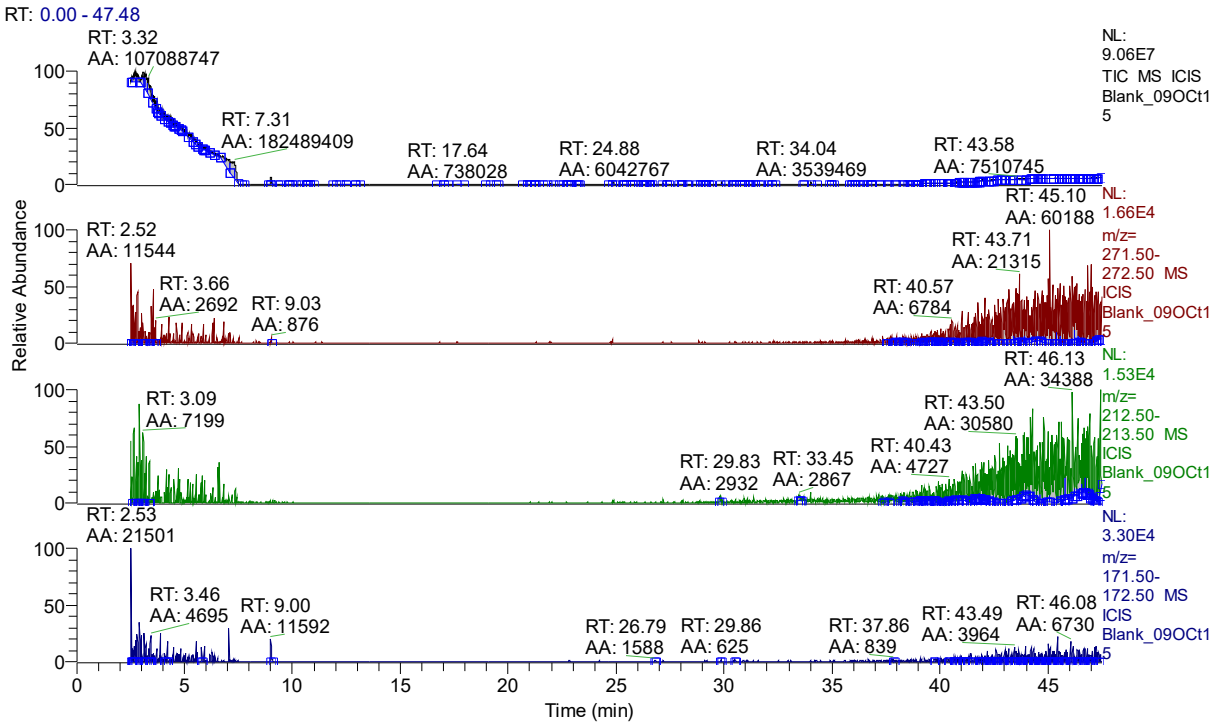


**Figure 5. Chromatogram for a blank run between environmental sample analysis for the 2015 samples ($RT_{DEET}$ ≈ 26.25min), ($RT_{17\text{-}\beta\ estradiol}$ ≈ 37.52min), ($RT_{\alpha\text{-}ethynyl\ estradiol}$ ≈ 38.05)**

## 2.4. Standard chromatograms

Retention time is the time measured from the point of injection to the point at which individual compounds completely elute through the column and are registered by the detection system (Harris, 2007). Retention times can be used as qualitative identifiers in the environmental samples when compared to the retention times determined from the standards/surrogates previously analyzed. The retention times for each standard fell within ± 2 minutes of the times determined by USGS method guiding this study. The retention times determined from this experiment for each standard are presented in Table II. The quantitation and two confirmation ions for the three standards and surrogate were provided by the USGS method and used as a qualitative identifier for this study.

**Table 2 Retention times from USGS methods, retention times determined in this procedure and mass-to-charge (m/z) ratios for quantitation ion and confirmation ions from USGS (Zaugg et al, 2002)**

| Compound Name | Retention time (min) USGS | Retention time(min) this study | Quantitation Ion (m/z) | Confirmation ion (m/z) | Confirmation ion (m/z) |
|---|---|---|---|---|---|
| N,N-diethyl-meta-toluamide (Deet) | 27.983 | ~26.25 | 119 | 190 | 91 |
| 17-beta-Estradiol | 39.574 | ~37.52 | 272 | 213 | 172 |
| α-ethynyl estradiol | 40.120 | ~38.05 | 213 | 296 | 160 |
| D-caffeine | 31.444 | ~29.90 | 197 | 110 | NA |

Retention times are determined by peak isolation for a given ion mass-to-charge ratio (m/z) for the quantitation and confirmation ions as they appear in chromatograms. There was only one confirmation ion given for deuterated caffeine in the USGS method (m/z = 110), and the three standards had two confirmation ions. The retention-time decrease indicated that the compounds were eluding off the column more quickly than the compounds studied in the USGS method. This time difference between the USGS and this method can be attributed to characteristics of the individual chromatography column used and the modified settings applied in this study.

A Thermo GC (Trace GC Ultra) and a Thermo ion trap mass spectrometer was used for this study, as it was the only one available, and an Agilent GCMS was used in the USGS study. The retention times for the quantitation ion in the compound of interest should be within 0.1minutes ($\pm$6 seconds) of the expected retention times as calculated from the relative retention time of the calibration standards and the retention time of the internal standard in the sample (Zaugg, 2002). Th Retention time for the standards and the environmental samples were determined by peak isolation from the given mass to charge ratios (m/z) provided by the USGS method.

Figure 6 is an example of the standard chromatogram for DEET. The top chromatogram displays the full mass spectral analysis of the DEET standard without peak isolation. The chromatogram directly below the full spectrum chromatogram isolates a mass range between 118.5-119.5 in order to isolate the quantitation ion for DEET. The two peaks isolated below the quantitation ion are the confirmation ions given for DEET and are used to qualitatively confirm the presence of the compound. All four peaks had a retention time at 26.27 minutes, which is less than the retention time published in the USGS method at 27.983 minutes, likely caused by different GCMS manufactured equipment used between the two studies.

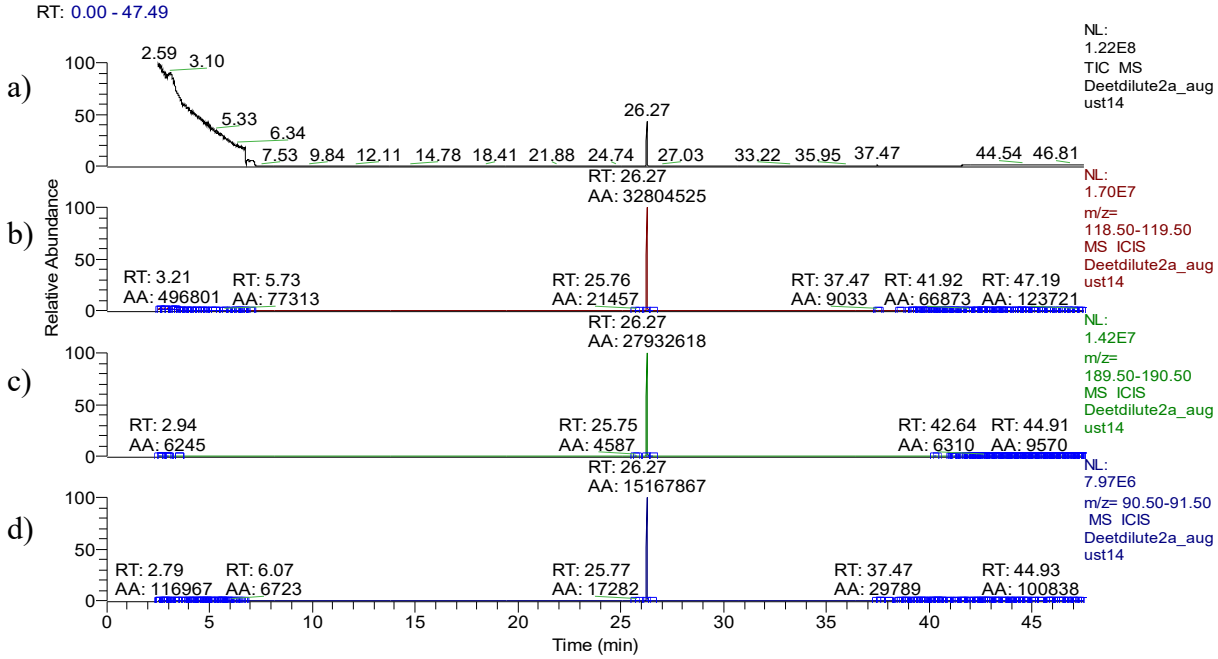


**Figure 6. a) The top chromatogram is the full spectrum for the DEET standard, b) chromatogram for the quantitation ion mass range 118.5-119.5 c) chromatogram for confirmation ion mass range 189.5-190.5 d) chromatogram for confirmation ion mass range 90.5-91.5. RT= retention time AA= Peak Area**

Chromatograms were used as a part of the calibration process and used to determine the limits of detection (LOD) based on decreasing peak areas as smaller standard concentrations were analyzed. The chromatograms used for standard analysis and environmental analysis followed the same format as the DEET chromatogram in Figure 6.

## 2.5. Calibration Curves & Method Detection

A calibration curve shows the response of an analytical method to known quantities of analyte. (Harris, 2007). The 10,000 ng/µL standards and surrogate concentrations were diluted from various volumes i.e. 1µL standard brought to volume with DCM in a 10 mL volumetric flask, this dilution method was chosen based on volumetric flasks available in the lab. The calibration ranges established by the guiding study ranged from 0.05 to 40.0 ng/µL. This process was used to determine sensitivity or method detection limits (MDLs). The lowest detected level from the standard analysis was for DEET, detecting concentrations as low as 1.07 ng/µL or PPM which is high compared to other studies. Due to time constraints, true instrument sensitivity and MDL's were not determined for the standards.

Calibration curves were constructed using the peak areas determined for the quantitation ions used as the dependent variable versus the known concentrations analyzed for each quality control standard/surrogate used as the independent variable. After construction, the calibration curves could be used to estimate concentrations for the compounds of interest when peaks at appropriate mass ranges appeared in the chromatograms from environmental sample analysis. The calibration curves were constructed using µg/L and the final concentrations are reported in ng/µL (ppm) as the USGS method did reported in the same units.

The $R^2$ coefficient of determination is a statistical measure of how well the regression line approximates the data. An $R^2$ of 1.0 indicates that the regression line perfectly fits the data. The $R^2$ value was determined for all of the standard calibration curves, as well as the curve determined for the surrogate D-caffeine. The $R^2$ values are discussed individually for each calibration curve starting with DEET. All of the $R^2$ coefficients extrapolated for the linear regression data were above 0.94, the USGS method did not give an acceptance criteria for $R^2$ and

the line equations derived by standard analysis were used to determine concentrations for quantitation peak areas that appeared during environmental analysis.

The calibration curve in Figure 7 was constructed from the analysis of the α-ethynyl estradiol standard. The equation determined from the calibration curve was used to determine the concentration of α-ethynyl compounds based on quantitation peak areas detected for α-ethynyl estradiol m/z range determined from the environmental sample analysis. The $R^2$ value for the α-ethynyl estradiol calibration curve was 0.94. The lowest concentration analyzed for α-ethynyl estradiol was 1.1 ng/µL (ppm).

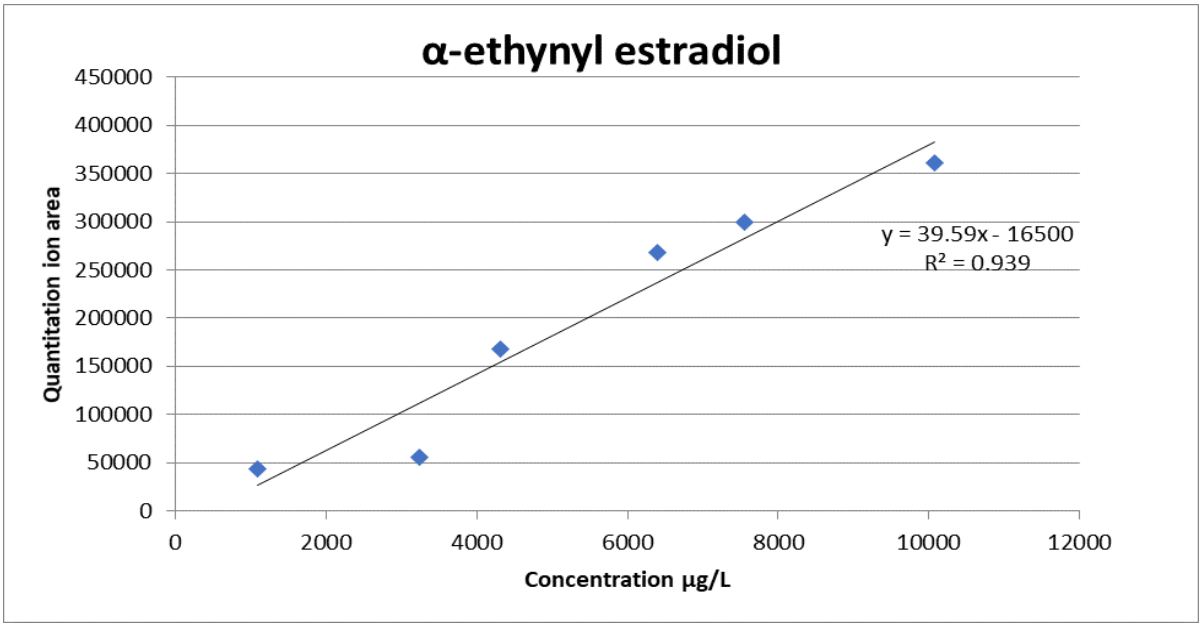


**Figure 7. Calibration curve for α-ethynyl estradiol including linear regression equation and $R^2$ value. Again X= concentration of ethynyl estradiol Y= area for the quantitation peaks**

The calibration curves for the other compounds of interest and the surrogate were also constructed the same way as the α-ethynyl estradiol calibration curve presented in figure 7. The calibration curves for the two additional standards and the surrogate D-caffeine can be found in the appendix. The linear equations determined by each calibration curve were used to determine

the approximate concentration of compounds when a quantitation peak could be identified for each compound of interest from the environmental sampling.

## 2.6. Extraction

Once the samples were salted and filtered, they were ready for solid-phase extraction (SPE). The extraction process for this study was performed with the assistance of the Bureau of Mines analytical lab located on the Montana Tech campus. The SPE cartridge extraction method was of particular use for this study because traditional extraction procedures normally employ liquid-liquid extraction methods. Performing the solid phase extraction method saves on solvent use, reduces solvent waste, and proves less expensive. (Zaugg et al 2002).

The SPE cartridge set up is displayed in Figure 8. The tubes were connected to the bottom end of the cartridge and the water samples were transferred from the bottles through the cartridges by vacuum. As the waste container filled, the vacuum would be turned off and spent water would be dumped in an appropriate waste stream provided in the MBMG lab. After the water was transferred through the cartridges they were wrapped in foil, placed in a baggy and allowed to dry overnight.

**Figure 8. The set-up for the SPE extraction cartridges with leur-lock fittings and tubes for transfer of water through the cartridges.**

After the cartridges were dried, glass Turbovap tubes were placed under the cartridge assemblage, and 15mL of 4:1 DCM:EE was pulled through the cartridge to elude the organic compounds off the solid phase of the cartridge. The sample bottles were rinsed with an additional 15mL of a 4:1 DCM:EE, pulled through the column by vacuum. The 15mL rinse insured that any hydrophobic compounds of interest are removed from the glass bottles. Once the solvent and solvent rinse from the bottles passed through the SPE cartridges, the Turbovap tubes were dried under nitrogen at 45ºC. The initial volume of solvent was condensed down to approximately 0.5mL, removed from the Turbovap where another 0.5 mL of 4:1 DCM:EE was added to rinse the tubes and bring the final volume to approximately 1.0mL based on marking on the bottom of the Turbovap tubes. A separate pipet for each tube was used to rinse and transfer the 1mL solvent from the tubes to GC vials. The vials were then stored in the freezer until GCMS analysis.

## 2.7. Gas Chromatography Mass Spectrometric Methods

After each batch went through the extraction process, the samples and blanks were analyzed using a Thermo Scientific Trace GC Ultra gas chromatograph and a Thermo ion trap mass spectrometer (Figure 9). The USGS method was written for an Agilent Technologies model 5973 GC/MS system. The Thermo Scientific GC/MS used in this procedure required slightly different adjustments and settings to meet acceptable performance criteria.


**Figure 9. Thermo scientific Trace GC Ultra gas chromatograph and Thermo ion trap mass spectrometer**

### 2.7.1. Recommended Gas Chromatography conditions from USGS

The USGS parameters for the oven were as follows: 40ºC hold for 3 minutes, ramp at 4ºC/min to 100ºC and 9ºC/min to 320ºC. The recommended temperature for the injection port was 290ºC with electronic pressure control set for a constant flow of helium carrier gas of 9 mL/min; injection volume, 2 µL, splitless injection. A splitless injection was chosen over a split injection because the analytes of interest are predicted not to be >0.01% of the sample. For trace analysis of analytes that are less than 0.01% of the sample, a splitless injection is appropriate. (Harris 2007)

### 2.7.2. Recommended Mass Spectrometric conditions USGS

Mass spectrometry is the detector of choice in chromatography. The mass spectrum is sensitive and provides both qualitative and quantitative information. Components in a complex chromatogram of poorly separated compounds can be readily measured with this instrument. (Harris, 2007) The following MS specifications were recommended by the USGS report: The Source analyzer in the USGS instrument was set at 200ºC; analyzer, 100ºC interface, held at 250ºC and programmed at 9ºC/min to 290ºC when the oven temperature surpasses 250ºC; electron-impact ionization mode. Full-scan mode extends from 45 to 450 atomic mass units in 0.5 seconds.

### 2.7.3. Actual Conditions used in Gas Chromatography Mass Spectrometry

Injection port temperature was set to 300ºC with a 10mL/min split flow and a splitless time of 2.00 minutes on the GC. The GC oven temperature was set to an initial temperature of 40ºC and held for 3.00 minutes, then ramped 100ºC by 4ºC/min with zero hold time and the last step ramped 9.0ºC/min until the temperature reached 320ºC and was held for 5 minutes. The ion source for the ITQ 900 mass spectrometer with one scanning event set to start 2.50 minutes after the GC started, since our compounds of interest did not elute off the GC column until after 20 minutes. An auto sampler was not part of the GCMS, so each sample was injected manually with a 7x DCM rinsed syringe.

The solvent used in this study, DCM:EE (dichloromethane: diethyl ether), and the analytes dissolved in the solvent eluted through a capillary column in the gas chromatograph. Molecules have different affinities for the stationary phase and come off the column at different retention times. The mass spectrometer then can capture, ionize, accelerate, deflect and detect the ionized molecules individually. A chromatogram can then be constructed by using different

mass-to-charge ratios resulting from the mass spectrometer. A chromatogram is a graph showing the detector response as a function of the elution time. The retention time for each component is the time needed after injection of the mixture onto the column until that component reaches the detector (Harris 2007).

# 3. Results

## 3.1. Hydrolab and Isotope Data

The data presented in Table 3, was intended to identify any potential trends with respect to the presence of the compounds of interest.

**Table 3. Summary of Hydrolab MS-5 Datasonde data collected 2014 and 2015. Alkalinity and carbon isotope data collected for the 2014 sample year only**

| August 29 2014 | Temperature (°C) | Specific Conductivity (µS/cm) | pH | DO %saturation | ORP mV | DO ppm | Alkalinity (mg/L as $CaCO_3$) | $\delta^{13}C$-DIC (‰) |
|---|---|---|---|---|---|---|---|---|
| **MT. Street** | 18.29 | 629.0 | 7.24 | 133.5 | 417 | 10.16 | 85 | -13.2 |
| **SBC-2** | 17.01 | 440.0 | 7.20 | 87.5 | 418 | 6.69 | 89 | -13.3 |
| **Miles Crossing** | 18.29 | 470.9 | 8.04 | 133.5 | 413 | 8.27 | 87 | -11.2 |
| **SBC-6** | 18.20 | 426.9 | 7.90 | 119.5 | 420 | 8.90 | 90 | -10.2 |
| **WSP** | 18.12 | 349.2 | 9.73 | 109.3 | 415 | 8.36 | 77 | -14.2 |
| **July 27 2015** | | | | | | | | |
| **MT. Street** | 12.48 | 320.3 | 7.05 | 109.3 | 424 | 9.39 | | |
| **SBC-2** | 16.10 | 520.5 | 6.61 | 83.1 | 474 | 6.61 | | |
| **Miles Crossing** | 16.29 | 576.0 | 7.61 | 135.0 | 413 | 10.72 | | |
| **SBC-6** | 16.73 | 633.5 | 8.15 | 150.9 | 395 | 11.83 | | |
| **WSP** | 19.88 | 360.8 | 9.76 | 106.4 | 349 | 7.81 | | |

The alkalinity for all the sites averaged between 77-90 mg/L $CaCO_3$ indicating the capacity to change the pH was within a normal range for surface waters during the 2014 sample collection.

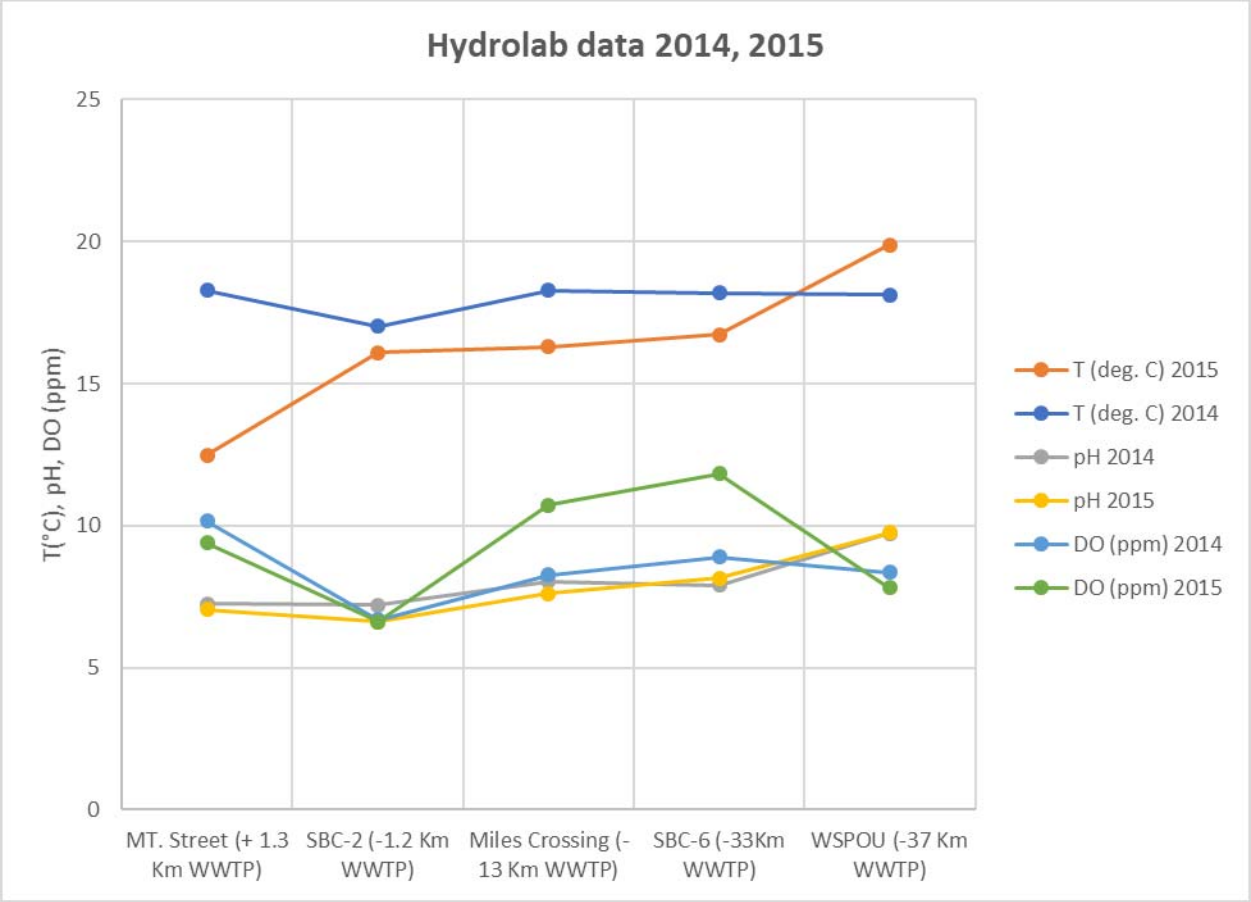


**Figure 10. Temperature, pH, and dissolved oxygen levels at each site for both years. Site upstream (+Km) and downstream (-Km) from the Wastewater Treatment Plant (WWTP)**

Hydrolab data trending is presented in figure 10. The pH was highest at the WSPOU site (pH = 9.73 for 2014 and pH = 9.76 for 2015) for both years. The higher pH at WSPOU was expected as the acidity is a known factor from regional mining activities. Lime is added to the water at this site during various times of the year to treat for metals in the water flowing into the ponds. SBC-2 (pH = 7.20), below the waste water treatment plant, had the closest to neutral pH (pH = 7) for the 2014 sample event. Montana street (pH= 7.05) had the closest to neutral pH for the 2015 sample event.

Temperature did not vary much as sampling for both years took place in the late summer. The warmest sample site was WSPOU for the 2015 sample event and at Montana street for the 2014 sample event. The temperature ranged more (12.48°C- 19.88°C) in 2015 than 2014. Both

collections occurred in from mid-morning to the afternoon one day and the temperature variations are most likely related to the time of the day.

The dissolved oxygen content varied between each site, Montana street and Miles crossing had the highest dissolved oxygen content (10.16 ppm) for the 2014 sampling event. The 2015 samples had the highest concentration (11.83ppm) at SBC-6 and the lowest concentration (6.61ppm) at the SBC-2 site. Dissolved oxygen content can indicate photochemical reactions occurring in the water. Dissolved oxygen generally decreases during daylight hours and increases during the night. (Parker, et al. 2013) Overall the water quality data was most similar at the SBC-2 site for both years.

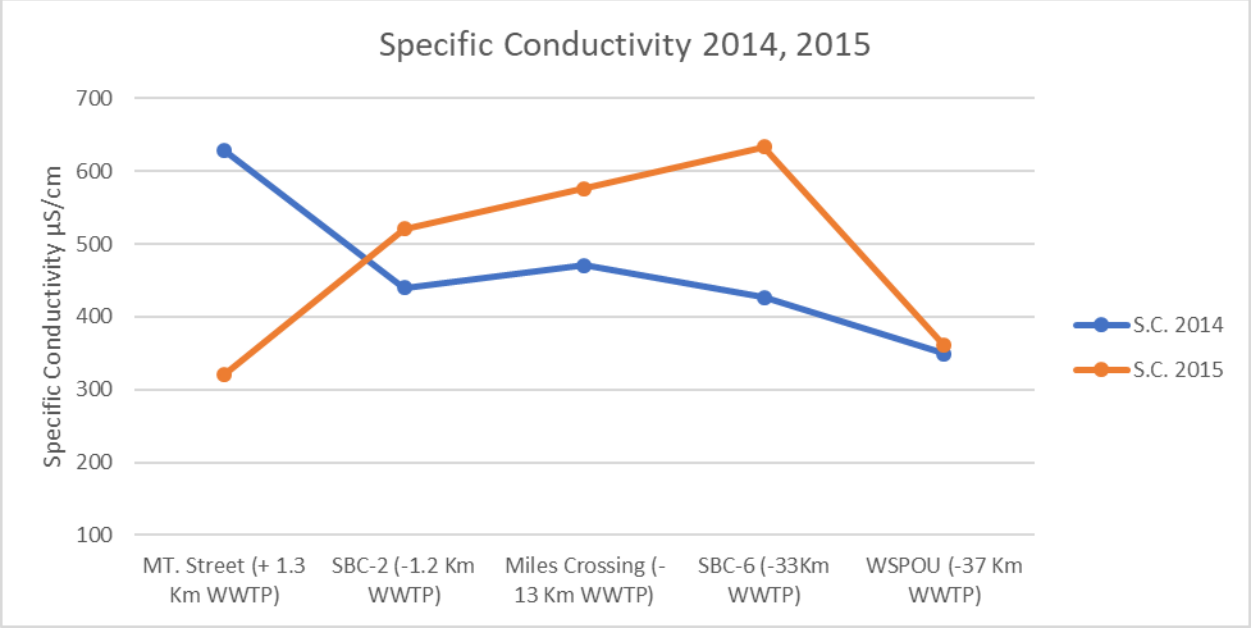


**Figure 11. Specific Conductivity trends for the 2014 and 2015 sample sites**

Specific conductivity for both years was plotted for each of the five sites and is presented in Figure 11. Specific conductivity indicates the ability of water to conduct electricity which indicates the ionic content of the water sample. Both years have varying specific conductivities at all sites tested 320.3 µS/cm - 629.0 µS/cm. SBC-6 had the highest specific conductivities for

the 2015 sample year, and Montana Street had the higher specific conductivities for the 2014 sample year, which also follows the dissolved oxygen trended for the same year.

Conductivity is inversely proportional to stream flow; slower stream flow normally will have a higher specific conductivity (Parker, 2013). It appeared that the 2015 stream flow was slow enough that it was not necessary to filter out particulates prior to extraction process.

There was not enough data to definitively determine a relationship between Datasonde data, isotope data and alkalinity with compound detection. The data does not appear to support any relationship between the data collected from the Hydrolab and the compounds of interest present in the water samples.

## 3.2. Chromatogram analysis

Chromatograms from the SBC-6 2015 sample for 17- β estradiol and is presented in Figure 12 to demonstrate the method. The retention time for the quantitation and confirmation ions appeared at 37.67 and 37.54 minutes. The quantitation peak area (27312) was used as the y value in the linear regression equation (y=59.669x-34319) determined from the standard addition curve constructed for 17-β estradiol. The concentration of 17-β estradiol in the SBC-6 sample was 1.03 ng/μL for the 2015 analysis. The chromatograms for all the environmental samples for each site can be found in appendix B.

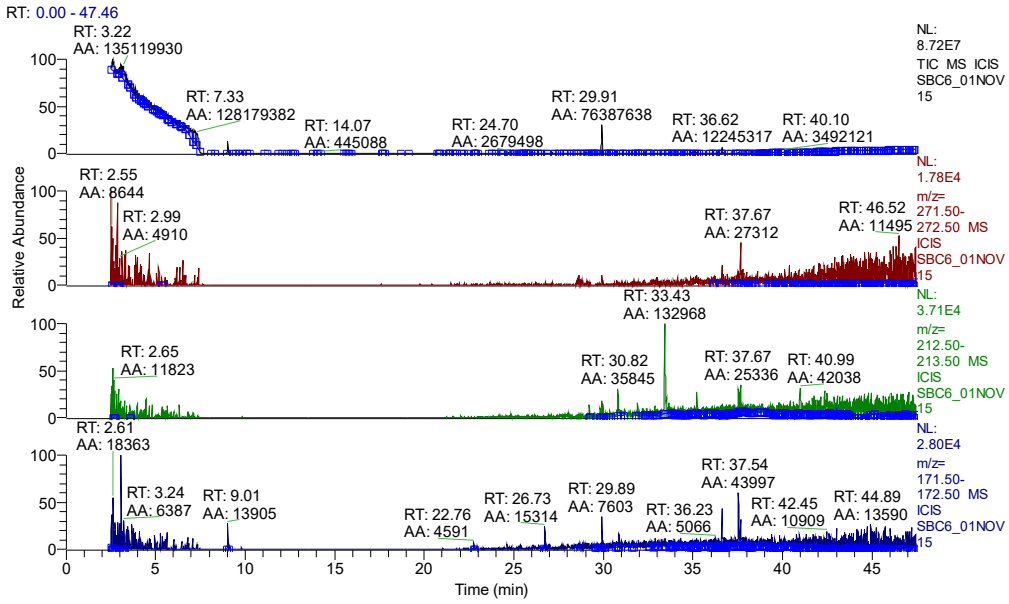


**Figure 12. Chromatograms for 17-β estradiol from SBC-6 analysis 2015 (RT= 37.67)**

## 3.3. Overall results

Table 4 summarizes the peak areas and retentions times derived from chromatograms produced by the water sample analysis for 2014 and 2015. The peak areas in Table 4 were used as the y variable for each linear regression equation derived from the standard calibration curves in appendix A. If a quantitation peak could not be isolated or visually seen it was considered to be below detection (BD). All identified peaks had retention times very close to the retention times determined by the standard analyses and were acceptable per the USGS acceptance criteria (±6 seconds).

**Table 4 Summary of Peak areas and retention times derived from chromatograms produced for both years. BD= below detection**

| Peak area and retention times for 2014 water samples | | | | |
|---|---|---|---|---|
| | **D-caffeine** | **DEET** | **17β-estradiol** | **α-ethynyl estradiol** |
| **Montana Street peak area** | 17986 | 146639 | 79751 | BD |
| **Retention Time (min)** | 30.39 | 26.27 | 37. | BD |
| **SBC-2 Peak area** | 50592 | 75906 | BD | BD |
| **Retention time (min)** | 30.39 | 26.27 | BD | BD |
| **Miles X Peak area** | 32839 | 60158 | BD | BD |
| **Retention time (min)** | 30.40 | 26.28 | BD | BD |
| **SBC-6 Peak area** | 36792 | 91875 | BD | BD |
| **Retention time (min)** | 30.38 | 26.27 | BD | BD |
| **WSPOU Peak area** | 38197 | 3313684 | 110788 | BD |
| **Retention time (min)** | 30.40 | 26.25 | 37.49 | BD |
| **Peak area and retention times for 2015 water samples** | | | | |
| | **D-caffeine** | **DEET** | **17β-estradiol** | **α-ethynyl estradiol** |
| **Montana Street peak area** | 32889393 | 626318 | 547226 | 95259 |
| **Retention Time (min)** | 29.91 | 26.42 | 37.65 | 38.24 |
| **SBC-2 Peak area** | 54274432 | 383632 | BD | 13129 |
| **Retention time (min)** | 29.93 | 26.42 | BD | 38.23 |
| **Miles X Peak area** | 27464898 | 242844 | 10254 | BD |
| **Retention time (min)** | 29.91 | 26.42 | 37.66 | BD |
| **SBC-6 Peak area** | 27468231 | 241331 | 27312 | BD |
| **Retention time (min)** | 29.91 | 26.42 | 37.67 | BD |
| **WSPOU Peak area** | 32094720 | 1581152 | 100563 | 173661 |
| **Retention time (min)** | 29.92 | 26.41 | 37.65 | 38.23 |

Table 5 summarizes the concentrations for the 2014 and 2015 samples using the peak area and the linear equations. The concentration for D-caffeine was also calculated based on the peak area isolated in the environmental samples for the 2015 sample analysis. The greatest

concentration for D-caffeine was 10.84 ppm this was made by adding 10 µL of a 10,840 µg/L weighed standard into a 10 mL volumetric flask. The results greatly exceeded the highest concentration analyzed (10,840 ppb (µg/L) or 10.84 ppm (ng/µL)) for the D-caffeine standard and could not be used to indicate a true percent recovery as they were double spiked. This made the data calculated for d-caffeine negligible for the 2015 sample set, as the concentrations of d-caffeine analysis were not comparable to the standard concentrations analyzed prior to environmental analysis. The 2014 sample chromatograms did contain peaks that were within the bracketing of the calibration curve established from surrogate analysis. The percent recoveries are presented in figure 5 for the 2014 samples. A surrogate concentration of 5.0 ppm (ng/µL) is expected from a 0.40-mL extract if 100 percent of the surrogate is recovered through the sample preparation for the USGS method. (Zaugg et al 2002). The percent recovery was based on the 5.0 ppm (ng/µL) and the final concertation of D-caffeine determined in the 2014 water samples. The samples were dried down to approximately 0.5-mL and the Turbovap tubes were rinsed with an additional 0.5-mL.

Blank samples consisting of 18MΩ water brought through the extraction process were analyzed prior to each site analysis and an example chromatogram is displayed in Figure 5. The blank analysis did not produce peaks based on ion m/z ratio isolation for the compounds of interest. This indicates that cross contamination or analyte carryover between each sample did not occur.

**Table 5. Summary of Concentrations for compounds of interest and the recovery of the surrogate added to each sample tested. BD = Below Detection limits.**

| Site | [DEET] ppm (ng/µL) | [17-βestradiol] ppm (ng/µL) | [α-ethynyl estradiol] ppm (ng/µL) | [D-caffeine] ppm (ng/µL) | D-Caffeine % recovery |
|---|---|---|---|---|---|
| MT. Street 2014 | 1.40 | 1.86 | BD | 1.51 | 30.16 |
| MT. Street 2015 | 2.84 | 9.75 | 2.82 | 1076 | |
| SBC-2 2014 | 1.19 | BD | BD | 2.55 | 50.92 |
| SBC-2 2015 | 2.11 | BD | 0.75 | 1775 | |
| Miles X 2014 | 1.14 | BD | BD | 1.98 | 39.62 |
| Miles X 2015 | 2.24 | 0.75 | BD | 899 | |
| SBC-6 2014 | 1.23 | BD | BD | 2.11 | 42.14 |
| SBC-6 2015 | 1.68 | 1.03 | BD | 851 | |
| WSPOU -2014 | 10.94 | 2.39 | BD | 2.15 | 43.04 |
| WSPOU-2015 | 5.72 | 2.26 | 4.80 | 994 | |

Warms Springs Ponds Operable Unit had the highest concentration of DEET at 5.72 ppm for the 2015 sample analysis and 10.94 ppm. 17-β estradiol was below the limits of detection at one site from the 2015 study and three sites from the 2014 study. 17-β estradiol was identified in both years at the Montana street site, with a high concentration (9.75 ppm) determined from the 2015 sample. 17-β estradiol was not detected at the SBC-2 site for both testing years but α-ethynyl estradiol was detected in the 2015 at this site. α-ethynyl estradiol was not detected in all five sites tested in 2014 and two of the sites tested in 2015. The 2015 Montana Street site and the 2015 WSPOU identified concentrations for all three compounds of interest. SBC-2 had the highest percent recovery (50.92%) for the 2014 sample analysis and Montana Street had the lowest percent recovery (30.16%).

Figure 12 graphically shows the concentration for the compounds of interest that were detected in the 2014 samples. It clearly shows that α-ethynyl estradiol was not detected at any sited for 2014. The highest concentration of 17-β estradiol (9.75ng/µL) was detected at the Montana Street site, the first site sampled in the town of Butte.

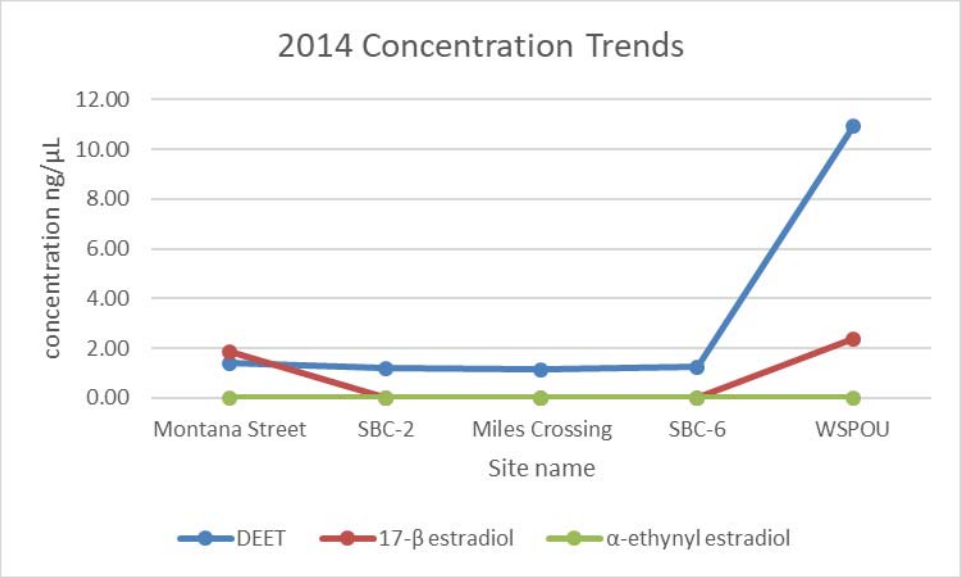


**Figure 13. Cocentration trends for DEET, 17-β estradiol and α-ethynyl estradiol for the 2014 sample analysis**

Figure 13 shows the concentration trends for the compound of interest for the 2015 samples. Miles Crossing and SBC-6 did not contain α-ethynyl estradiol and SBC-2 did not contain 17-β estradiol in the 2015 samples. DEET was detected in every test site. Overall the compounds of interest were qualitatively identified in 63% of the chromatograms for the combined years. For the 2014 samples 47% quantitatively contained the compound of interest, were 0% of the sites tested for 2014 did not have any peaks for α-ethynyl estradiol. 80% of the waters tested in 2015 had peak areas identified for the compounds of interest and the concentrations were determined from the calibration curves.

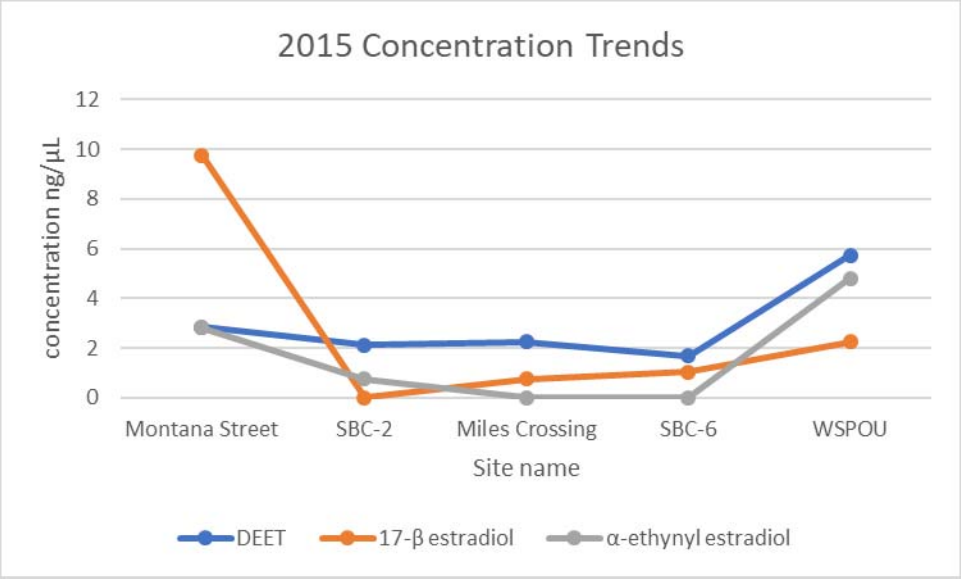


**Figure 14. Cocentration trends for DEET, 17-β estradiol and α-ethynyl estradiol for the 2015 sample analysis**

# 4. Discussion

## 4.1. Organic wastewater compounds in Silver Bow Creek.

Figure 13 shows the sampling sites and the concentrations for the compounds of interest for both the 2014 and 2015 samples. It is important to note that although there were percent recovery issues with the surrogate, D-caffeine, the compounds of interest were present and qualitatively identified in the water collected from the sites. DEET is a commonly used insect repellent and not surprisingly was detected at all sites tested in this study. The 2015 samples appeared to have larger peak areas for DEET than the 2014 samples (except WSPOU) and can be observed in the chromatograms in appendix B. All but WSPOU 2014 had higher concentrations of DEET for the 2015 samples. The 2014 WSPOU site had the highest concentration (10.94 ng/µL) of DEET for both years. Montana Street and WSPOU 2015 samples both had higher concentrations for the compounds of interest, compared to the three sites (SBC-2, Miles Crossing, and SBC-6) in between them.

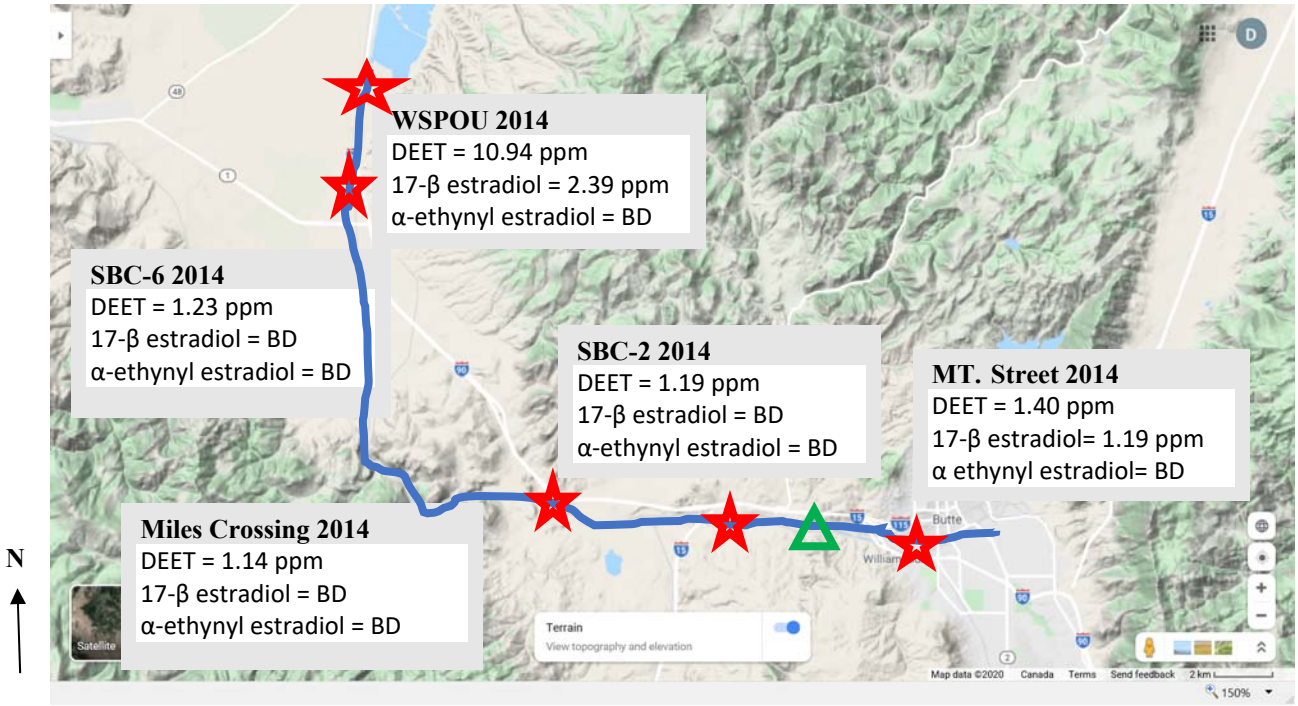


**Figure 15. Sampling sites and concentrations of the compound of interest in 2014.**

The estradiol compounds appeared to be better detected in the 2015 sample analysis. 17-β estradiol was identified in both years at the Montana street site. This indicates that there may be some sewage water entering Silver Bow Creek before entering the waste water treatment plant. SBC-2 did not show a presence of 17-β estradiol for both years but did show a presence of α-ethynyl estradiol for the 2015 sample. SBC-2 is the first site downstream from the waste water treatment plant which may have treated for the estradiol compounds and thus have lessened their presence at the SBC-2 site. In 2016 major upgrades were implemented at the Waste Water Treatment Plant. It would be worth repeating this study to see if OWC concentrations at SBC-2 and downstream have been reduced.

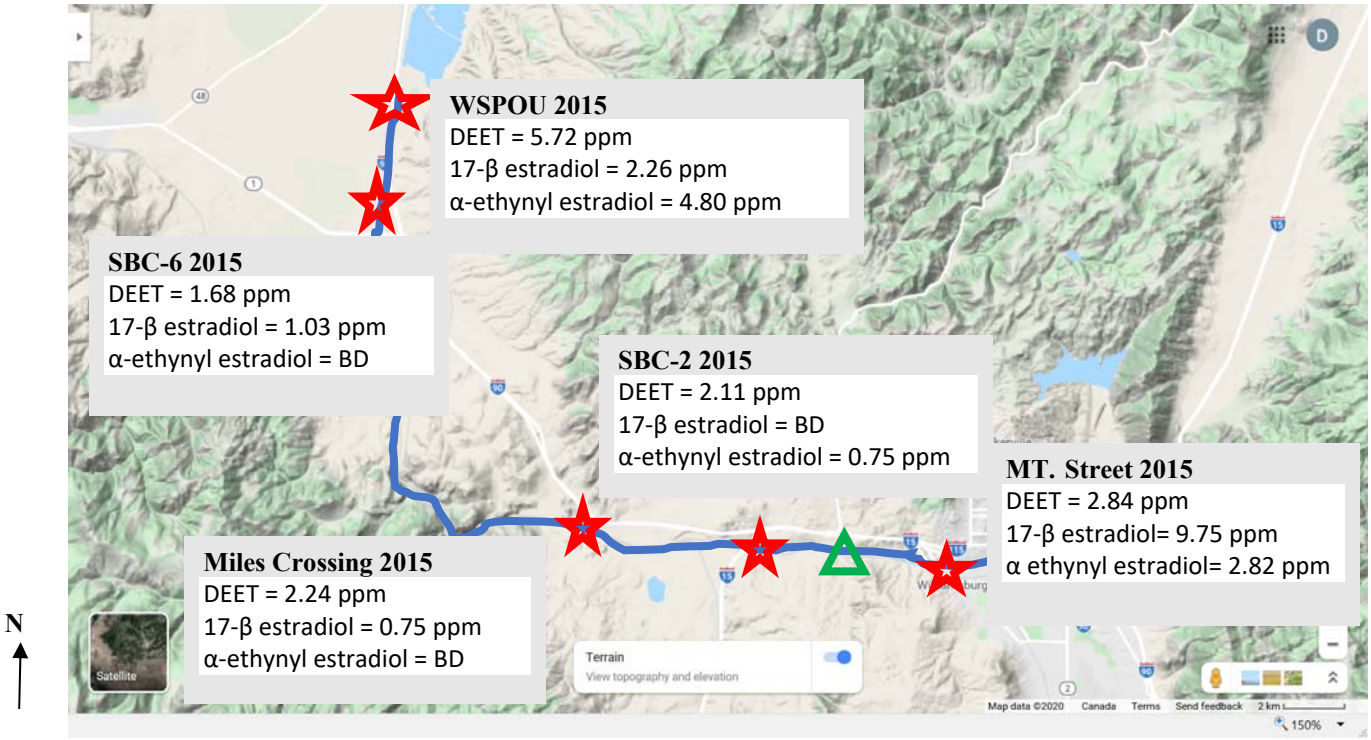


**Figure 16. Sampling sites and concentrations of the compound of interest in 2015**

The 2015 analysis from the Montana Street site and WSPOU did identify quantitation peaks for all three compounds of interest. Both sites do appear to have more human presence in the area that may contribute to the peaks for all three compounds on interest. The 2015 concentrations for the compounds of interests are presented in Figure 13 and 14. Miles Crossing and SBC-6 produced measurable amounts of 17-β estradiol and SBC-2 had a measurable concentration of α-ethynyl estradiol. The results of this study are higher than what other studies normally find in surface waters. The Gallatin County Study determined 2.25 and 9.05 ng/L (ppb) of 17-β estradiol in wastewater samples and as high as 79 ng/L (ppb) of DEET in surface water samples (Icopini et al, 2016). All the sites in both years showed a high presence of DEET. DEET was specifically not used or applied to clothing during water collection and should not have been a source of DEET in this study. The main contributing factor for the higher than normal concentrations most likely were derived from errors during standard analysis as the

standards were analyzed sporadically and did not preserve well in the freezer. This may have led to inaccurate line equations that may not have correctly bracketed compound concentrations derived for the construction of the calibration curves.

The 2014 surrogate, D-caffeine did produce peaks that fell within the standard calibration curve. The final concentration derived from the quantitation peak analysis of D-caffeine in the 2014 samples was used to determine percent recovery and can be seen in table 5. The highest percent recover for the 2014 samples was 50.92% from SBC-2. Not salting the water samples in 2014 may have resulted in less compound preservation for the analysis.

## 4.2. Trending

SBC-2 had a higher specific conductivity in 2015 than in 2014, which may associate with 17-β estradiol detection for 2015. The higher specific conductivity in 2015 may indicate a higher percentage or wastewater in Silver Bow Creek

SBC-6 had a higher concentration of dissolved oxygen in 2015 than in 2014 but a lower concentration of the compounds of interest based on quantitation peak area. Otherwise the rest of the sites had similar dissolved oxygen reading for both years. It cannot be definitively concluded that there are any relationships between dissolved oxygen and compound concentrations considering all the logistic issues encountered during sample preparation, i.e. double spiking the 2015 samples and not adding salt to the 2014 samples right away. It is interesting to observe that WSPOU did have similar concentrations (2.39 ng/μL, and 2.26 ng/μL) for 17-β estradiol and the pH was lowest at this site. The water quality data may not directly relate to the compounds themselves, and further studies would be necessary to establish any relationships.

## 4.3. Experimental Limitations

Due to time constraints and instrument availability, most calibration standard concentrations were only analyzed once. Unlike the USGS method the lack of standard replicates and time delays between standard analysis increased error of establishing true and accurate method detection limits (MDL's). The MDLs for this study are based on peak area and the concentration from a single run. The standard analysis was performed sporadically, because the GCMS used in this study was single injection and each analysis took about 4 hours. After the standards were prepared, they were placed in a freezer until the next dilution was prepared.

Comparing the D-caffeine chromatograms produced by the analysis in 2014 and 2015, the 2015 chromatograms appear to have a much larger peak area and less noise compared with the 2014 chromatograms. The D-caffeine peak areas (27468231) greatly exceeded the areas used to construct the calibration curve for the 2015 analysis. The largest area (329688) used to determine the calibration curve for D-caffeine for the 2015 analysis as presented in Figure 3. It was suggested in the USGS method that, if the calculated concentration of a compound exceeds the highest concentration point of the calibration curve by 20 percent or more, one should add higher concentration calibration standards to the curves or dilute the extract to bring the compound response within the range of the calibration curve. (Zaugg et al, 2002). Due to time constraints, re-analysis of the standards for D-caffeine to higher concentrations, or another collection and extraction of the environmental samples did not take place. The surrogate D-caffeine may not have been appropriately added to the water samples because 1-mL was added to the samples at least two times and not 100µL as suggested by the USGS method. There were several challenges finding appropriate pipettes and lab equipment to match the scale of the USGS method.

The 2014 samples were spiked with D-caffeine, but peak areas for the 2014 samples produced low percent recoveries. Although the percent recoveries were low the quantitation peak areas were within the area range used to construct the calibration curve for the 2014 samples. Percent recovery for of D-caffeine could not be quantitatively determined for the 2015 samples. It was acceptable to proceed with the analysis because "Concentrations reported by the NWQL for compounds and surrogates in environmental samples are never corrected for spike or surrogate recoveries." (Zaugg et al, 2002). This means that regardless of whether the D-caffeine had a percent recovery, it would not have been used as a correction factor for the environmental samples.

## 4.4. Recommended experimental and sampling improvements

There are several key learning points from this study. Limits with instrumentation, not having an autosampler, and having to analyze standards, samples, and blanks sporadically did have had an impact on the study. The preparation of the standards would take place one day and maybe analyzed on another day. It was observed that the longer a standard was stored the more likely the estradiol standards would precipitate out of solution. The USGS method was likely meant for a standard calibration curve to be analyzed within a 24-hour period of sample prep.

It was observed that precipitates formed in the estradiol vials stored in the freezer. This made it challenging to get an $R^2$ value over 0.98. The USGS method described using DCM as the solvent for the standards. If this study is repeated a more polar solvent i.e. diethyl ether may be considered to dissolve the estradiol standards used in this assay, to keep the powdered standards (17-β estradiol, and α-ethynyl estradiol) in solution and the use of an autosampler could resolve many issues.

Now that there has been familiarization with this method, performing this test could be a less challenging knowing the limitations and using more appropriate equipment to determine more accurate concentrations for the water samples that had peak areas appear for the compounds of interest. Better sample preparation and quality control analysis would have contributed to a more robust and comprehensive study. Although there were challenges with standard analysis, determining true MDLs for the GCMS, and sample preparation issues, the presence of the compounds of interest were at least qualitatively identified in the water samples. It would be worth repeating this study in the future to more accurately determine the concentration of these compounds at the same sites tested here, as the contaminants were found in numerous locations.

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## Appendix A: Calibration curves derived by standard analysis for the compounds of interest and DEET.

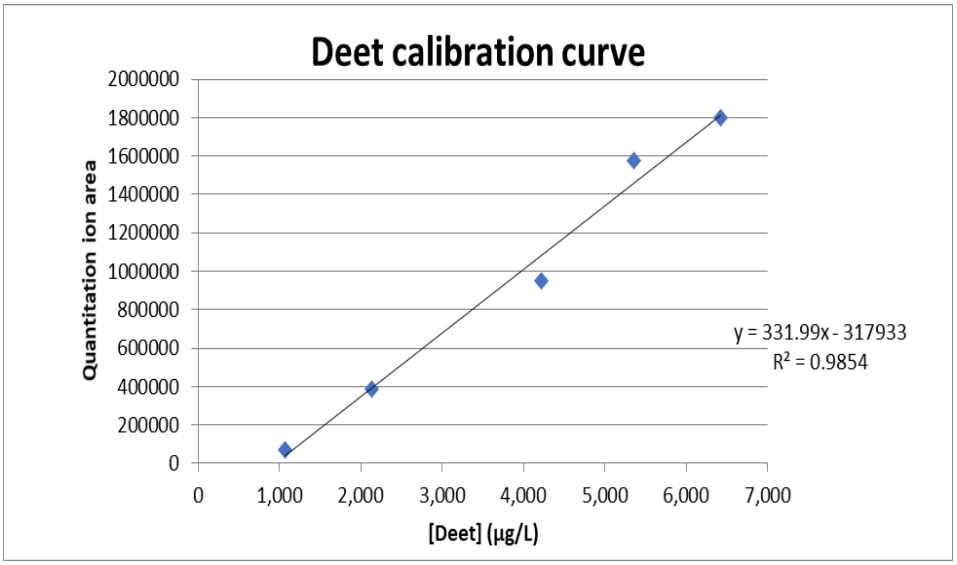


Figure 17: Standard calibration curve for DEET

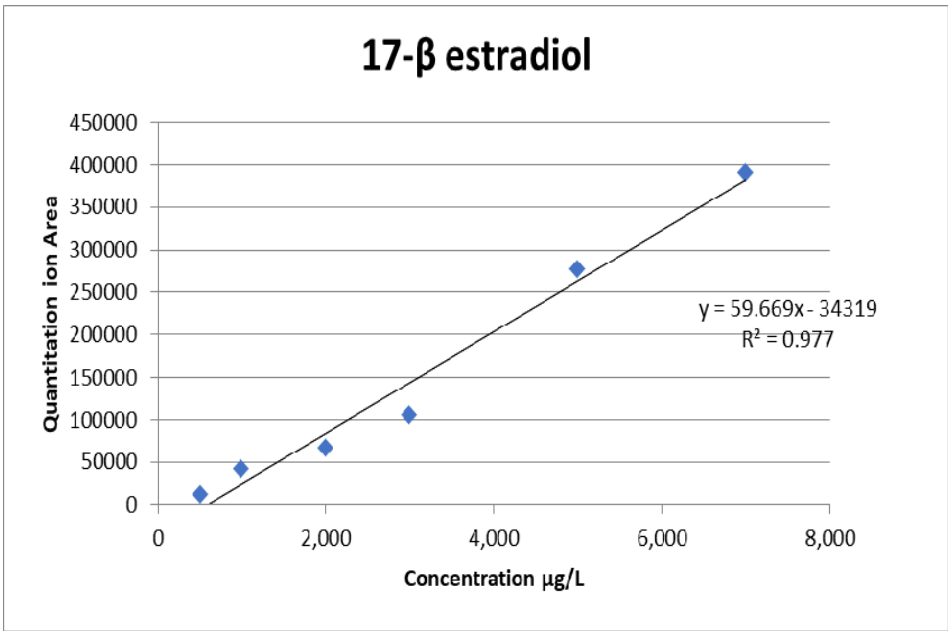


Figure 18: Standard calibration curve for 17-β estradiol

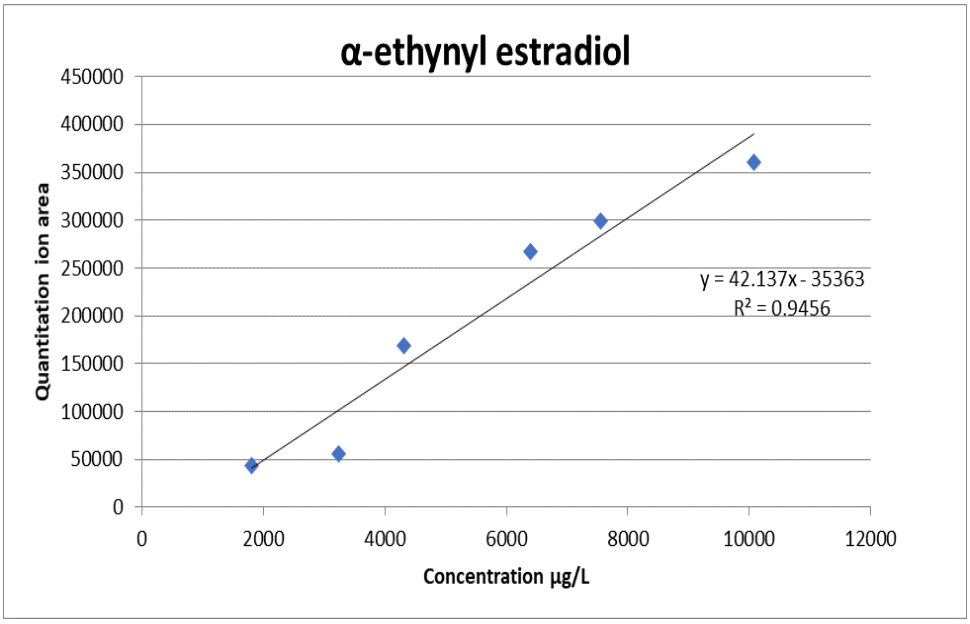


Figure 19: Standard calibrtion curve for α-ethynyl estradiol

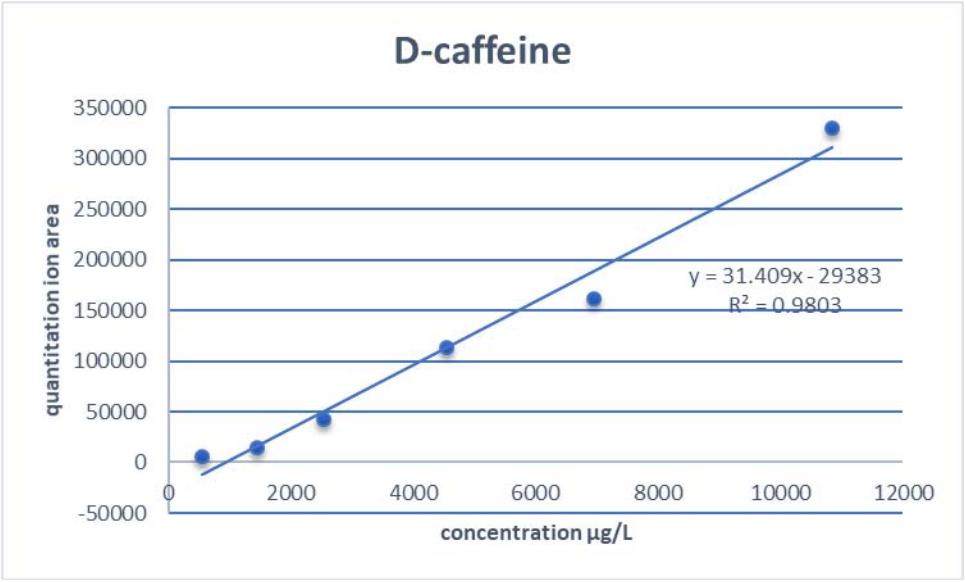


Figure 20: Standard calibration curve for D-caffeine

# Annex B: Environmental sample chromatograms for the 2014 and 2015 sample analysis

## 1. Montana Street

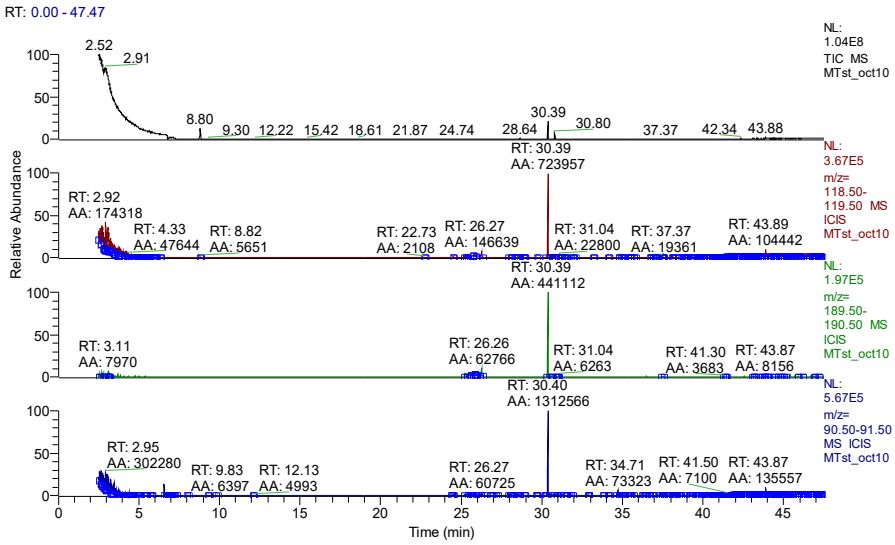


Figure 21: Montana street (2014) chromatogram isolated for DEET (RT = 26.27min)

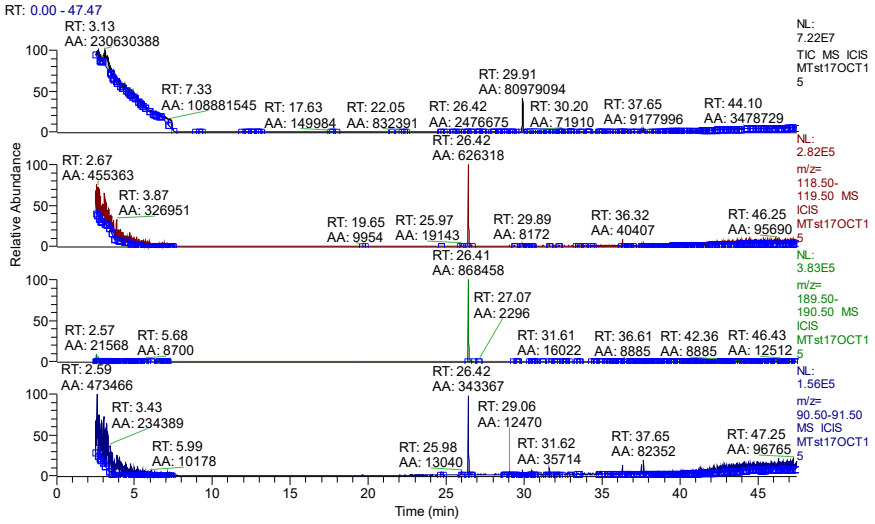


Figure 22: Montana street (2015) chromatogram isolated for DEET (RT = 26.42min)

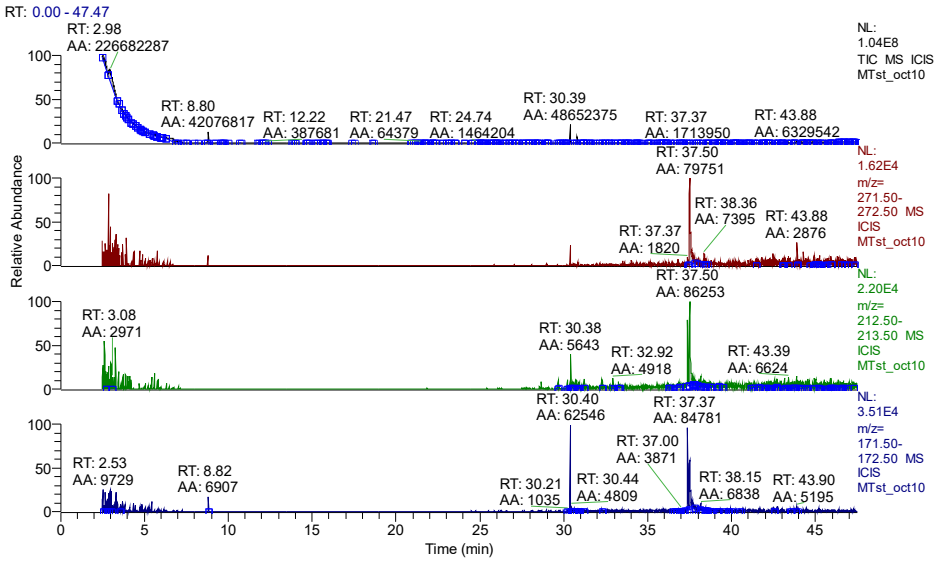


Figure 23: Montana street (2014) chromatogram isolated for 17-β estradiol (RT = 37.50min)

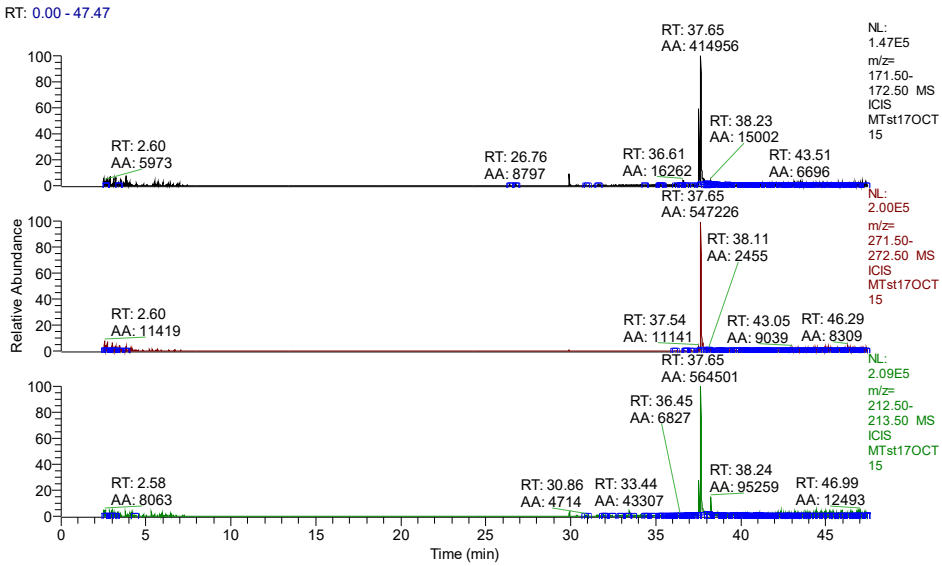


Figure 24: Montana street (2015) chromatogram isolated for 17-β estradiol (RT = 37.65min)

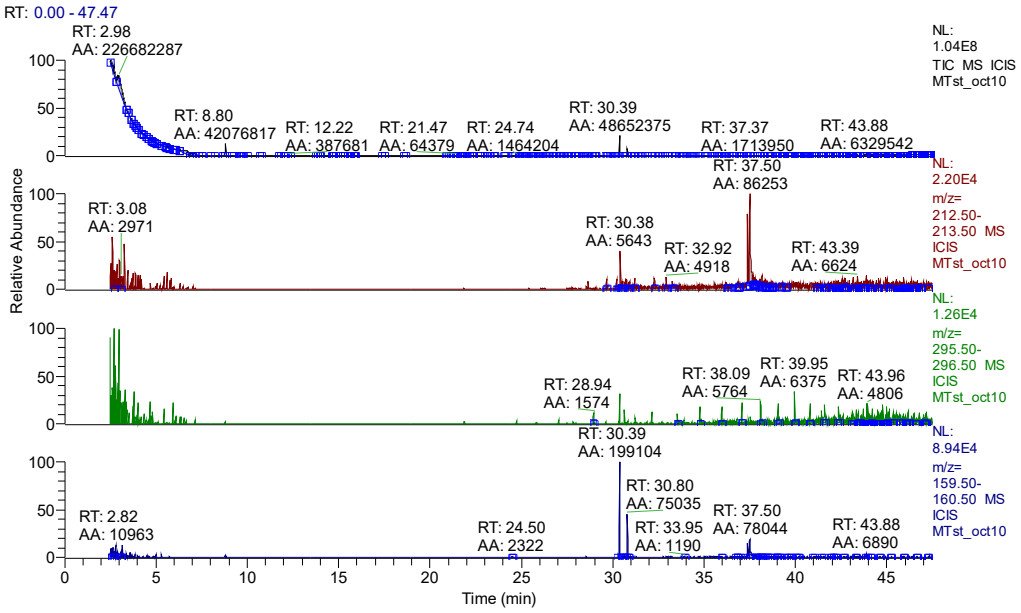


Figure 25: Montana street (2014) chromatogram isolated for α-ethynyl estradiol (BDL)

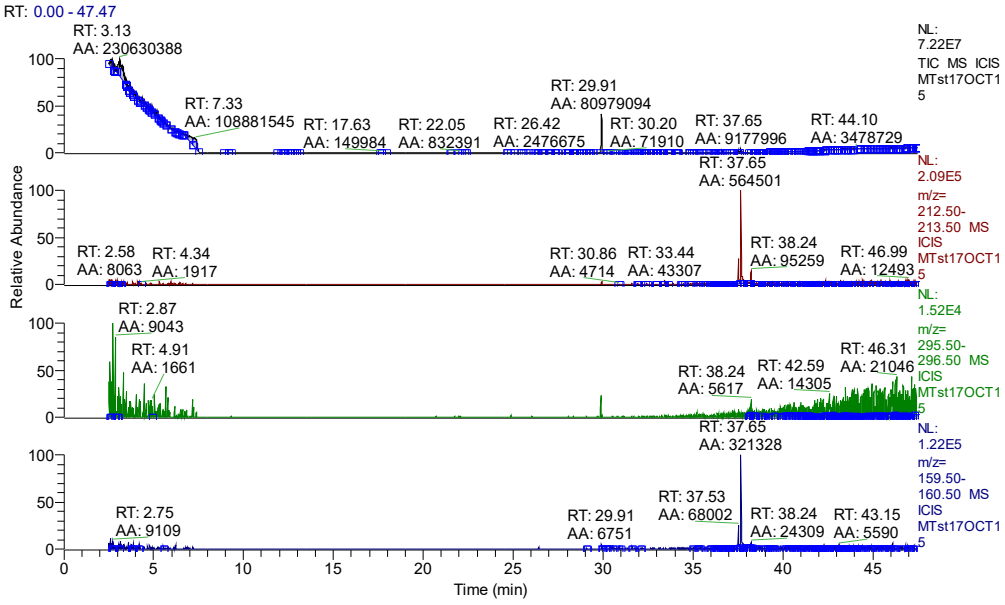


Figure 26: Montana street (2015) chromatogram isolated for α-ethynyl estradiol (RT = 38.24min)

## 2. SBC-2

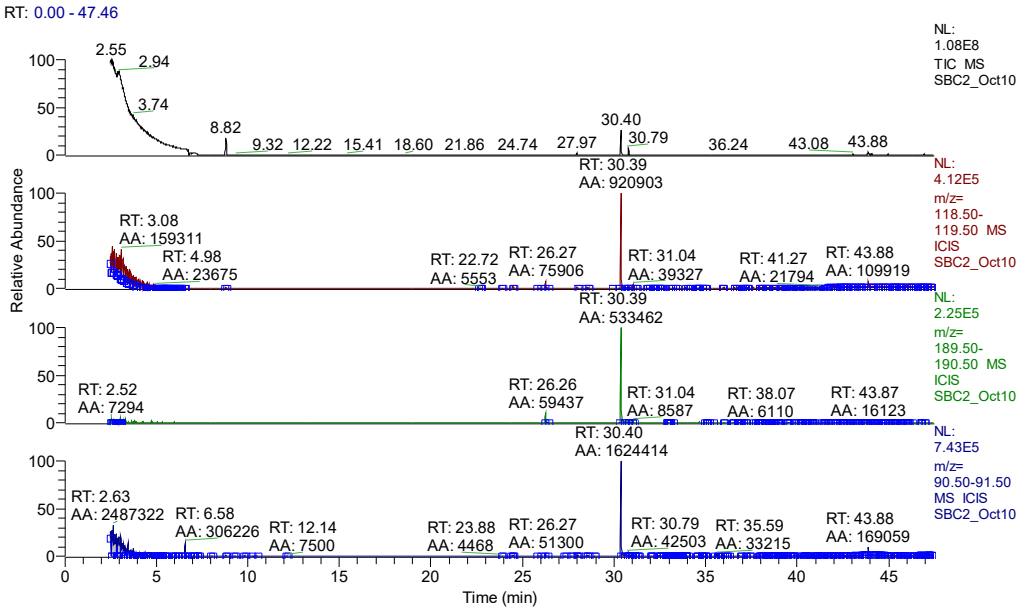


Figure 27: SBC-2 (2014) chromatogram isolated for DEET (RT = 26.27min)

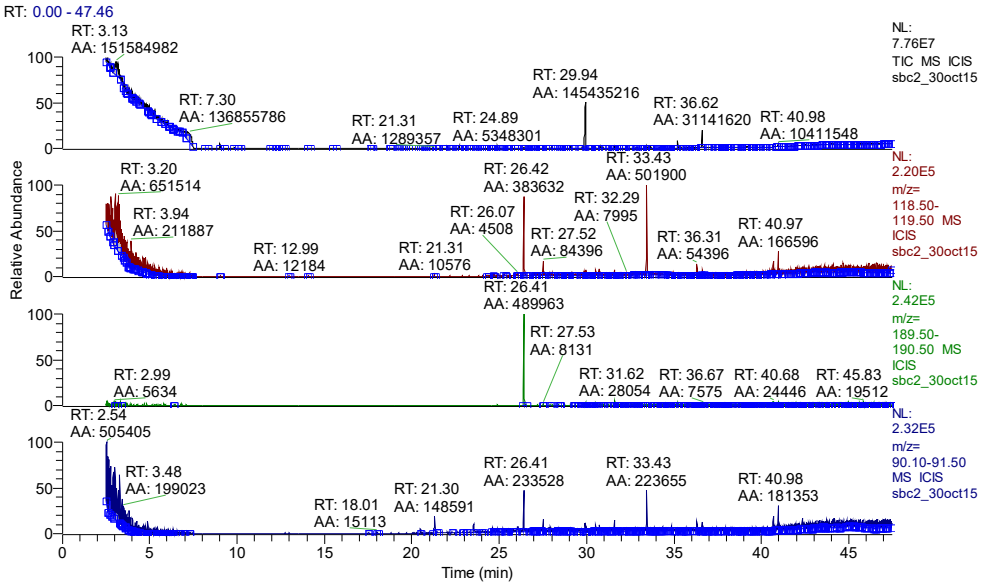


Figure 28: SBC-2 (2015) chromatogram isolated for DEET (RT= 26.42)

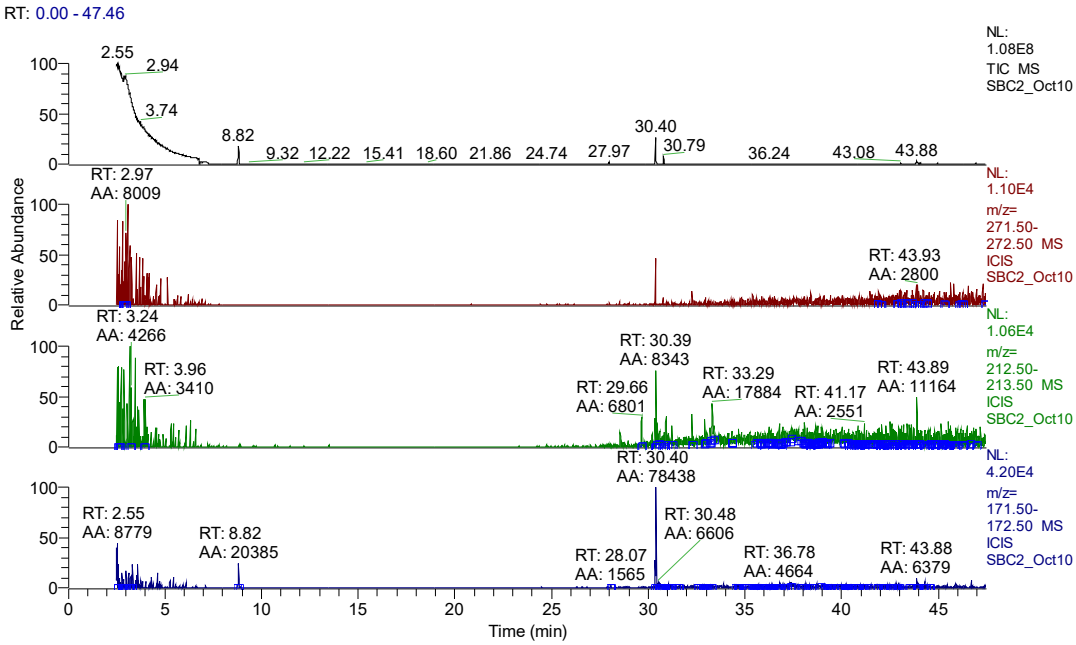


Figure 29: SBC-2 (2014) chromatogram isolated for 17-β estradiol (BDL)

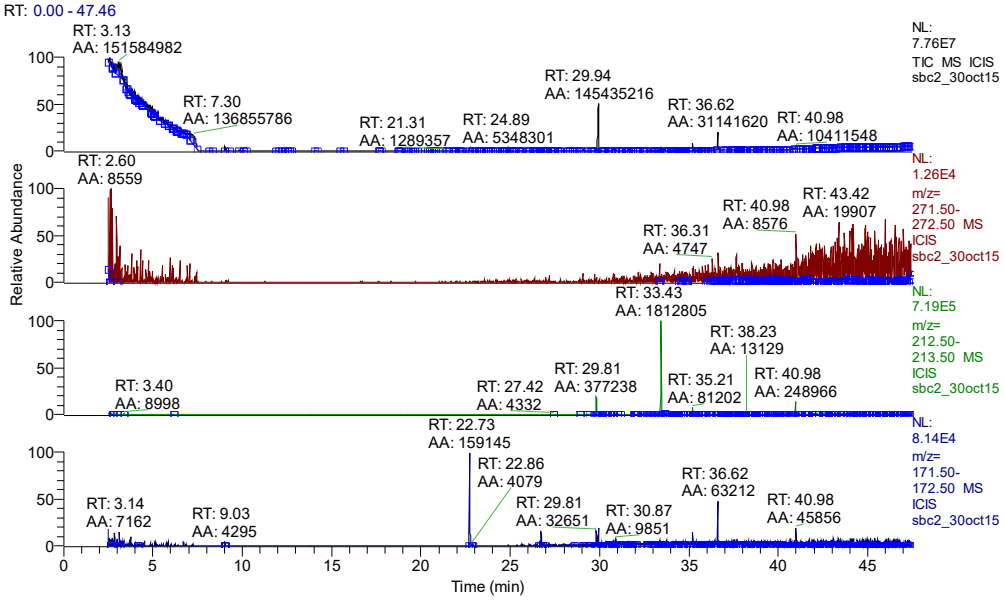


Figure 30: SBC-2 (2015) chromatogram isolated for17-β estradiol (BDL)

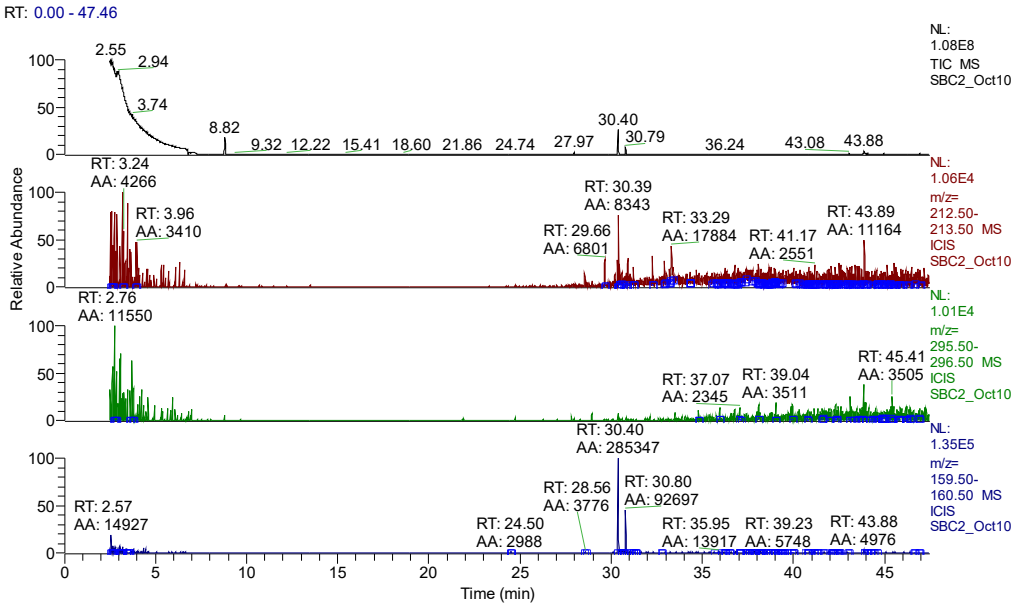


Figure 31: SBC-2 (2014) chromatogram isolated for α ethynyl estradiol (BDL)

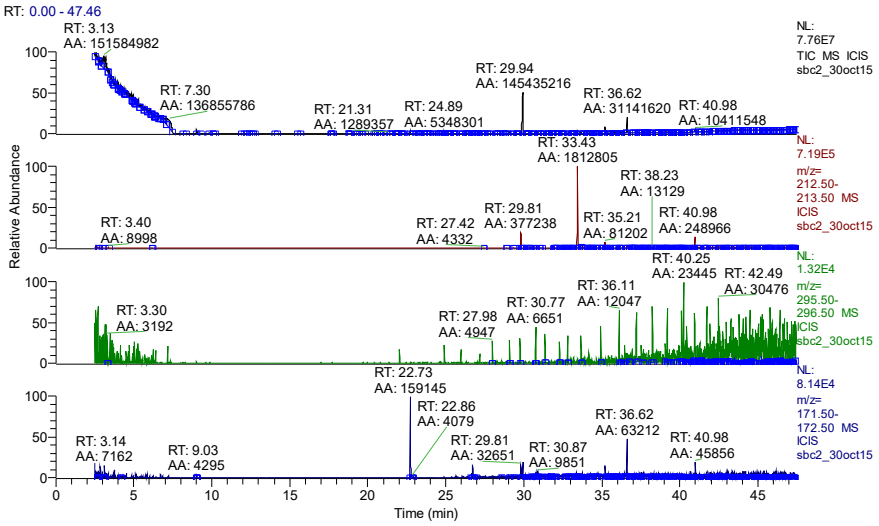


Figure 32: SBC-2 (2015) chromatogram isolated for α ethynyl estradiol (RT= 38.23)

3. Miles Crossing

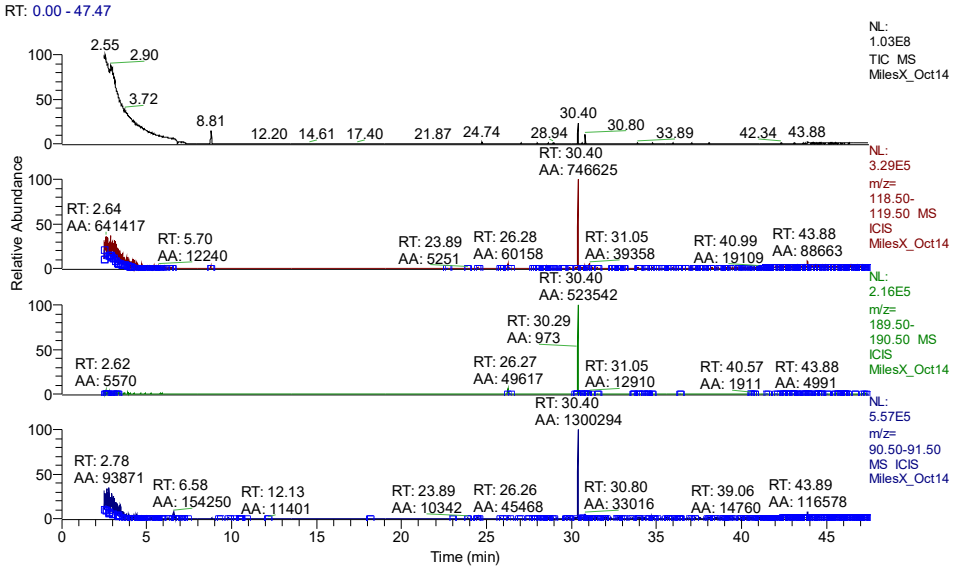


Figure 33: Miles Crossing (2014) chromatogram isolated for DEET (RT= 26.28)

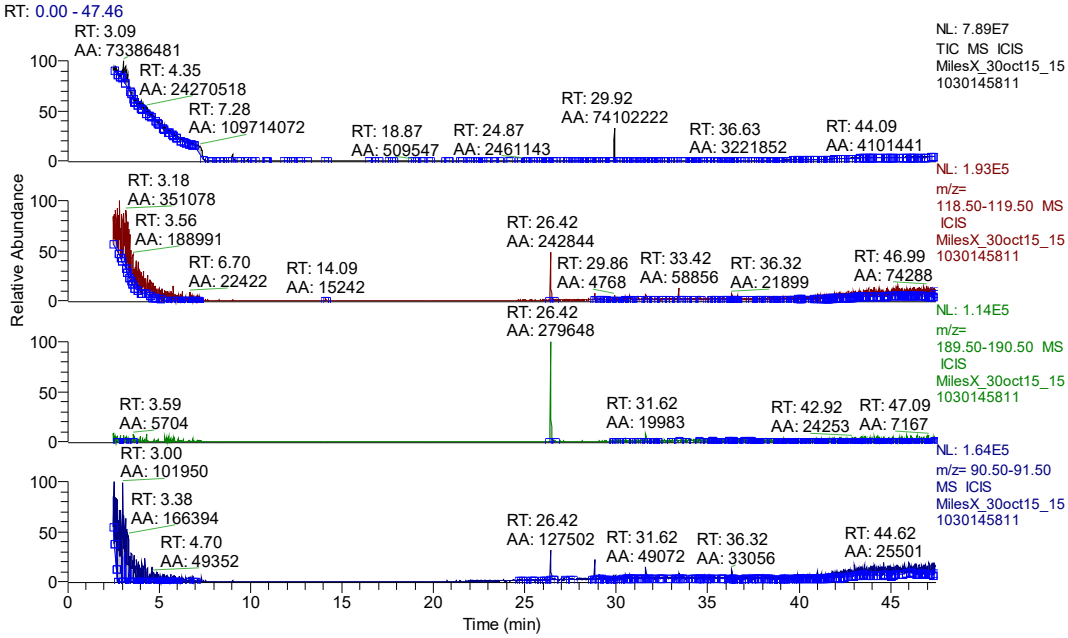


Figure 34: Miles Crossing (2015) chromatogram isolated for DEET (RT= 26.42)

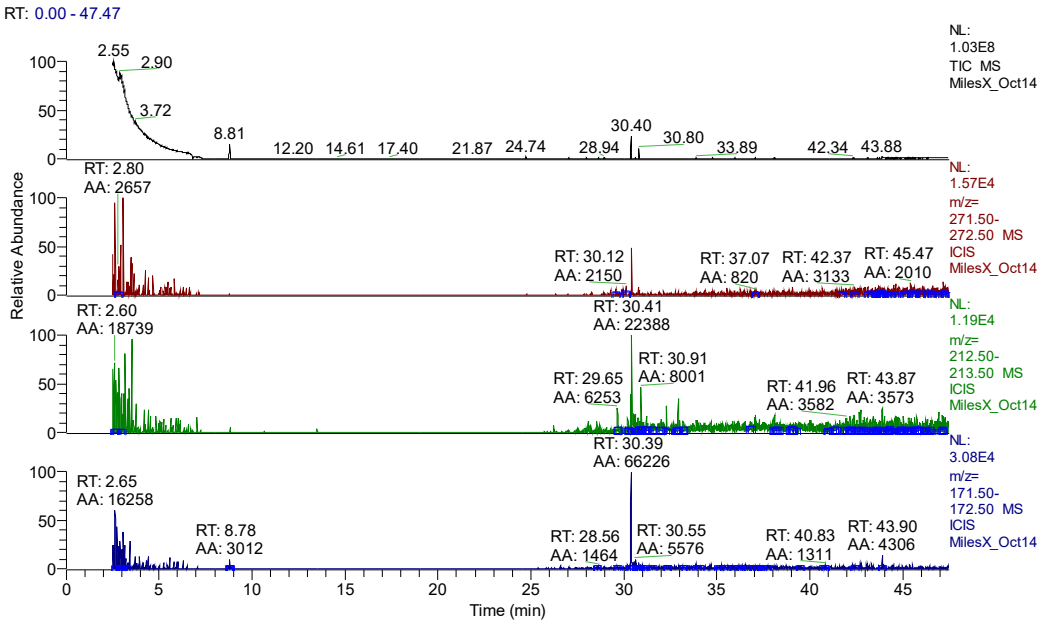


Figure 35: Miles Crossing (2014) chromatogram isolated for 17-β estradiol (BDL)

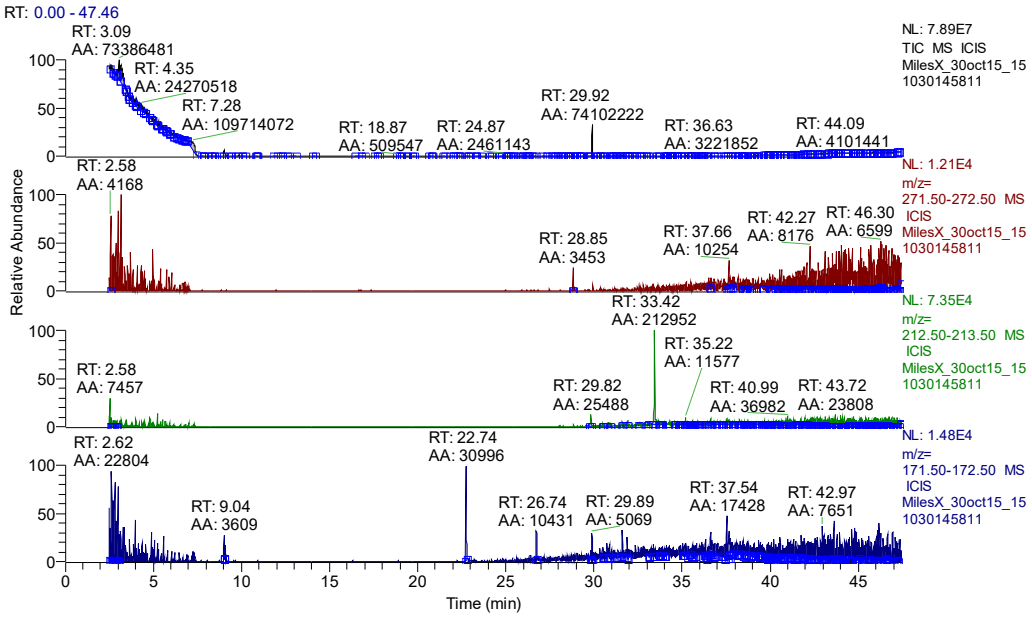


Figure 36: Miles Crossing (2015) chromatogram isolated for 17-β estradiol (RT= 37.66)

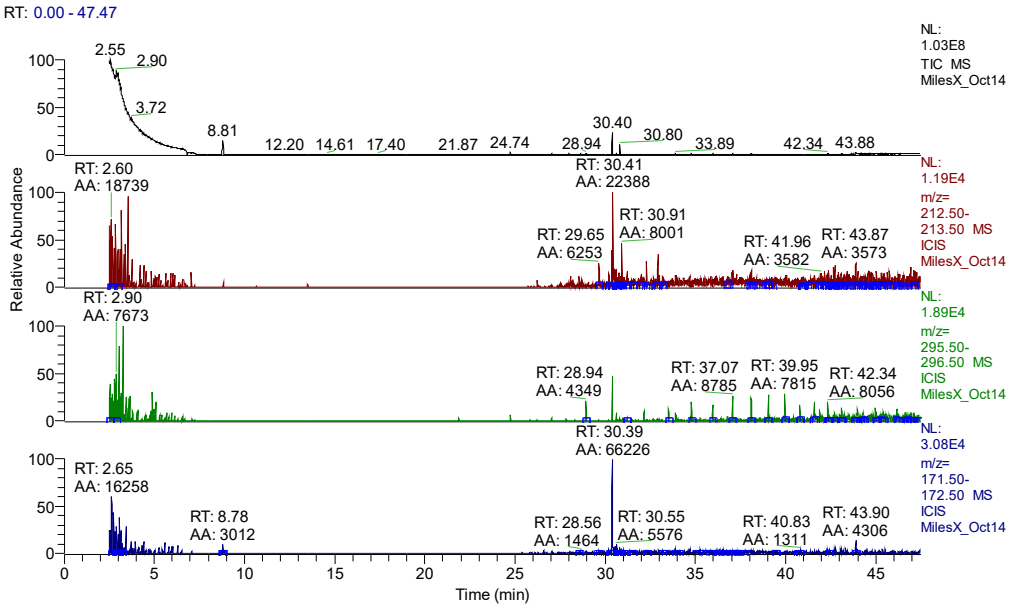


Figure 37: Miles Crossing (2014) chromatogram isolated for α ethynyl estradiol (BDL)

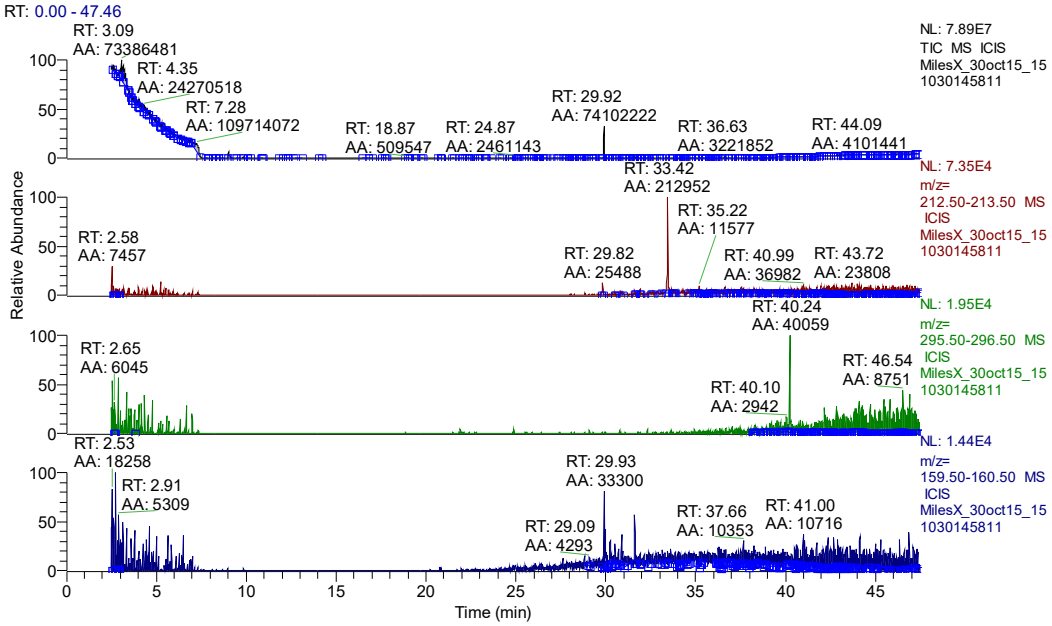


Figure 38: Miles Crossing (2015) chromatogram isolated for α ethynyl estradiol (BDL)

## 4. SBC-6

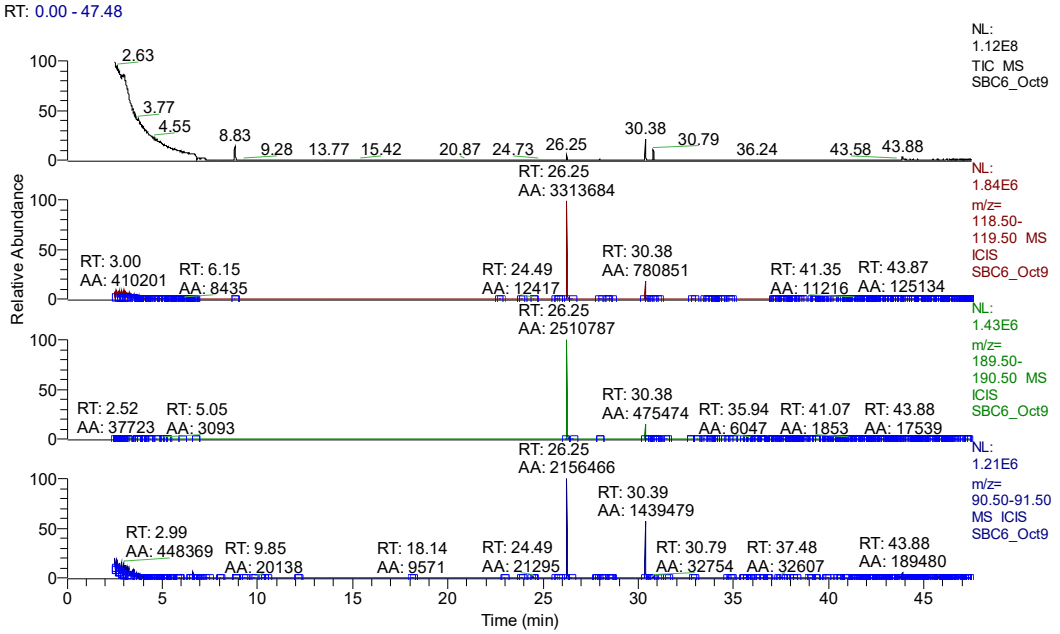


Figure 39: SBC-6 (2014) chromatogram isolated for DEET (RT= 26.25)

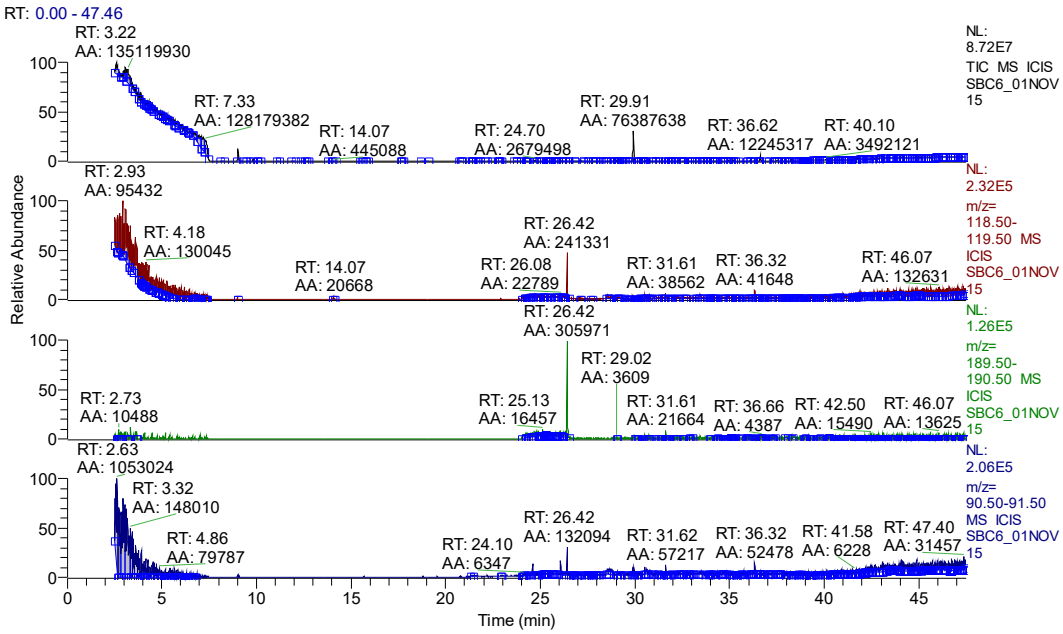


Figure 40: SBC-6 (2015) chromatogram isolated for DEET (RT= 26.42)

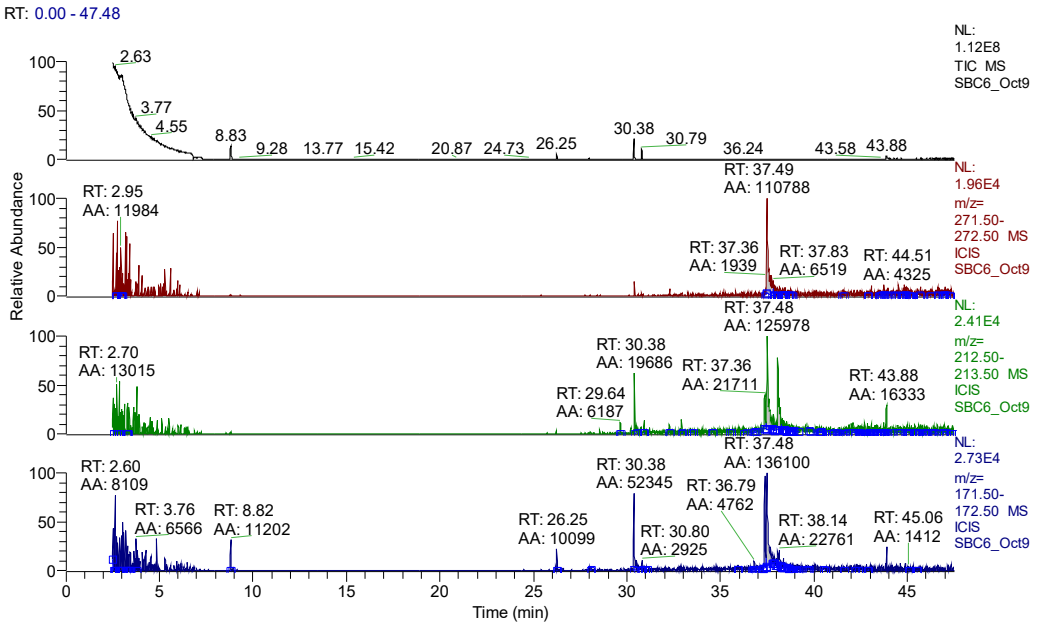


Figure 41: SBC-6 (2014) chromatogram isolated for 17-β estradiol (RT= 37.49)

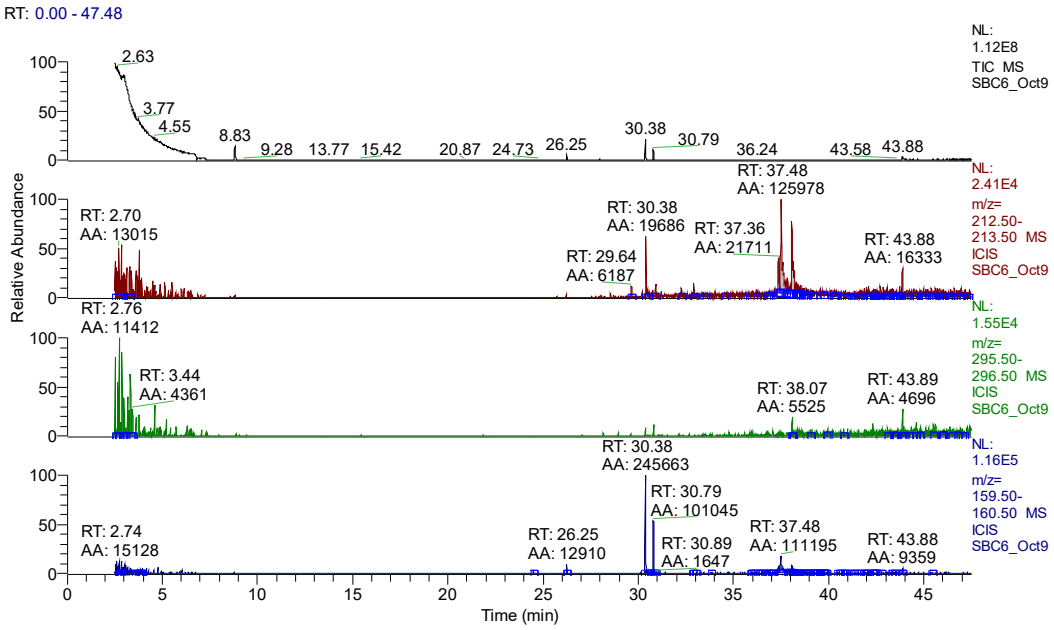


Figure 42: SBC-6 (2014) chromatogram isolated for α ethynyl estradiol (BDL)

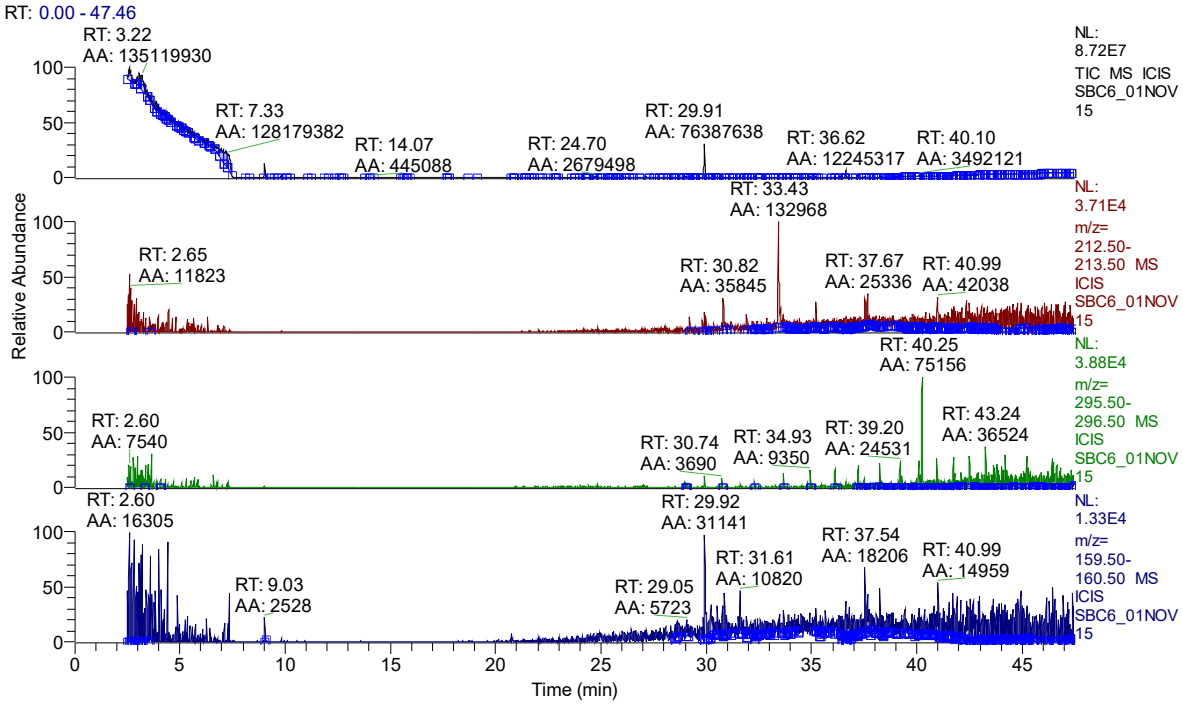


Figure 43: SBC-6 (2015) chromatogram isolated for α ethynyl estradiol (BDL)

5. WSPOU

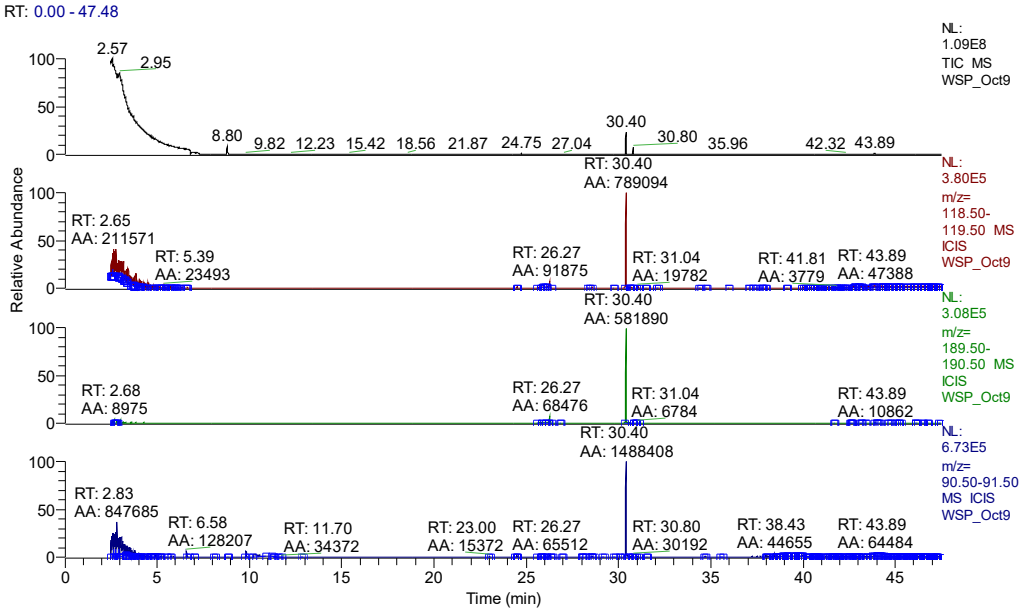


Figure 44: WSPOU (2014) chromatogram isolated for DEET (RT= 26.27min)

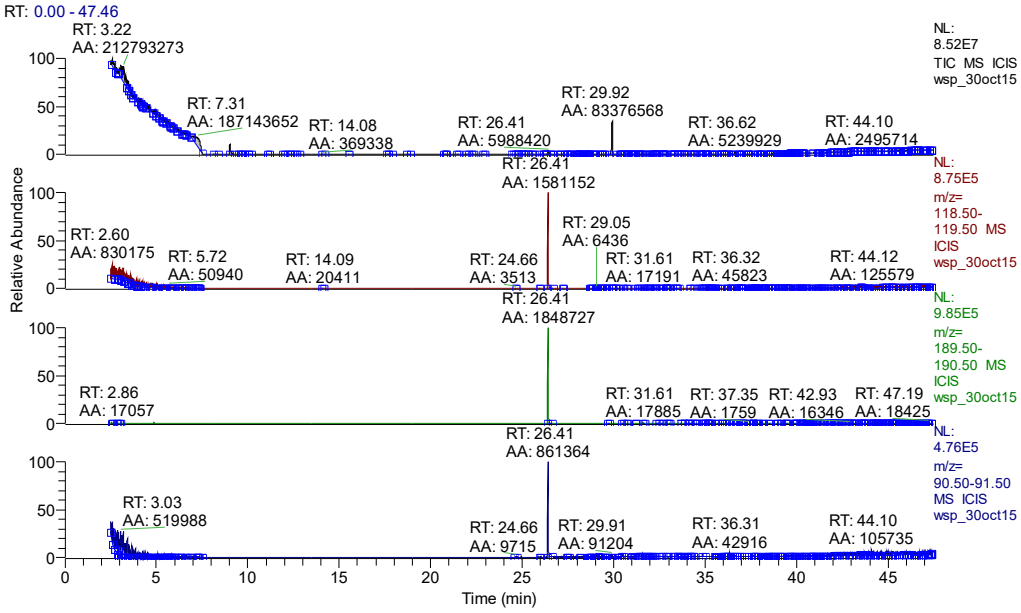


Figure 45: WSPOU (2015) chromatogram isolated for DEET (RT= 26.41min)

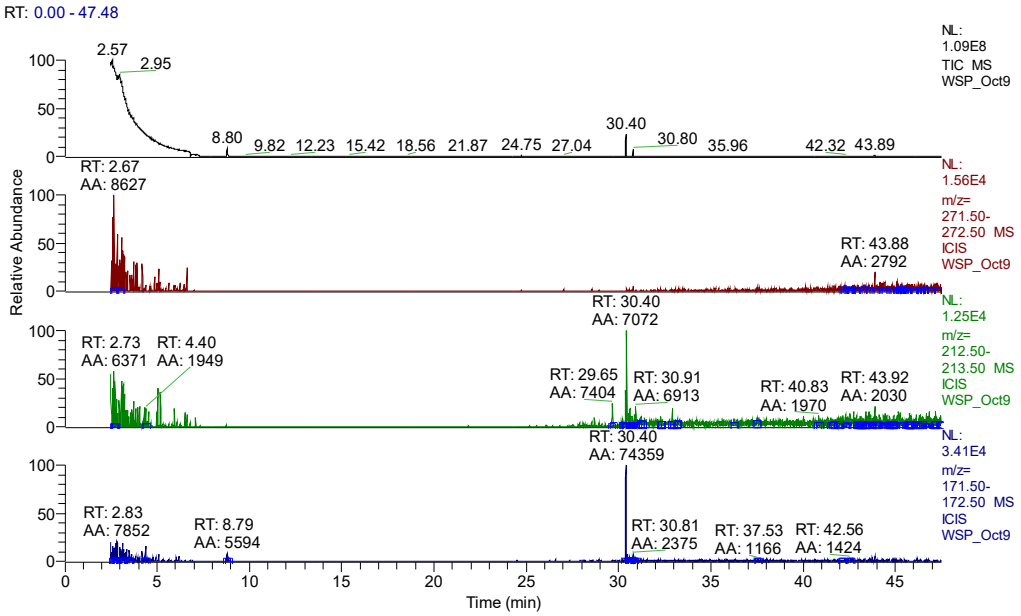


Figure 46: WSPOU (2014) chromatogram isolated for 17-β estradiol (BDL)

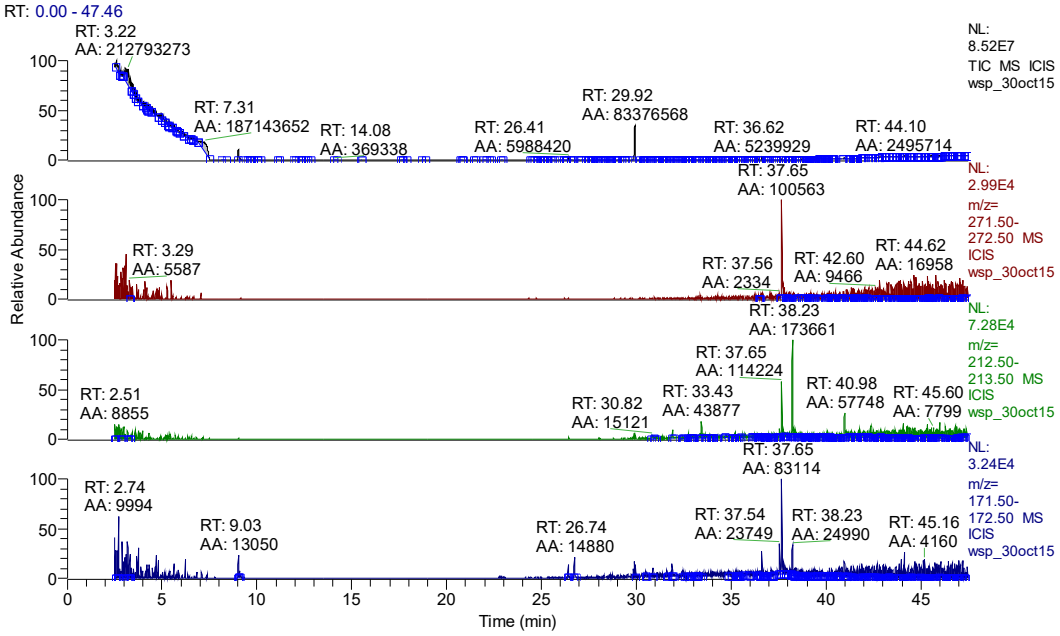


Figure 47: WSPOU (2015) chromatogram isolated for 17-β estradiol (RT= 37.65)

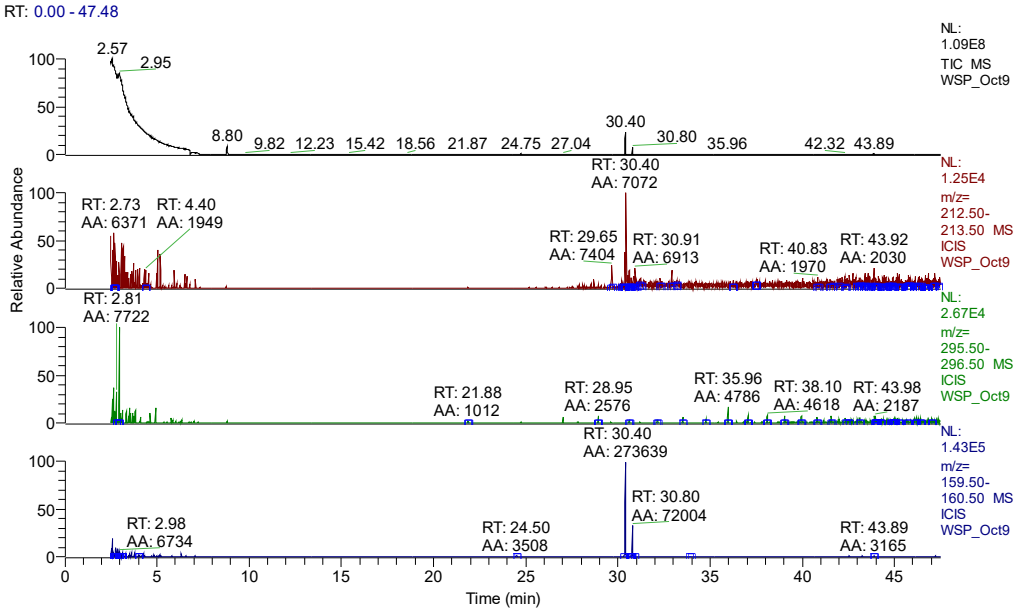


Figure 48: WSPOU (2014) chromatogram isolated for α ethynyl estradiol (BDL)

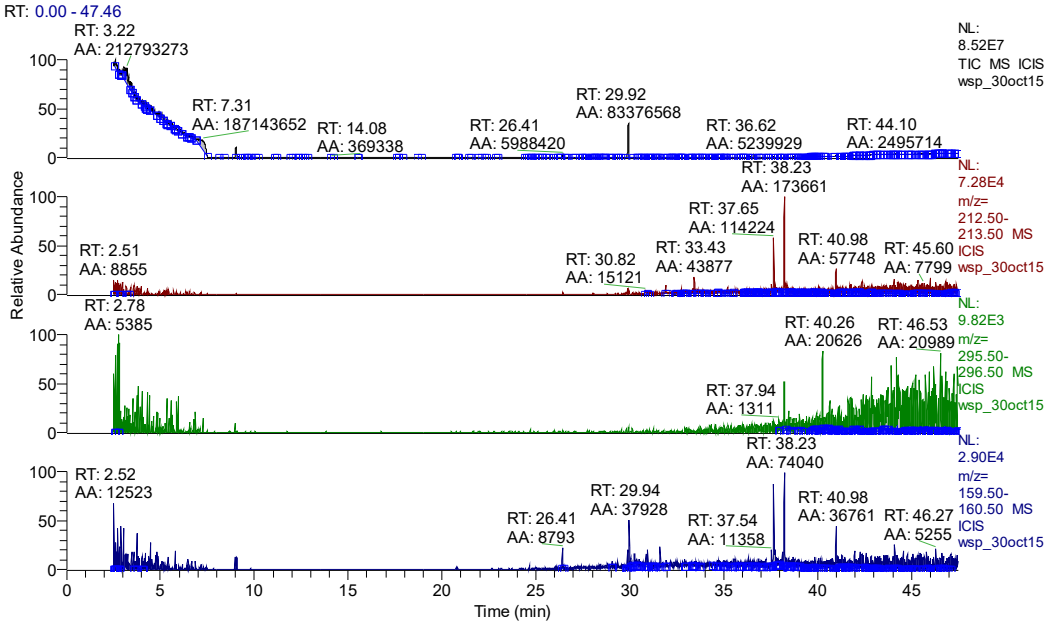


Figure 49: WSPOU (2015) chromatogram isolated for α ethynyl estradiol (BDL)