Method development to determine indicator analytes for pharmaceuticals and personal care products in surface waters including biosolid leachates

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1.0 Abstract

The land application of biosolids is an emerging field of study, but it is controversial amongst researchers. This is primarily due to concerns about the environmental impact of potential contaminants in biosolids. The risk of contamination is notably a local issue in the Thompson-Nicola region (BC Ministry of Environment 2016). One class of contaminants of concern that have been shown to leach from biosolids is Pharmaceuticals and Personal Care Products (PPCP) (Hydromantis 2009). The goal of this project is to develop a method to determine three PPCP analytes in aqueous samples so that it could be applied to surface waters and biosolid leachates. Further, this work would allow these samples to be investigated locally. The analytes chosen for screening were triclocarban, triclosan, and naproxen as they are good indicators of the presence of PPCP in environmental samples. The instrumentation used was High Performance Liquid Chromatography (HPLC) paired with Diode Array Detection (DAD). Numerous variations of solid phase extraction (SPE) were investigated in order to improve detection limits. The optimum SPE technique was coupled with HPLC-DAD, and applied to spiked and unspiked water samples.

2.0 Introduction

Biosolids are the resulting mass of organic materials that accumulate from wastewater treatment (Anekwe 2017). They are chemically and biologically treated with a goal of removing any pathogens or hazardous compounds, and contain vital nutrients for a variety of practical land applications. Examples of such include optimization of crop growth in addition to mine reclamation (DEQ 2014). In fact, the utilization of biosolids in agriculture aids in the reduction of greenhouse gas emission by the recycling of organic wastes (BC Ministry of Environment 2016). However, applications are strictly regulated for usage and health implications, as trace amounts of toxins can have a major impact on the surrounding ecosystem (BC Ministry of Environment 2016).

Detection of contaminants strictly from wastewater treatment is a difficult task; the use of E.coli as a marker has been used in the past, but it is hard to discriminate between human or animal sources (Lim et al. 2017). Chemical markers, such as those that originate from Pharmaceuticals and Personal Care Products (PPCP), are good indicators of human origin (Lim et al. 2017). In addition, PPCP are good indicators due their common presence in waste as well as their biodegradability resilience. (Lim et al. 2017). These PPCP compounds are also monitored in biosolids (Hydromantis 2009).

One of the key concerns about application of biosolids is the potential for PPCP and other compounds to leach from biosolids into the environment (Anekwe 2017). This has been a concern nationally, but very relevant locally (LRCS 2016). The primary environmental concern is the toxicity to aquatic organisms. Recently, studies have demonstrated that exposure to specific amounts of PPCP can be lethal to a variety of algae species, and can cause disruption to the endocrine systems of fish (Lim et al. 2017). The determination of biosolid leachate composition is critical to ascertaining the affects on the environment. Additional analysis of aqueous matrices, such as surface waters, would also give an indication to biosolid presence and potential toxicity. Therefore, having the ability to screen and quantify these PPCP would improve the land application approach for the recycling of wastewater treatment products.

The goal of this project is to investigate the development of an analytical method using Solid-Phase Extraction (SPE) with High Performance Liquid Chromatography and Diode-Array detection (HPLC-DAD) for the determination of triclosan, triclocarban, and naproxen in aqueous matrices such as leachate from biosolids (Figure 1). These analytes have been chosen as marker compounds for the presence of other PPCP compounds due to their frequent usage. Triclosan and triclocarban have antibacterial properties, and are commonly included in soaps and toothpaste (Unilever 2018). Naproxen is a frequently used, non-steroidal anti-inflammatory drug that can be purchased over the counter to treat mild to moderate pain. Other literature methods for detecting these three analytes are illustrated in Table 1.

Table 1: Comparison of analytical methods, found in published literature, that aim to detect triclosan, triclocarban, and naproxen. The reference article listed in the references at the end of this report.
Note: MP is mobile phase.

Reference	Analytes	Instrument	Parameters	Extraction Method	Detection Limit
Al-Rajab (2015)	Triclosan Triclocarban Naproxen	**HPLC**	**Triclosan MP** -> methanol to water in an 80:20 ratio **Triclocarban MP** -> methanol to water in an 80:20 ratio **Naproxen MP** -> acetonitrile to 10mM ammonium acetate at a 30:70 ratio	Series of treatments and centrifugation	ppb
Alavrez-Duran (2015)	Triclosan Naproxen	Photolysis Assay and GC separation and Mass Spec	Column: fused silica Carrier Gas: Helium Temperature Ramp		ppm
Amdany (2014)	Triclosan Naproxen	HPLC, UV, FED (UV used for triclosan, FED for naproxen)	MP: acetonitile:water at 70:30 v/v	POCIS preceding Solid Phase extraction	Naproxen: 0.2 µg/l Triclosan: 4.1 µg/l
Baranowska (2011)	Triclosan Triclocarban	HPLC/DAD	MP: methanol (A) and water (B) with gradient elution: 0 min 57% A, 2 min 90% A, 3 min 100% A, 6 min 100% A, 10 min 57% A	Solid Phase Extraction	Triclosan: 1.9ng/ml Triclocarban: 1.0ng/ml
Kim (2013)	Triclosan	HPLC/DAD	MP: acetonitrile: acetic acid, 10 mM aqueous solution (70:30, v/v, isocratic elution)	Solid Phase Microextraction	1ng/L
Klein (2010)	Triclocarban	HPLC/MS	MP: methanol and water: The gradient consisted of an initial 2 min hold at 30% methanol, then increasing from 30 to 100% methanol over 5 min followed by a 5-min hold at 100% methanol and 2-min of equilibration at 30% methanol.	Stir Bar Sorptive Extraction	1ng/L
Pedrouzo (2010)	Triclosan Triclocarban	HPLC/MS	A binary mobile phase **gradient** was used. Solvent A was Milli-Q water with acetic acid (pH 2.8) and solvent B was Methanol.	Stir Bar Sorptive Extraction	5-10 ng/L
Zheng (2015)	Triclosan	HPLC	MP: methanol to water. 80:20	Liquid-Liquid micro Extraction	2-20 ng/L

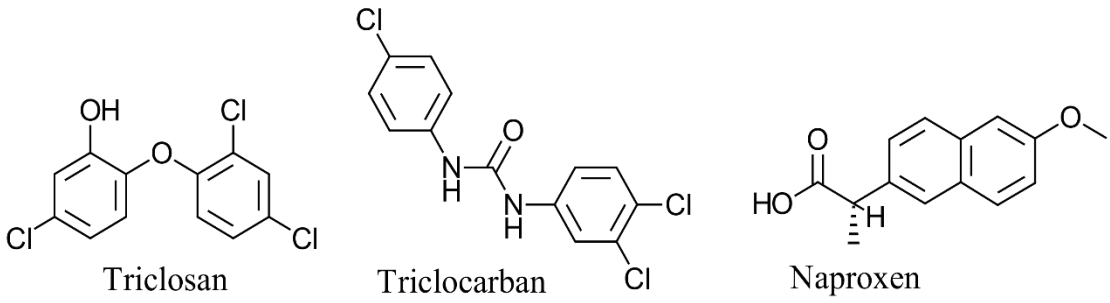

Figure 1: Structure of analytes examined in this project

3.0 Experimental

3.1. Chemicals and Reagents

Triclosan (TCS), triclocarban (TCC), and naproxen (NPRX) were purchased from Sigma-Aldrich. Their respective chemical structures are illustrated in Figure 1. It notable that TCS has a pKa of 7.9, TCC has a pKa of 11.4, and NPRX has a pKa of 4.15 (Pubchem 2018). HPLC grade LC-MS water was purchased from Omnisolv, and the HPLC grade methanol was purchased from BDH VWR Analytical. The three analytes were dissolved in methanol to prepare stock solutions, and were stored in a refrigerator for the duration of the research. These stored stock solutions were diluted for daily use.

3.2. Instrumentation: HPLC

3.2.1. Parameters

The development and optimization of a reverse phased HPLC method was necessary to accurately detect the three indicator analytes. Analysis was performed on an Agilent 1220 HPLC instrument paired with a G1315 C diode array detector. During the method development phase, a Phenomenex Kinetex EVO C18 column (2.6 µm particle size, 100 x 3.0 mm) was used, as well as the Phenomenex Kinetex F5 column (2.6 µm particle size, 150 x 4.6 mm). The mobile phase was a mixture of 0.5% glacial acetic acid and methanol. The exact ratios varied throughout method development, and are discussed later. The diode-array detector monitored wavelengths of 230nm, 258nm, 265nm, 270nm, 273nm, and 280nm; these were chosen from values stated in previous literature (Baranowska and Wojciechowska 2011)

3.2.2. Method Development

Stock solutions of TCS, TCC, and NPRX were prepared in methanol at 1000 mg/mL, and were stored in a refrigerator. Diluted solutions that were approximately 20 mg/mL were prepared and transferred to autosampler vials for analysis. Initially, these dilute solutions were made up in HPLC-grade water. Yet, it is notable that there were solubility issues with TCC; in water, the solubility of TCC is $2.37x10^{-3}$ mg/L at 25ºC (Pubchem 2018). In subsequent calibration trials, methanol was used for solutions during HPLC analysis. The acidic composition of the aqueous mobile phase (0.5% acetic acid) was chosen to ensure that NPRX would be in neutral form to interact with the column. This analyte had the lowest pKa value. Method development started with a C-18 column for the stationary phase. Further experimentation resulted in the use of a Kinetex-F5 column due to better analyte resolution paired with shorter runs.

Table 2: Method Development for HPLC-DAD

Method #	Mobile Phase	Column	Other Parameters	Results
1	**80:20** methanol: acetic acid (0.5%)	**C-18**	**Flow rate:** 0.5 ml/min **Wavelengths (nm):** **282:** triclosan 2**58:** triclocarban **273**: naproxen **Injection volume:** 5.00 µL **Run times:** 8 mins	Analytes were visible, yet resolution needs to be improved. Order of elution = naproxen, triclosan, triclocarban
2	**60:40** methanol: acetic acid (0.5%)	**C-18**	**Flow rate:** 0.6 ml/min **Wavelengths (nm):** **282:** triclosan 2**58:** triclocarban **273**: naproxen **Injection volume:** 5.00 µL **Run Times:** 15 mins	Analytes were better resolved; however, triclosan and triclocarban are still within a minute of each other. This could preferably be improved.
3	**60:40** methanol: acetic acid (0.5%)	**Kinetex F5**	**Flow rate:** 0.6 ml/min **Wavelengths (nm):** **282:** triclosan 2**58:** triclocarban **273**: naproxen **Injection volume:** 5.00 µL **Run Times:** 20 mins	Only naproxen was detected; triclocarban and triclosan did not produce peaks. Inconclusive results; need to evaluate a mobile phase change, with more methanol.

4	**90:10** methanol: acetic acid (0.5%)	**Kinetex F5**	**Flow rate:** 0.6 ml/min **Wavelengths (nm):** **282:** triclosan 2**58:** triclocarban **273**: naproxen AND **265, 230 nm** **Injection volume:** 5.00 µL **Run Times:** 20 mins	Each analyte was detected, with adequate resolution; peaks were optimally shaped. -naproxen needs to be more separated from blank peaks that appeared at start Will try an aim to further separate the three; as each all are within
5	**80:20** methanol: acetic acid (0.5%)	**Kinetex F5**	**Flow rate:** 0.6 ml/min **Wavelengths (nm):** **282:** triclosan 2**58:** triclocarban **273**: naproxen AND **265, 230 nm, 270 nm** **Injection volume:** 5.00 µL **Run Times:** 10 mins	Analytes are well resolved, peaks are optimally shaped. Run time is respectable. Results were reproducible
6	**50:50** methanol: acetic acid (0.5%)	**C18**		Pressure was very high at around 400 barr, and analytes were not detected. Perhaps too much aqueous solution for the column.

3.2.3. HPLC Calibration

To determine a detection limit for the optimized method, a series of standards were prepared in methanol, as outlined in Table 2. Calibration curves for the three analytes were created separately. The detection limit was evaluated by conducting multiple HPLC runs on the standard solutions, and examining what peak areas were distinguishable from the baseline.

Table 3: Concentrations of the stock and standard solutions

	Stock (mg/L)	**Standard 0 (mg/L)**	**Standard 1 (mg/L)**	**Standard 2 (mg/L)**	**Standard 3 (mg/L)**	**Standard 4 (mg/L)**	**Standard 5 (mg/L)**
Triclosan	992.00	19.84	9.92	5.95	1.98	0.50	n/a
Triclocarban	1024.00	20.84	10.24	6.14	2.05	0.51	0.2048
Naproxen	998.00	19.96	9.98	5.99	2.00	0.50	0.1996

3.3. Solid Phase Extraction

To improve the detection limit of the developed HPLC-DAD method, spiked water samples were subjected to SPE prior to analysis. Three different SPE approaches were conducted to determine an optimal method. Bakerbond SPE Cartridges composed of octadecyl (C-18, 3 mL, 500mg/column) bound to silica gel were first utilized to extract the analytes. The inclusion of TCC in the spiked water solution required larger volumes; thus, manually pumping the 250 mL spiked water sample through the discs was an inefficient approach. The method is depicted in Figure 2.

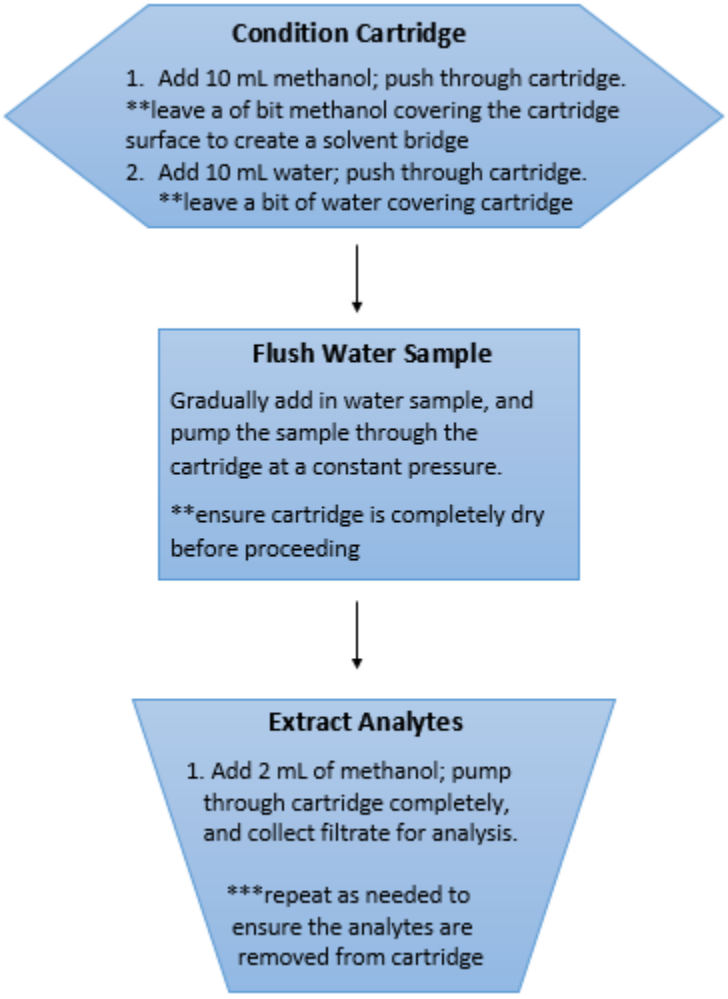

Figure 2: SPE cartridge method

Solid Phase Microextraction (SPME) was the second approach examined using a PDMS fibre, and a PDMS/DVB fibre. SPME was an efficient approach to perform; however, the resulting extraction for both fibres was not optimal. Peaks had to be manually integrated to

calculate a fraction of the expected milligram recovery. Therefore, this was not examined in detail and no data was collected.

Solid Phase Extraction with C-18 discs was the final approach examined. A vacuum filtration apparatus was assembled in a fume-hood and was powered through a pump. A pressure gauge, which measured in mmHg, was included to consistently control pressure during the stages of extraction. The system also included a 1 L collection flask, a filter, and a pouring reservoir clamped to the top. A visual of the set up is provided in Figure 4. This approach was deemed as optimal for subsequent trials, and a distinct method was developed (Figure 3). The amount of collections was dependent on the water sample. Small changes to this general procedure were made once data was evaluated, and will be discussed further in the results section.

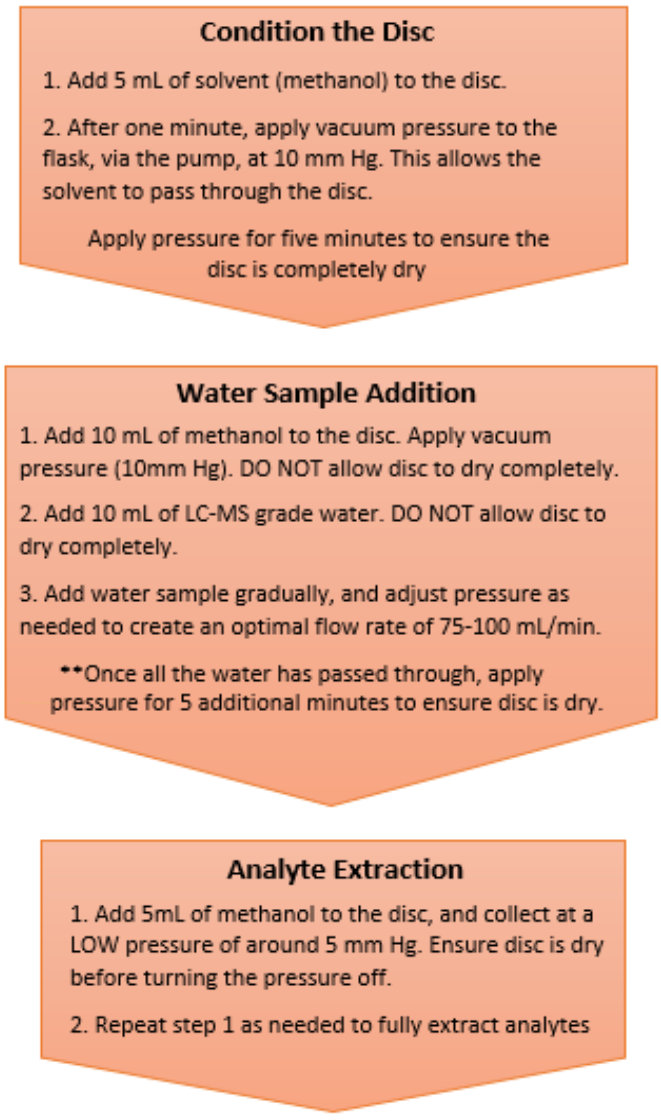

Figure 3: SPE disc experimental method

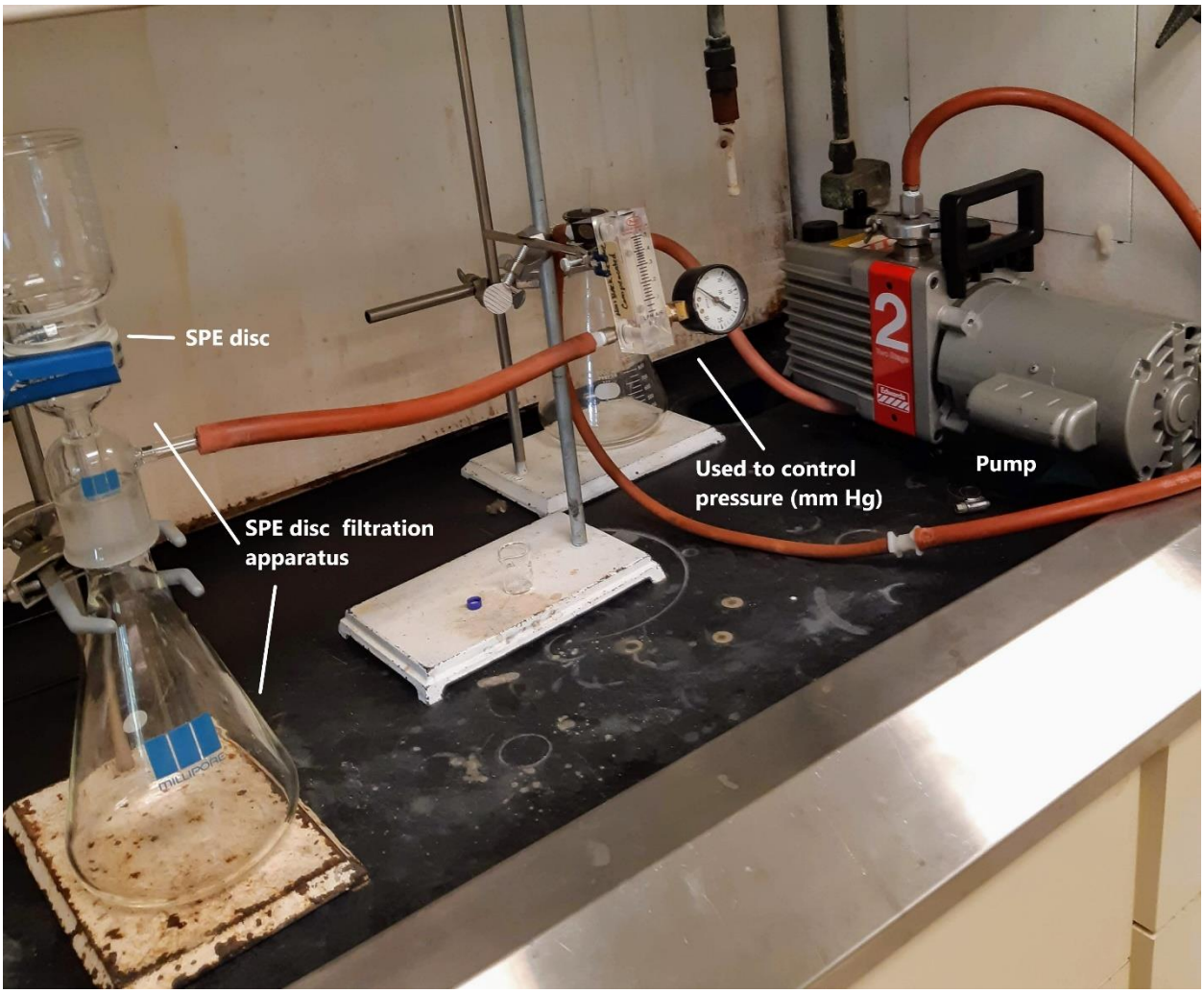

Figure 4: SPE disc method set-up.

A real water sample was subsequently collected and examined with the developed HPLC-DAD, SPE disc method. The sample was taken from the local Thompson River, around a meter from the shoreline at Pioneer Park. This location is notably upstream from the Kamloops Wastewater Treatment plant. Two 250 mL replicates of unspiked and spiked river water were completed 24 hours apart. The spiked water samples had stock solution additions of 20 uL TCS, TCC, and NPRX. This addition of TCC is notably slightly higher than its solubility in water; however, river water has suspended particulates that create allowance for more TCC to stay in solution.

4.0 Results

4.1. HPLC

Analyses were conducted of a mixed standard in order to develop the HPLC-DAD portion of the method. Initial runs were done on the C18 column. The first experimental method had an efficient run time of eight minutes, and the order of elution was determined; naproxen was eluted first, followed by TCS, and TCC exited the column last. However, the resolution of the three analytes was poor. Therefore, the second experimental method had a higher ratio of aqueous phase to organic phase. This would increase the interaction time between the analytes and the stationary phase. At a run time of fifteen minutes, the second method still did not produce fully resolved peaks, and analytes eluted later (Figure 3). However, there was a slight improvement from trial 1.

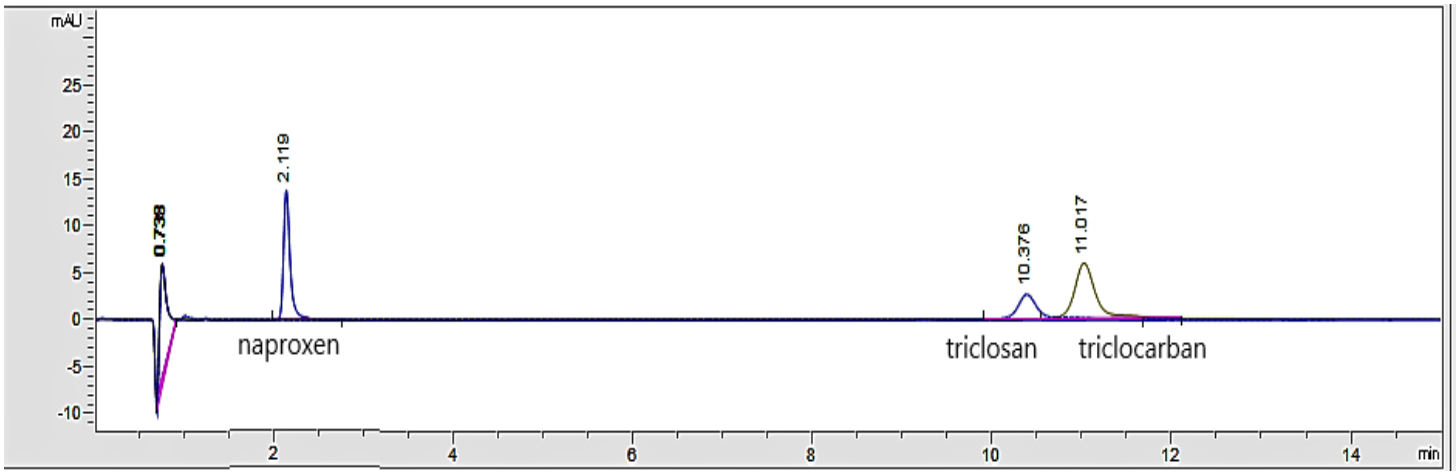

Figure 5: Chromatograph of method development at experimental condition 2, which is outlined in Table 2.

The C-18 column was switched out for the third experimental method for a Kinetex-F5 column. The F5 column is designed to handle higher ratios of aqueous phase, and could increase separation due to the more polar nature of the column. Thus, further developing a method on this column would allow for greater experimentation. The ratio of mobile phase in trial two was repeated in trial three to compare the differences in the two columns. Naproxen was the only analyte detected in the third method, which was deemed as inconclusive results for the new column. Since naproxen is the first analyte to exit the column, trial four introduced a higher ratio of organic to aqueous phase at 90:10 compared to the 60:40 ratio in trial three. This was speculated to decrease the run time and detect all three analytes. Likewise, the results from the fourth experimental method did detect TCC, NPRX, and TCS with adequate resolution. However, the analytes were all within a minute of each other, and naproxen was not well resolved from the peaks appearing in the blank chromatograph. Therefore, the fifth experimental method had an adjusted ratio of organic to aqueous mobile phase at 80:20 to further separate all peaks. As a result, TCC, NPRX, and TCS were well resolved, and the ten-minute run time was efficient, as depicted in Figure 6. The fifth method was repeated to investigate reproducibility, and the results were congruent with the initial runs.

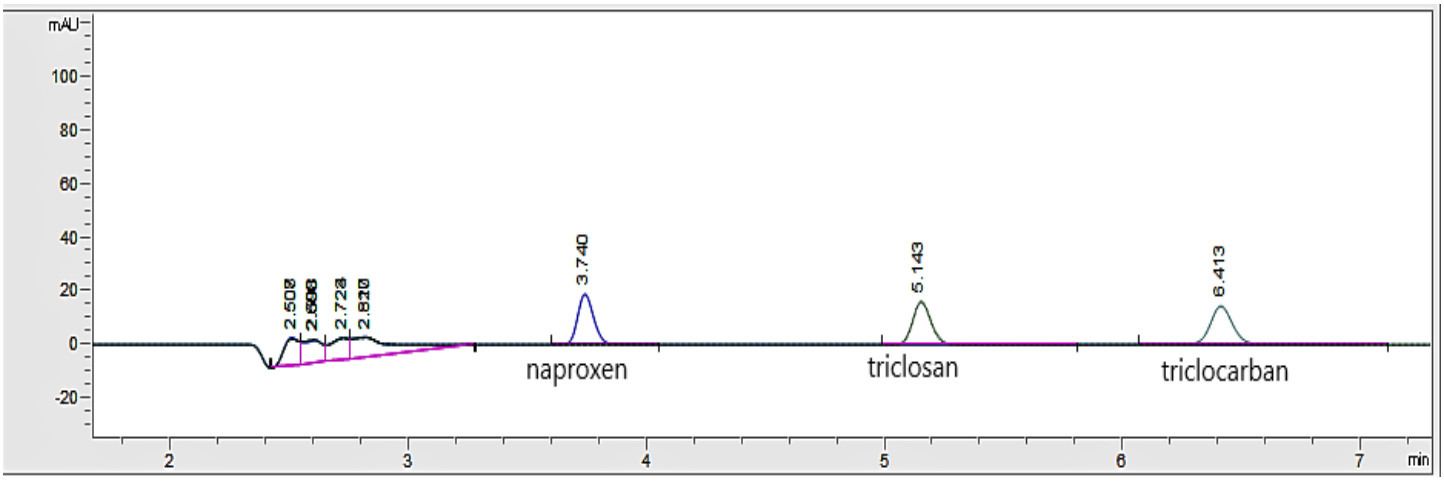

Figure 6: Chromatograph of method development at trial 5

Before selecting the fifth experimental method as the optimal HPLC-DAD method, a sixth method was conducted with the C-18 column. In addition to this switch, a ratio of organic to aqueous phase of 50:50 was chosen to see if the peaks could be separated further. However, this approach produced high pressures of 406 barr, and only naproxen was detected. Perhaps this 50:50 mobile phase ratio had too much aqueous phase for this C-18 column to handle proficiently. Hence, the fifth experimental method was selected as the optimal method for subsequent research in this project. As shown in Table 1, the optimized method included a flow rate of 0.6 mL/min. The diode-array detector monitored wavelengths of 230nm, 258nm, 265nm, 270nm, 273nm, and 280nm.

After finalizing an HPLC-DAD method, three calibration curves were constructed from a series of six standards. Triplicate runs of each standard were performed, and the curves utilized peak area for the y-axis. Since a variety of wavelengths were analyzed on the DAD, optimal wavelengths for each analyte were chosen to consistently select peak areas. NPRX and TCS peak intensity was greatest at 230nm, while TCC has greatest peak intensity at 265 nm. From these runs, a range for the retention time of each analyte was calculated based on the first run of every standard. Hence, NPRX ranged 3.640-3.688 minutes, TCS ranged 4.889-5.001 minutes, and TCC ranged 5.991-6.248 minutes. The coefficient of determination values showed little variation between the variables, as values ranged from 0.9991-0.9995. From the calibration curves, detection limits were calculated for TCS, TCC, and NPRX to be 0.496, 0.205, and 0.0998 mg/L, respectively.

4.2 SPE cartridge

All three analytes were detected using this SPE approach; however, extraction yields were not ideal. For the first attempt, the percent recovery of TCS, TCC, and NPRX were 102.4%, 2.719%, and 43.79%, respectively. These values were obtained from the calibration

curve data, in addition to subsequent calculations. In comparison to literature, acceptable recoveries are roughly between 70-130% (Shen et al. 2012, Wu et al. 2010). Since a very small amount (1 µL) of TCC was added to the spiked 500 mL water sample, a variable and low percent recovery was expected. For the second attempt with the SPE cartridge, the percent recovery of TCS, TCC, and NPRX were 52.18%, 37.88%, and 36.73%, respectively. These results were quite distinctly different from the first trial, which signifies that this cartridge method was not reproducible. Further development of this method was not investigated, therefore the extraction efficiency of TCS, TCC, and NPRX with this C-18 cartridge is inconclusive.

4.3 SPE disc

Using the discs was the best extraction approach investigated in this project. Three methods (A-C) of varied concentrations of analytes were conducted, and each included minor changes to improve percent recovery. Percent milligram recovery values are illustrated in Figure 7 for each method.

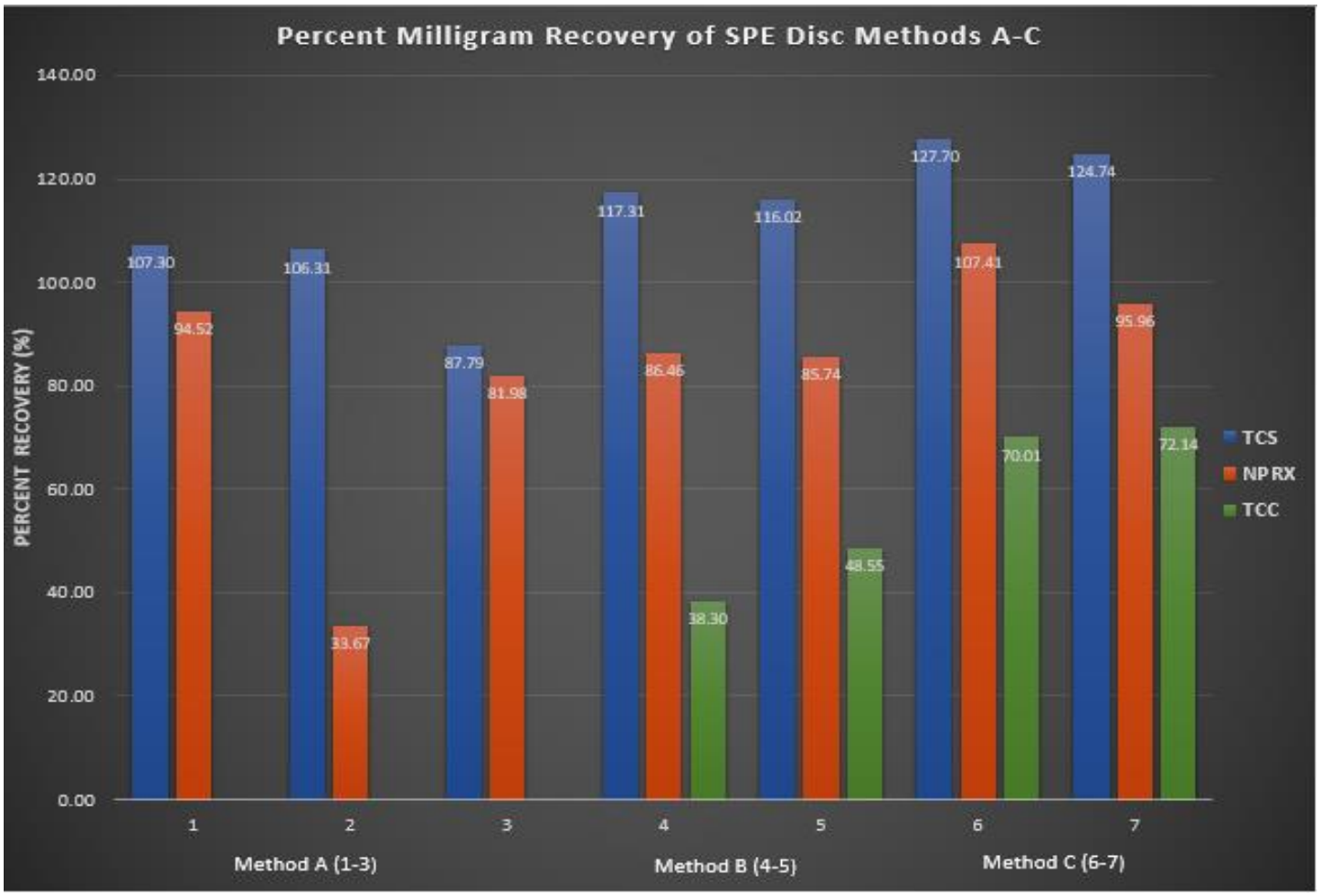

Figure 7: Percent milligram recoveries for TCS, NPRX, and TCC from the SPE disc approach. Methods A-C mainly differ in spiked analyte concentrations, which decrease from A-B-C. Other details are further explained in the results section.

Method A had the highest concentration of spiked analytes. It is notable that TCC was excluded for this method, as NPRX and TCS could be added at higher amounts to better investigate disc efficiency for the first few extractions. Method A had three replicates, where the first run of TCS and NPRX had very respectable recoveries (107.30, 94.52%). The second replicate had a noticeably lower recovery for NPRX (33.67%). Since a new jug of HPLC-grade LC-MS water was used during that replicate, it was speculated that the pH could have been higher, consequently affecting NPRX extraction (pKa = 4.15). For the third replicate, three drops of HCl was added to the water sample, lowering the pH to 2.48, which resulted in a significantly higher NPRX recovery (81.98%). This is due to the neutral form of naproxen being abundant, and therefore interacting with the stationary phase. The differences within the first SPE collections are illustrated on the chromatographs in Figure 8. Subsequent methods included adding three drops of HCl to each water sample.

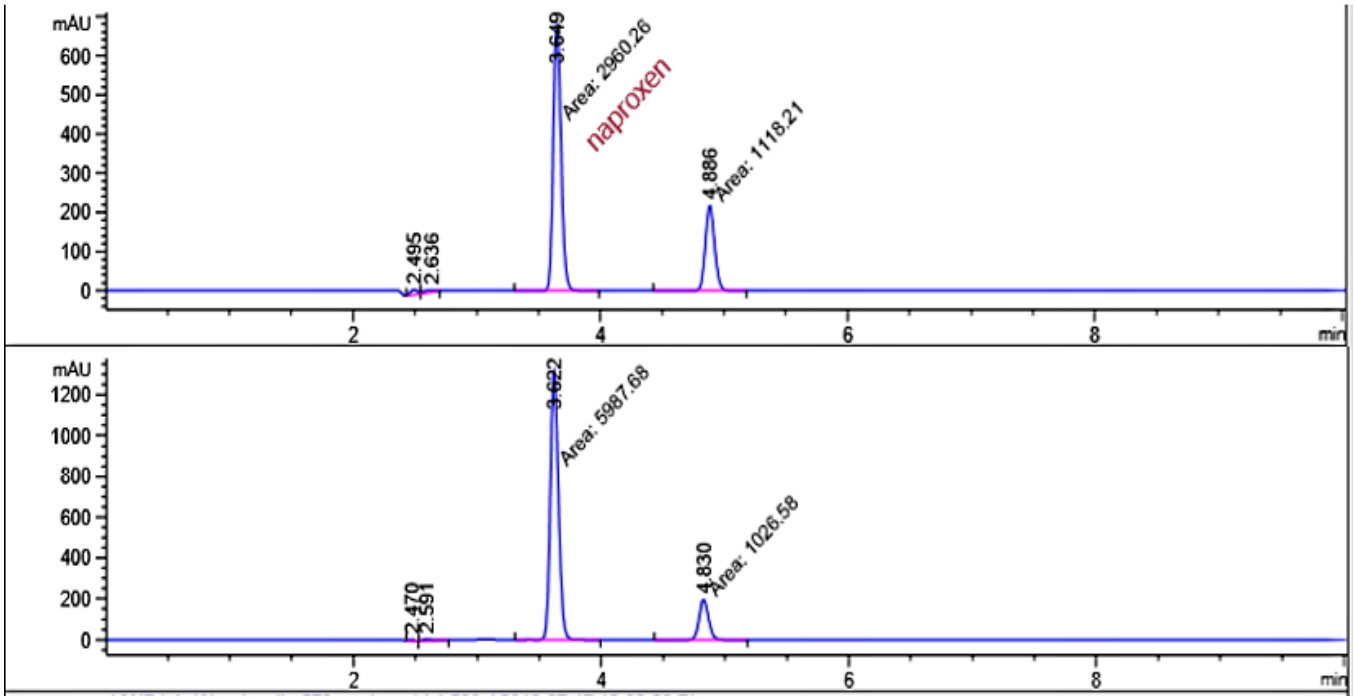

Figure 8: Method A; differences in naproxen extraction of the first SPE collection at 230 nm. The top chromatograph shows when no HCl was added to the water sample (NPRX peak area = 2960.26). The bottom chromatograph shows that when HCl was added to the water sample, NPRX extraction was drastically improved (NPRX peak area = 5987.68).

Method B investigated extraction with lower analyte concentrations. TCC was included at a considerably lower concentration than TCS and NPRX to account for its low solubility. The first replicate had higher recoveries than in Method A for TCS (117.31%) and NPRX (86.46%). The extraction of TCC was seemingly poor at only 38.30%; however, the initial addition of only 5 µL of TCC stock solution has a larger amount of error associated with the smaller aliquot of analyte. Alternatively, 25 µL of TCS and NPRX were added to the 250 mL water sample. The

second replicate closely resembled the results from the first replicate for TCS, NPRX (116.02, 85.74%). TCC had a notable increase in percent recovery (48.55%), which can be seen in Figure 7.

Method C increased the water sample size from 250 mL to 500 mL to further investigate the effects of lowering analyte concentrations, but maintaining manageable spike aliquots of 20 µL for NPRX and TCS. TCC was spiked at 8 µL. This method further improved from the results of Method B for TCS, NPRX, and TCC (127.70, 107.41, 70.01%). The evident increase in extraction efficiency for TCC is comparable to other methods published in literature (Shen et al. 2012). The second replicate of Method C was similar to the first replicate for TCS, NPRX, and TCC (124.74, 95.96, 72.14%). An example of the entirety of an extraction is given in Figure 9 (showing NPRX and TCS) and in Figure 10 (showing TCC).

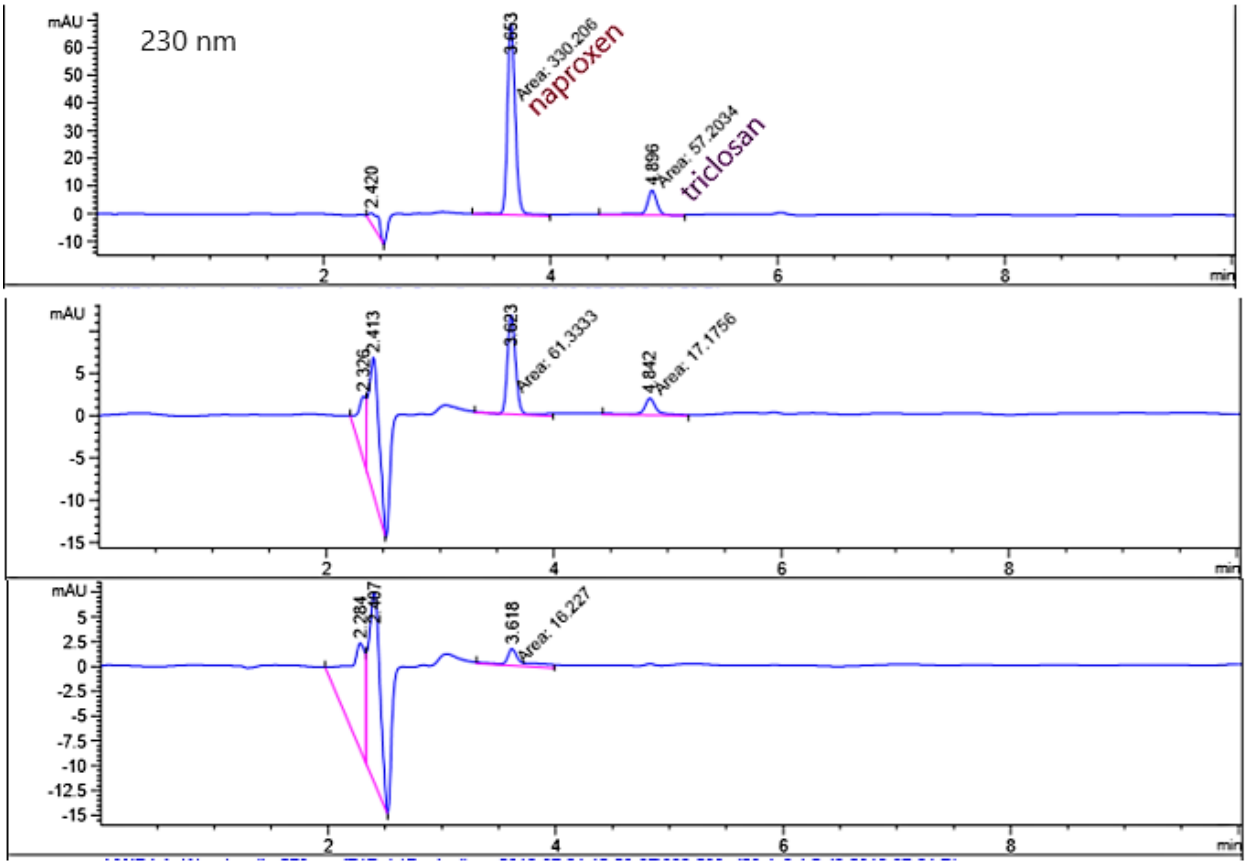

Figure 9: The three SPE collections that make up the entirety of the extraction for NPRX and TCS at 230 nm.

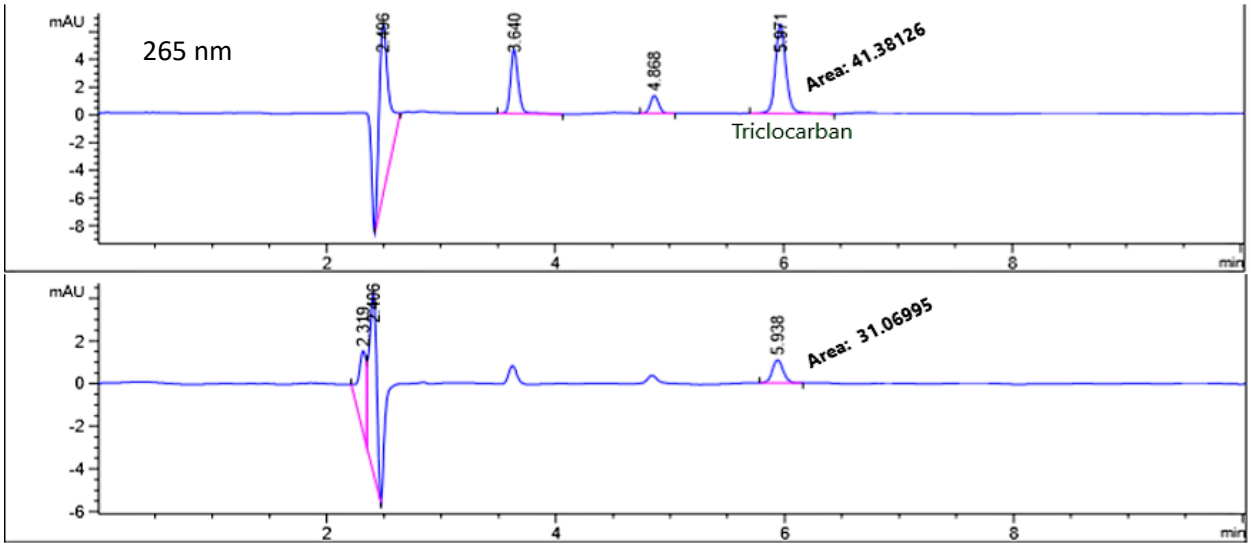

Figure 10: The two SPE collections that make up the entirety of the extraction for TCC at 265 nm.

Since the SPE discs were producing high extraction efficiencies in spiked water samples, a real water sample was chosen to investigate. A 500 mL river water sample was subjected to the same general SPE disc procedure, as well as the addition of 3 drops of HCl. The first unspiked water sample showed no detectable TCS, NPRX, or TCC in any of the collections. However, a replicate of the unspiked water sample performed 24 hours after the first run showed two new distinct peaks. The appearance of these peaks on the second day of extractions could be due to the settling of river sediments in the water sample, as analytes interact and stick to particulate matter. Figure 11 compares chromatographs of the first collections for days 1 and 2. In reference to day 2, the first peak (3.643 mins) is within the calculated range of retention times for NPRX (3.640-3.688 mins). The second peak (4.882 mins) falls just short of the calculated range of retention times for TCS (4.889-5.001 mins).

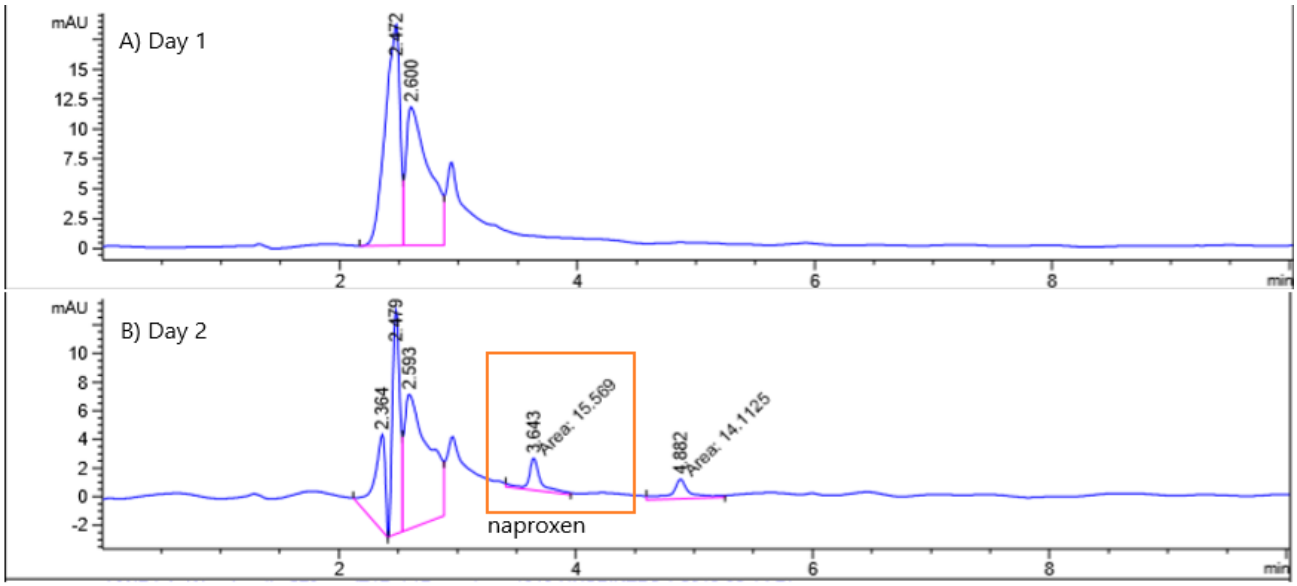

Figure 11: Unspiked river water samples ran 24 hours apart (230 nm)

Therefore, this research is indicative that NPRX is present in the river water, but does not support the presence of TCS. Yet, further investigation and more replicates could provide more conclusive results. TCC was not detected on either day.

Two replicates of a spiked river water sample were also conducted 24 hours apart. Table 4 shows the percent recovery for TCS, TCC, and NPRX for both days. It is notable that recoveries on the second day were in fact slightly larger than the first day for NPRX and TCS. Since the unspiked sample on the second day produced the two peaks, these results are indicative that both TCS and NPRX being present in the river water.

Table 4: Recovery of spiked water samples (%)

	DAY 1	DAY 2	Difference
Analyte	Percent milligram Recoveries (%)		Day 1 → Day 2
TCS	105.90	110.28	+ 4.37%
NPRX	91.70	93.39	+ 1.69%
TCC	71.57	71.25	- 0.32%

5.0 Conclusion

An HPLC-DAD method was successfully developed to detect the three selected indicator analytes. In addition, an SPE disc method was optimized to extract TCS, NPRX, and TCC with highly respectable recoveries (124.74, 95.96, 72.14%). Average detection limits for the analytes were 0.496, 0.205, and 0.0998 mg/L. Combining the two methods achieved detection of all analytes in spiked surface waters, which shows that the developed method was successfully applied to real water samples. Detection of analytes in the unspiked river water warrants further investigation. Applying this HPLC-DAD SPE disc method to biosolid leachates would be the ultimate test to see if we can detect a diverse list of PPCP in treated wastes. Other areas of future work for the developed method include lowering the detection limit, testing for other PPCP, and analyzing surface waters downstream of the Wastewater Treatment Plant.

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