DEVELOPING AND TESTING A METHOD FOR THE ANALYSIS OF CHEMICAL HUMAN WASTE MARKERS IN GROUNDWATER AND IDENTIFYING SOURCES OF NITRATE CONTAMINATION

By

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ABSTRACT

Groundwater supplies residents in Central Wisconsin with their drinking water. This water is susceptible to contamination by nitrate and other compounds that pose health risks to consumers. The contamination may stem from both agricultural and human waste treatment practices. Understanding the relationship between nitrate and other contaminants in groundwater can direct remediation efforts, as well as future residential planning.

This research identified and quantified multiple chemical indicators of nitrate contamination in groundwater. A reliable analytical method for identifying septic system tracers was developed to simultaneously analyze a group of pharmaceuticals, personal care products, and artificial sweeteners. The selection of septic waste tracers was based on chemical characteristics, such as mobility in groundwater and water solubility, as well as their common use. Method development included instrument optimization and designing a sample preparation technique. Solid phase extraction (SPE) was used to improved analyte detection. Four SPE methods were compared to determine which method yielded the best and most consistent recoveries for the most analytes.

Once the analytical method was determined, groundwater samples were taken from five wells in a suburban subdivision in Central Wisconsin and analyzed. Nitrate concentrations in these wells ranged from less than half the drinking water standard of 10 mg/L to more than five times the standard. The method for septic system tracers was used with existing laboratory methods for indicators of agricultural contamination. With these methods, the presence of specific contaminants could be identified and their

concentrations quantified. The analytical results were used to identify the likely sources of nitrate contamination in those wells.

1. PURPOSE

Residents in rural and suburban areas often obtain their drinking water from private wells. Unlike municipal sources, private wells are not regulated to ensure that drinking water standards are met. These wells are susceptible to groundwater contamination, which may occur without knowledge to the consumer and pose potential health risks.

There are several challenges for private well owners. For example, they can have their water analyzed by a laboratory, but that is typically for just a few possible contaminants. In addition, it can be difficult to understand the implications of private well results when the water has low or intermediate concentrations of contaminants such as nitrate. Further, the concentrations may vary over time or may simply be indicators of other potential contaminants. Because private well owners usually do not have an understanding of the source of contamination, it is difficult to determine the potential health risks or the best approach to remedy the problem.

The objective of this study was to develop a method for the simultaneous analysis of a group of pharmaceuticals, personal care products, and artificial sweeteners for identifying sources of nitrate contamination in private wells. Reliable methods have been established for pesticides and pesticide metabolites, but the method for common septic system tracers in groundwater needed to be improved. A method for the simultaneous analysis of multiple compounds would be more effective in identifying the source of contamination in wells. Both chemical characteristics and use within a community determine the occurrence of septic system indicators in groundwater. A variety of compounds were evaluated to determine the most useful tracers.

2. LITERATURE REVIEW

The following is a review of literature on sources of nitrate contamination and analytical methods for identifying chemical indicators of septic waste contamination in groundwater.

Nitrate in groundwater

Nitrate (NO_3^-) is a naturally occurring chemical compound often found at low levels in groundwater. Concentrations greater than 3 mg/L generally indicate contamination (Madison and Burnett, 1985). Anthropogenic sources of nitrate include applications of nitrogen-containing fertilizers and manure, as well as discharge of septic tank effluent (WI DNR, 1999). Hydrologic, chemical, and biological processes control the extent to which these sources impact groundwater. Hydrologic influences include water table elevation, groundwater flow direction, rainfall and irrigation (Ritter et al., 2007). Chemical processes control the mobility of nitrate in the soil profile (Stackelberg et al., 1997; Allred, 2007). Nitrification is a biological process that can convert the ammonia into nitrate (Avtar et al., 2013; Lee et al., 2006; Sprent, 1987).

Nitrate in groundwater is a health concern. The United States Environmental Protection Agency (EPA) and the Wisconsin Division of Public Health (WI DNR, 2010) list a primary drinking water standard of 10 mg/L (ppm) for nitrate-N. Since 2000, almost 1 in 6 private water supply wells tested in Portage County had nitrate-nitrogen concentrations that exceeded this standard (Portage County, 2011). This standard is set to prevent methemaglobinemia. Methemaglobinemia, also known as blue baby syndrome, can occur when an individual ingests high levels of nitrate. Nitrate is converted to nitrite. Infants are especially at risk due to their high stomach pH, which

increases this conversion. Nitrite oxidizes the ferrous (Fe^{2+}) iron in hemoglobin to ferric (Fe^{3+}) iron producing methemoglobin. Red blood cells use hemoglobin to transport oxygen throughout the body. Methemoglobin cannot bond with oxygen, thus reducing the oxygen available to the body. This can lead to symptoms such as bluish skin. In extreme cases it can affect breathing and heart function, and even lead to death (Fan et al., 1987; National Academy of Science, 1995). The drinking water standard of 10 mg/L is not limited to infants. The Wisconsin Division of Public Health recommends that "people of all ages avoid long-term consumption of water" that exceeds the drinking water standard for nitrate. Nitrate consumption has been associated with increased risk of thyroid disease, diabetes, and certain types of cancer, which may be exacerbated in individuals with existing health conditions, such as inherited enzyme defects or cancer (WI DNR, 2010).

The relationship between nitrate and co-contaminants

Two of the most likely sources of nitrate contamination are agricultural practices and septic systems. Both sources can have other co-contaminants such as pesticides, pesticide metabolites, pharmaceuticals and personal care products. Some of these co-contaminants may be used in identifying the source of nitrate in a well. Some co-contaminants have also their own health concerns.

Agricultural activities are the largest non-point sources of elevated nitrate concentrations in groundwater (Madison and Burnett, 1985). This includes applications of fertilizers and manure. Stackelberg et al. (1997) evaluated a network of 72 monitoring wells in New Jersey for nitrate and pesticides. Wells were categorized as new urban, with development less than 30 years old (n=30), old urban, with development more than

30 years old (n=14), agricultural (n=15), or undeveloped (n=13). Sixty percent of the agricultural wells had nitrate concentrations exceeding the drinking water standard of 10 mg/L nitrate-N. These wells also had the highest median nitrate concentration at 13.0 mg/L. The herbicide metolachlor was detected in 75 percent of the agricultural wells at concentrations ranging to 0.466 ug/L. In contrast, median nitrate concentrations for the new and old urban wells were 2.6 and 3.5 mg/L, respectively. These wells were more likely impacted by domestic fertilizers and sewage waste. Only one of the 44 urban wells had nitrate exceeding 10 mg/L. Fewer than 50 percent of the urban wells had detectable concentrations of metolachlor, all less than 0.03 ug/L. Nitrate concentrations were less than 1.0 mg/L for all samples from wells in undeveloped areas. About 10 percent of these had detectable concentrations of metolachlor.

Septic systems discharge wastewater to the groundwater and can be a source of nitrate and other contaminants. Septic systems are a common method of treating human waste in rural and suburban areas. Ammonia and organic nitrogen in septic tank effluent may result in total nitrogen concentrations of 50 - 100 mg/L (Shaw, 1994). As these nitrogen sources move through the subsurface they are converted into nitrate (Madison and Burnett, 1985). While nitrogen from septic effluent may only account for 10 percent of the total nitrate leached into groundwater in Wisconsin, it can significantly impact individual wells (Shaw, 1994). Some pharmaceuticals, personal care products, and artificial sweeteners are compounds unique to human use. The presence of these septic waste markers can indicate that excess nitrate in a well is coming from a septic system.

Pesticides

Chemical analyses for pesticides and their breakdown products can help determine if a well is being impacted by agricultural practices. Alachlor, metolachlor, and acetochlor are three herbicides widely used in Wisconsin to control weed growth in growing crops such as corn and soybeans (Rheineck and Postle, 2000). Known as chloroacetanilide herbicides, the parent compounds can be transformed by microbial activity in the soil into ethane sulfonic acid (ESA) and oxanilic acid (OA) metabolites. The metabolites are more water mobile and persistent than the parent compounds in groundwater (Thurman, 1996).

Postle et al. (2004) surveyed private drinking water wells from 336 sampling locations in Wisconsin in 2001. They analyzed for alachlor, metolachlor, acetachlor, and their ESA and OA metabolites. Of the 336 samples, the ESA metabolite was detected in the greatest number of wells, followed by OA metabolites. The only parent herbicide detected was alachlor, and it was only found in one well. Alachlor ESA was detected in 103 wells at concentrations ranging from 0.101 – 14.8 ug/L. Table 2.1 shows the data from this study.

Table 2.1 Chloroacetanilide herbicide and metabolite results from Postle et al., 2001 survey.

Compound	Number of Detects (N=336)	Concentration Range (ug/L)
Alachlor	1	0.690
Alachlor ESA	103	0.101-14.8
Alachlor OA	16	0.145-13.5
Metolachlor	0	--
Metolachlor ESA	88	0.103-10.2
Metolachlor OA	23	0.103-5.89
Acetochlor	0	--
Acetochlor ESA	10	0.104-0.809
Acetochlor OA	1	0.155

Domestic Wastewater

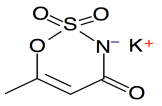
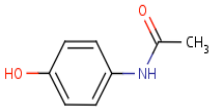
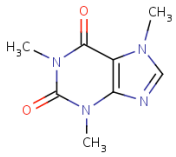
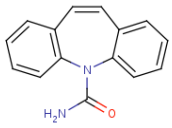
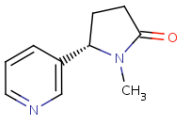
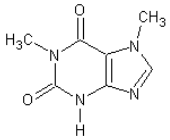
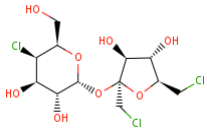
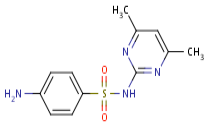
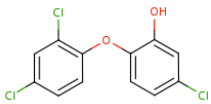
Many products used as pharmaceuticals, personal care products (PPCP), and food additives, such as artificial sweeteners, can be used as indicators of contamination from septic systems. Chemical compounds such as caffeine, its metabolite paraxanthine, and the nicotine metabolite cotinine are potential indicators, as they are mobile in groundwater and the parent compounds are commonly used. Acesulfame and sucralose are popular low calorie sweeteners found in diet sodas and food products. Their wide use and persistence in groundwater makes them good candidates as chemical waste markers (Buerge et al., 2009; Scheurer et al., 2009; Van Stempvoort et al., 2011). Carbamazepine, while not as widely used as caffeine and nicotine, does not appear to be removed while passing through soil (Nakada et al., 2008) and is one of the most frequently detected pharmaceuticals (Fram et al., 2011). Variable occurrence and reaction in the soil and aquifer suggest it is important to consider a wide range of chemical compounds as waste indicators. Identifying these types of compounds in association with high nitrate concentrations could indicate septic contamination in groundwater.

Researchers have investigated pharmaceuticals, personal care products and artificial sweeteners as markers of human waste. The focus in many studies has been on wastewater and surface water. Glassmeyer et al. (2005) analyzed samples from 10 wastewater treatment plants (WWTPs) in the United States for a suite of compounds that included acetaminophen, caffeine, carbamazepine, cotinine, sulfamethazine, and triclosan. Although sulfamethazine was not detected at any of the sample sites, the other five compounds were detected in at least 50% of the samples in that study. A study in Japan also evaluated concentrations of carbamazepine and triclosan (Nakada et al., 2008).

Concentrations in Japan were found to be approximately one order of magnitude lower compared to rivers in Europe and the United States. These results likely reflect regional differences in product use. The artificial sweetener sucralose was found in European surface waters (Loos et al., 2009). Scheurer et al. (2009) evaluated multiple artificial sweeteners in German waste water and surface waters, and may have been the first to consider sweeteners other than sucralose. Van Stempvoort et al. (2011) assessed artificial sweeteners, including acesulfame and sucralose, in urban areas in Canada. Another recent study evaluated the distribution of pharmaceuticals in over 1000 samples from untreated groundwater used for public drinking-water supplies in California (Fram et al., 2011). Acetaminophen, caffeine, carbamazepine, and paraxanthine were each detected in at least one of the samples.

Chemical characteristics of compounds affect their fate and transport in groundwater. *Koc* is the soil organic carbon water-partitioning coefficient, a value that can be used to predict the mobility of organic soil contaminants. Compounds with higher *Koc* values are less mobile in groundwater. *Solubility* in groundwater is another factor that can fate and transport of a compound. The greater the solubility of a compound, the more likely it will travel through an aquifer (Lawrence, 2006).

Table 2.2 Chemical characteristics of compounds that may affect their fate/transport in groundwater (from the Hazardous Substances Data Bank, 2006-2012.

Compound	Structure	MW (g/mol)	Koc	Solubility mg/L @25°C
Acesulfame		163.15	4	$5.88x10^5$
Acetaminophen		151.17	42	$1.4 x10^4$
Caffeine		194.19	22	$2.16x10^4$
Carbamazepine		236.27	510	18
Cotinine		176.22	130	$1x10^6$
Paraxanthine		180.16	--	
Sucralose		397.64	10	$2.27x10^4$
Sulfamethazine		278.34	49-208	$1.5x10^3$ (at 29°C)
Triclosan		289.54	2400-15,892	10 (at 20°C)

Wastewater Tracer Analysis

One of the challenges to using pharmaceuticals and personal care products as tracers is the difficulty of analysis. The best markers are very water-soluble and do not react with natural solids. They are also usually found at low concentrations. This presents an analytical challenge. It makes it necessary to concentrate samples, but because they are so water soluble, that can be difficult to do. In addition, the analytes of interest may need to be separated from matrix interferences before analysis.

Extraction techniques

Solid phase extraction (SPE) is a sample preparation technique that can improve analytical results by concentrating samples. This can greatly lower detection limits and remove interferences from a sample matrix. Solid phase extraction has many benefits over liquid-liquid extraction, in that it requires less organic solvent waste and no expensive, breakable glassware. The SPE process can be automated, thus decreasing analyst time and increasing reproducibility. It also prevents incomplete phase separation associated with liquid-liquid extraction (Sigma-Aldrich, 1998).

Scheurer et al. (2009) evaluated several types of SPE sorbents as well as the effect of sample pH in extracting artificial sweeteners from water samples. They spiked 50 mL samples at 200 ng/L (0.2 ppb). They achieved recoveries of 0 – 77% for acesulfame, and 52 – 91% for sucralose, depending on the cartridge type and pH of the sample. Table 2.3 lists results for the three highest recoveries for acesulfame and sucralose in the Scheurer study.

Table 2.3 Comparison of percent recoveries for the artificial sweeteners acesulfame and sucralose using various solid phase extraction cartridges and pH adjustments to samples (Scheurer et al., 2009).

Acesulfame (n=3)				Sucralose (n=3)			
Cartridge	Sample pH	% Recovery	Standard Deviation	Cartridge	Sample pH	% Recovery	Standard Deviation
IST Isolute SDB 1 (200 mg)	2	77	0	IST Isolute SDB 1 (200 mg)	2	91	1
IST Isolute SDB 1 (200 mg)	3	75	1	Waters Oasis WAX (60 mg)	5	89	1
Waters Oasis WAX (60 mg)	7	65	13	IST Isolute SDB 1(200 mg)	7	88	3

In addition to Scheurer, other studies have evaluated methods for identifying the chemical waste indicators proposed in this study (Table 2.4). Many of these studies used Water Oasis HLB cartridges for extracting samples. In most cases, this was performed without adjusting the pH of the samples, but certain analyte recoveries seem to be enhanced by acidifying a sample prior to extraction. For example, methods for sulfamethazine and triclosan analyses describe acidifying a sample to pH 2 and 3 prior to extraction, respectively (Thurman et al., 2000; Hua et al., 2005). Spike recoveries in many studies are reported at 60% or greater, though for some studies recoveries were based on as few as three samples.

Table 2.4 Reported analyte recoveries from studies evaluating the chemical waste indicators proposed in this study.

Compound	Cartridge	pH	Samples (N=)	% Spike Recovery	Author
Acetaminophen	Oasis HLB 500 mg (6 mL)	7	8	78 (±8.2)	Cahill et al. (2004)
Caffeine	Sep-Pak tC_{18} Plus (Waters)	6-8	4	70 (±21)	Nakada et al. (2008)
Cotinine	Oasis HLB 500 mg (6 mL)	7	8	108 (±9.5)	Cahill et al. (2004)
Sucralose	Oasis HLB 200 mg (6 mL)	7	6	62 (±9)	Loos et al. (2009)
Triclosan	Oasis HLB 500 mg (6 mL)	3	3	82 (±8)	Hua et al. (2005)

Analyte detection

Several field studies were considered in determining acceptable method detection limits and for evaluating concentrations previously detected in surface and groundwater samples (Table 2.5). Glassmeyer et al. (2005) screened for sulfamethazine in surface water samples, but did not find detectable concentrations. Fram et al. (2011) detected carbamazepine in groundwater at concentrations up to 0.42 ug/L, while Nakada et al. (2008) detected it at 34.7 ug/L in a surface water sample. Van Stempvoort et al. (2011) found sucralose and acesulfame at concentrations of 24 and 33.6 ug/L, respectively, in Canadian groundwater samples. The method detection limit for acesulfame was 0.008 ug/L, but it was three orders of magnitude higher for sucralose at 5 ug/L. This likely accounts for the greater frequency in which acesulfame was detected in that study.

Table 2.5 Summary of method detection limits and maximum concentrations detected in previous studies (GW = groundwater, SW = surface water).

Compound	Method detection limit (ug/L)	Maximum concentration detected (ug/L)	Matrix	Author
Acesulfame	0.008	33.6	GW	Van Stempvoort et al. (2011)
Acetaminophen	0.06	1.89	GW	Fram et al. (2011)
	0.036	1.78	SW	Glassmeyer et al. (2005)
Caffeine	0.10	0.29	GW	Fram et al. (2011)
	0.016	7.99	SW	Glassmeyer et al. (2005)
Carbamazepine	0.03	0.42	GW	Fram et al. (2011)
	0.011	0.27	SW	Glassmeyer at al. (2005)
	--	34.7	SW	Nakada et al. (2008)
Cotinine	0.023	1.03	SW	Glassmeyer et al. (2005)
Paraxanthine	0.06	0.12	GW	Fram et al. (2011)
Sucralose	5	24	GW	Van Stempvoort et al. (2011)
Sulfamethazine	0.1	No detect	SW	Glassmeyer et al. (2005)
Triclosan	0.6	59.1	SW	Nakada et al. (2008)

3. METHODS

A method for simultaneously analyzing a group of contaminants found in human waste would be useful for understanding human impacts to groundwater, but such a method needs to overcome the challenges of low concentration and polar chemistry of the compounds. This section describes the development of such an analytical method and the application to a suburban area that might have mixed sources of groundwater contamination.

Waste Marker Analytical Method Development

Compound Selection

Ten chemical compounds were chosen for the suite of waste indicators. These compounds were chosen based on chemical characteristics, such as likely mobility in groundwater inferred from *Koc* and water solubility, as well as common use. This suite included nine compounds unique to human use and one compound indicative of animal waste (Table 3.1).

Table 3.1 Chemical waste analytes of interest in this study.

Chemical Compound	Use
carbamazepine	pharmaceutical mood stabilizer, anti-seizure medication
acetaminophen	analgesic
triclosan	antimicrobial agent
caffeine	stimulant
paraxanthine	caffeine metabolite
cotinine	nicotine metabolite
acesulfame	artificial sweetener
sucralose	artificial sweetener
benzoylecgonine	cocaine metabolite
sulfamethazine	veterinary antibiotic

Standard preparation

Calibration standards containing the ten waste indicators were prepared in methanol at the concentrations shown in Appendix A. Appendix A also shows the analytes that had deuterated standards available as internal standards. Deuterated standards were not available for acetaminophen and paraxanthine, so acesulfame-D4 and caffeine-D9 were used, respectively, for quantitation. Although benzoylecgonine was not expected to be found in great frequency in rural and suburban groundwater samples and therefore not considered a useful indicator of septic contamination, its good SPE recovery and detection by ESI-LC/MS/MS suggest that benzoylecgonine-D3 has potential as an effective surrogate standard for determining the efficiency of the extraction process. Benzoylecgonine was kept in the intermediate standard mix, and was quantified using sucralose-D6 as the corresponding internal standard.

A linear regression was determined based on the concentrations of seven calibration standards plotted against the response for each analyte. To qualify as a useful indicator, each analyte in the waste indicator mix required a goodness of fit, or R^2, value of 0.990 or greater. This is the quality control value designated for organic compound analysis in the Wisconsin Administrative code NR 149.14. The percent relative standard deviation was calculated for internal standard areas in the calibration standards to evaluate reproducibility and reliability of results.

Analysis and instrument optimization

Analysis of the indicators was performed using an Agilent 1200 series high performance liquid chromatograph coupled to an Agilent 6430 triple quadrupole mass spectrometer with an electrospray ionization source. Twenty uL of sample was injected and carried through the LC column (Zorbax Eclipse XDB-C8 column, 4.6 × 50 mm; 1.8 μ) (Scheurer et al., 2009) by a mobile phase of 15 mM acetic acid in reverse osmosis (RO) water (mobile phase A) and 15 mM acetic acid in methanol (mobile phase B). An Agilent 1200 series LC pump was used to provide a pre-programmed gradient at a flow rate of 0.5 mL/minute. With electrospray ionization, the sample and mobile phase are drawn to the tip of a capillary tube where a voltage is applied. The sample is nebulized into a fine aerosol to create charged droplets. As the solvent evaporates with a counter flow of heated nitrogen gas, the electric field on the droplet surface increases until the droplet bursts, forming smaller droplets. The process continues until ions are released into the gas phase. These ions can then be analyzed by mass spectroscopy (Cech and Enke, 2001).

The triple quadrupole mass spectrometer was run in multiple reaction-monitoring (MRM) mode for both positive and negative ions. This allowed for multiple precursor ions to be selected for and monitored in the same analytical run. While sensitivity, determined by peak shape and area, was satisfactory for many of the analytes of interest, the peak areas for others were less than desired. For example, a peak area of less than 5000 for sucralose was considered low. An effort was made to manually optimize the instrument conditions for the analysis of acesulfame, sucralose, and triclosan. This process began with determining the exact mass of the correct precursor ion in MS2 Scan

mode. Next, the appropriate fragmentor voltage was determined by scanning in MS2 selected ion monitoring (SIM). A product ion scan was performed to identify product ions and the collision energy that provides the greatest abundance for those product ions. Finally, an MRM scan was run to determine the cell accelerator voltage for each product ion. This process required an extensive process of optimization, but resulted in noticeable increases in peak area for some analytes. Table 3.2 shows the instrument conditions before and after optimization. The gas flow and nebulizer pressure were also optimized. Table 3.3 shows the peaks areas for certain analytes before and after optimization.

In an effort to increase detection sensitivity, the analytical run was divided into time segments. Instead of scanning for all analytes during the entire method run, specific MRM transitions are monitored only during a specific time segment. There are fewer transitions to monitor during each scan. The dwell time, or the amount of time analyzing for a single MRM transition, was maximized, which improved signal-to-noise ratios and increase sensitivity. Table 3.4 shows the optimized conditions for the analytes of interest and internal standards. It also indicates which analytes are monitored in each time segment.

Table 3.2 Binary pump timetable before and after optimization was performed.

Initial		Optimized	
Time (minutes)	% of mobile phase B	Time (minutes)	% of mobile phase B
0	10	0	10
10	80	6.5	95
12	80	8.5	95
13	10	9	10
Post run time (minutes)		Post run time (minutes)	
2		1.5	
Total run time (minutes)		Total run time (minutes)	
17		12	

Table 3.3 Comparison of peak areas before and after optimization. Prior to optimization areas are the average of the last five analytical runs prior to optimization. After optimization areas are an average of the first two runs after optimization was performed.

	Calibration Standard #1 (Low)		Calibration Standard #7 (High)	
Analyte	**Prior to Optimization**	**After Optimization**	**Prior to Optimization**	**After Optimization**
Acesulfame	751	3391	69524	271394
Sucralose	1078	1122	57201	80052
Triclosan	439	1238	33096	100221

Table 3.4 Optimized conditions for analytes in the chemical waste marker suite. IS? = Indicates if the analyte is used as an internal standard; Prec Ion = Precursor Ion; Prod Ion = Product ion; FV = Fragmentor Voltage; CE = Collision Energy; CAV= Cell Accelerator Voltage; IM = Ion Mode; TS = Time Segment

Analyte	IS?	Prec Ion	Prod Ion	Dwell	FV	CE	CAV	IM	TS
Acesulfame	No	162.1	162.1	90	80	0	2	-	2
Acesulfame	No	162.1	82.1	90	80	8	2	-	2
Acesulfame-D4	Yes	166.2	166.2	90	80	0	2	-	2
Acesulfame-D4	Yes	166.2	86.2	90	80	8	2	-	2
Acetaminophen	No	152	110	42	90	15	3	+	2
Acetaminophen	No	152	65	42	90	35	3	+	2
Cotinine	No	177	98	42	90	20	5	+	2
Cotinine	No	177	80	42	90	24	5	+	2
Cotinine-D4	Yes	181	98	42	90	20	5	+	2
Cotinine-D4	Yes	181	84	42	90	24	5	+	2
Paraxanthine	No	181	124	42	100	35	3	+	2
Paraxanthine	No	181	69	42	100	35	3	+	2
Caffeine	No	195	138	36	110	15	2	+	3
Caffeine	No	195	110	36	110	25	2	+	3
Caffeine-D9	Yes	204	144	36	110	25	2	+	3
Caffeine-D9	Yes	204	116	36	110	30	2	+	3
Sucralose	No	419	239	80	129	15	2	+	3
Sucralose	No	419	221	80	129	15	2	+	3
Sucralose-D6	Yes	427	245	80	129	15	2	+	3
Sucralose-D6	Yes	425	243	80	129	15	2	+	3
Sulfamethazine	No	279	156	36	90	15	2	+	3
Sulfamethazine	No	279	186	36	90	15	2	+	3
Sulfamethazine-D4	Yes	283	160	36	90	15	2	+	3
Sulfamethazine-D4	Yes	283	96	36	90	15	2	+	3
Benzoylecgonine	No	290	168	36	100	24	2	+	3
Benzoylecgonine	No	290	105	36	100	24	2	+	3
Benzoylecgonine-D3	No	293	171	36	100	24	2	+	3
Benzoylecgonine-D3	No	293	105	36	100	24	2	+	3
Carbamazepine	No	237	194	65	125	18	2	+	4
Carbamazepine	No	237	179	65	125	38	2	+	4
Carbamazepine-D10	Yes	247	204	65	125	18	2	+	4
Carbamazepine-D10	Yes	247	202	65	125	38	2	+	4
Triclosan	No	289	35	105	105	70	4	-	4
Triclosan	No	287	35	105	105	70	4	-	4
Triclosan-D3	Yes	290	35	105	105	70	4	-	4
Triclosan-D3	Yes	292	35	105	105	70	4	-	4

Solid phase extraction

A Dionex Autotrace 280 (Thermo Scientific) unit was used for automated solid phase extraction (SPE) of samples. SPE is a four-step process of conditioning, loading, drying and eluting the solid phase cartridges. When the SPE process was complete, the eluted samples were dried down, and then brought up to the desired volume in a solution that matches the mobile phase initial make-up. This study evaluated different methods for the solid phase extraction. Two sorbents were evaluated. Their properties are listed in Table 3.5. Several different processing methods were compared. Table 3.6 outlines the variations in the SPE methods tested. All four methods used a sampling loading volume of 100 mL and a final volume of 500 uL. This should yield a 200-fold increase in analyte concentration, demonstrated in Equation 3-1.

Equation 3-1 Calculation showing that the solid phase extraction methods evaluated in this study had the potential to increase analyte detection by a factor of 200.

Concentration of analyte in HW intermediate mix		Volume of HW intermediate		Volume of spiked sample		Concentration of carbamazepine in spike mix
100 ng/mL	x	50 uL	=	1000 mL	x	5 ng/L

Sample volume loaded onto SPE cartridge		Concentration of carbamazepine in spike mix		Volume of sample eluted		Concentration in eluted sample
100.0 mL	x	5 ng/L	=	5.0 mL	x	100. ng/L

Volume of sample eluted		Concentration in eluted sample		Final volume of extract		Concentration in extract
5.0 mL	x	100.0 ng/L	=	500 uL	x	1 ug/L (= 1000 ng/L)

$$\frac{\text{Final concentration}}{\text{Initial concentration}} = \frac{1000 \text{ ng/L}}{5 \text{ ng/L}} = \textbf{200-fold increase in analyte concentration}$$

Statistical comparison of the SPE methods used a non-parametric test to evaluate which method would yield analyte recoveries closest to 100%. Analyte recoveries for two methods were ranked from smallest to greatest by the absolute value of difference between the recovery and 100%. Under the null hypothesis that there are no differences between methods, the sum of ranks for each method should be similar. The probability was calculated that there was a difference in the rank sums. This test was useful, as methods were not run with an equal number of trials. This test also minimizes the effects of outliers. The program was run to calculate the probability that one method would have recoveries significantly closer to 100% than another method. The statistical program R (x62 3.0.1) was used to create the box plots and perform a Mann-Whitney U-test. The box plots show the median and inner quartiles within the box (Williamson et al., 1989; Yau, 2014).

Table 3.5 Description of solid phase cartridges evaluated in this study.

Cartridge	Sorbent	Designed Use
Waters Oasis HLB 6 cc (200 mg)	Hydrophilic-lipophilic-balanced (reversed-phase)	wide range of acidic, basic, and neutral compounds
UCT C8+Aminopropyl CUNAX2 6 mL (1000 mg)	Hydrophobic plus Aminopropyl Copolymeric	neutral and charged compounds

Table 3.6 Description of the solid phase extraction schemes evaluated. The first letter in the method abbreviation signifies the cartridge (H=HLB, N=NAX). The second letter indicates how the method was varied (A=sample acidified; U=no pH adjustment; D=sample taken to total dryness).

	NU	HU	HA	HD
Sample pH	No adjustment	No adjustment	Adjusted with H_2SO_4 (1:1) to <2	No adjustment
Cartridge	UCT C8+ Aminopropyl CUNAX2 6 mL (1000 mg)	Waters Oasis HLB 6 cc (200 mg)	Waters Oasis HLB 6 cc (200 mg)	Waters Oasis HLB 6 cc (200 mg)
Cartridge conditioning	5.0 mL of methanol at 5.0 mL/minute	5.0 mL of methanol at 5.0 mL/minute	3.0 mL of acetonitrile at 10.0 mL/min.	5.0 mL of methanol at 5.0 mL/minute
	5.0 mL of RO water at 5.0 mL/minute	5.0 mL of RO water at 5.0 mL/minute	3.0 mL of RO water adjusted with H_2SO_4 (1:1) to <2 at 10.0 mL/min.	5.0 mL of RO water at 5.0 mL/minute
Loading	100.0 mL of sample at 5.0 mL/minute	100.0 mL of sample at 5.0 mL/minute	100.0 mL of sample at 1.0 mL/minute	100.0 mL of sample at 5.0 mL/minute
Drying with nitrogen gas	5 minutes	30 minutes	1 minute	30 minutes
Elution	5.0 mL of 2.5% NH_4OH in methanol at 5.0 mL/minute	5.0 mL of methanol at 5.0 mL/minute	5.0 mL of acetonitrile at 1.0 mL/minute	5.0 mL of methanol at 5.0 mL/minute
Drying down	40˚C to 200 uL	50˚C to less than 100 uL	50˚C to 50 uL	50˚C to total dryness

Field Application

Study Site

The field test of the indicator suite was performed in the Town of Hull in Portage County, Wisconsin. Residents in the Town of Hull rely on private wells for their drinking water, making groundwater quality a major concern for the community. The study site was located in the Conifer Estates Subdivision in the Town of Hull (SE ¼ of the SE ¼ section 35, T24N-R8E). This area was selected because it has two possible sources of nitrate contamination. It has a relatively high density of wells and septic systems, with most of the lots being less than one acre in size. There is agricultural land east of the subdivision and groundwater flows from east to west.

Monitoring wells and private water supply wells were used in the study. Three monitoring wells had been installed by the University of Wisconsin – Stevens Point (W1, W2, and W3). These wells were constructed from polyvinylchloride (PVC) pipes. The private wells were six inch, drilled wells with metal casings and screens. One well had been drilled in 2013 (W4) and the other in 2000 (W5). The lower portion of Figure 3.2 indicates the locations of the wells in the subdivision.

The five wells in the study varied in depth from 28 to 54 feet. The top portion of Figure 3.2 illustrates the relative elevation of the wells. Static water level and total depth of the monitoring wells was measured using a popper. Static water level and depth of the private water supply wells were taken from well construction reports (Table 3.7).

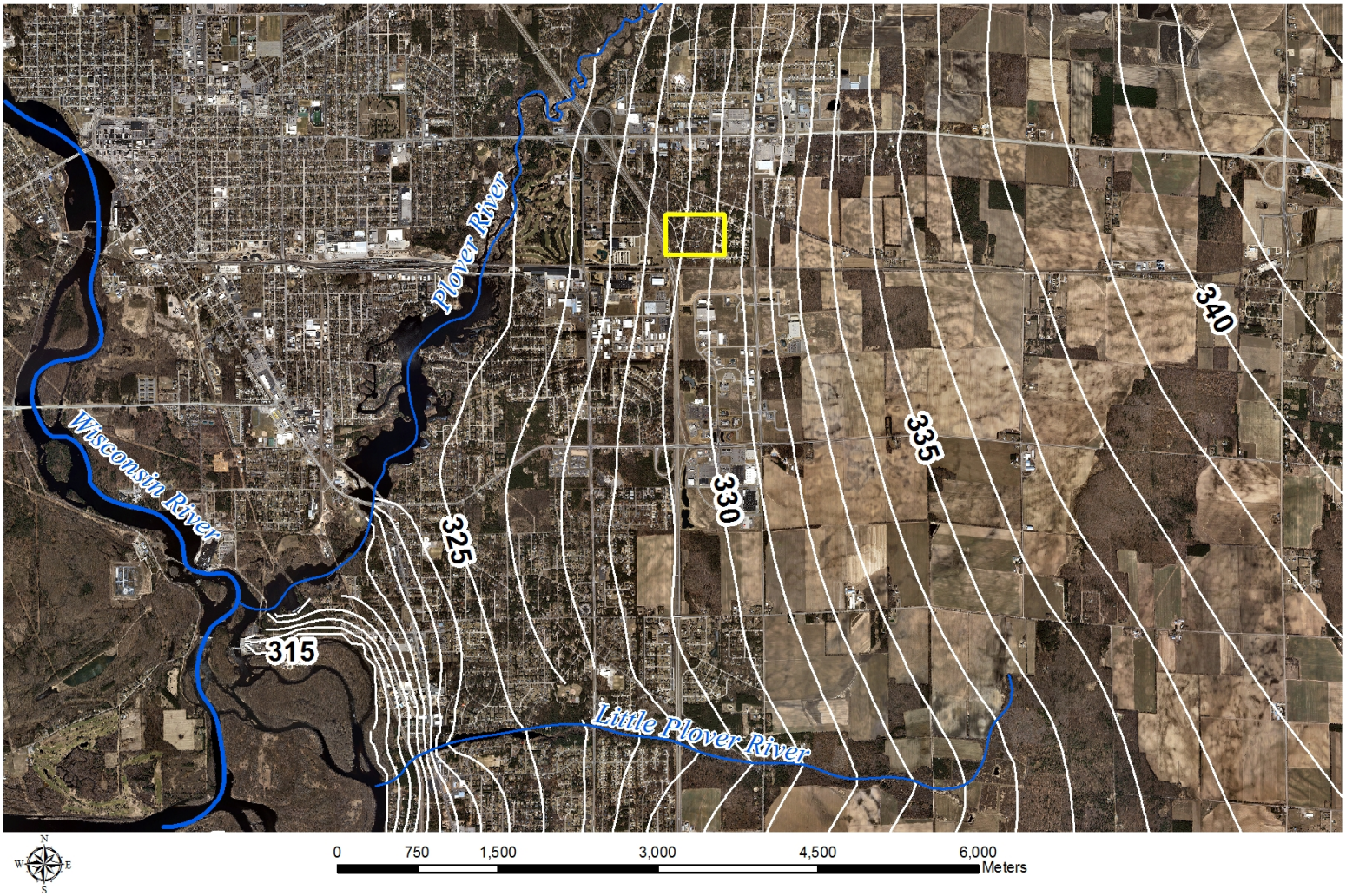


Figure 3.1 Field study location. Groundwater contours shown in meters. (Groundwater contour mapping provided by Dave Mechenich, UWSP Center for Watershed Science and Education and assistance with map formatting provided by Eric Englund.

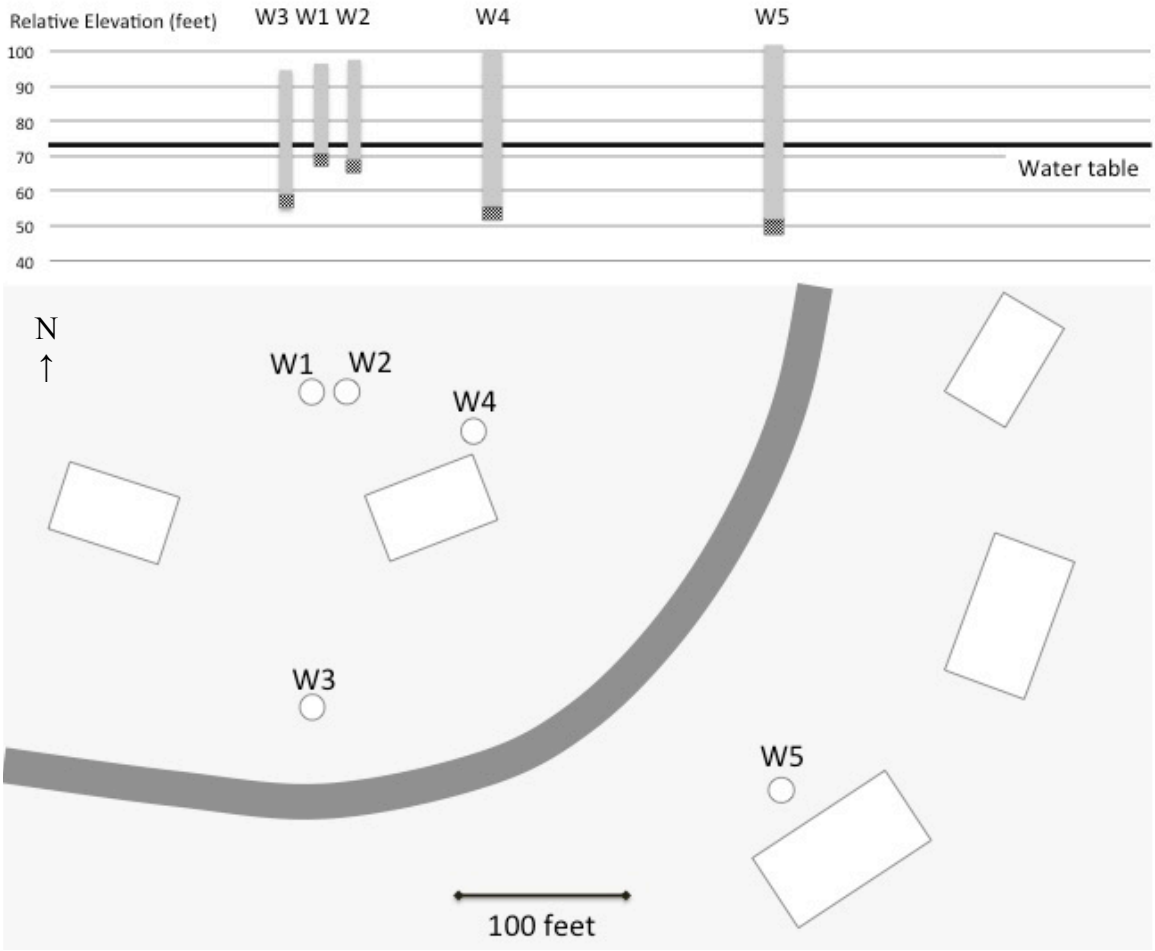


Figure 3.2 Cross-sectional view depicting relative elevations of wells (upper). Crosshatched areas on the well profile show approximate well screens locations, but are not to scale. Plan view of field study sites (lower) indicating the positions of wells, houses, and the road in the subdivision (not to scale).

Table 3.7 Well information for the field study site. (* = no record, assumed length)

Well ID	Well construction material	Depth to static water from top of casing (feet)	Depth of well bottom (feet)	Screen Length (feet)
W1	PVC	25.1	28	3*
W2	PVC	25.3	32	3*
W3	PVC	23.5	41	3*
W4	metal	27.5	48.5	3
W5	metal	31.0	54	4

Well Sampling

Groundwater samples from the monitoring wells were collected by pumping through polyethylene tubing. Approximately four well volumes were purged from the wells before samples were taken. W4 was sampled from an outside faucet and W5 was sampled from a pressure tank. Samples for chloroacetanilide herbicide metabolites (CAAM) and human waste indicator analyses were collected in one-liter, amber glass bottles. Samples for nitrate and chloride analyses taken from the monitoring wells were field filtered using a 0.45 um membrane filter, and stored in high-density polyethylene (HDPE) bottles acidified with sulfuric acid. Samples for phosphorus and metals analyses taken from the monitoring wells were field filtered using a 0.45 um membrane filter, and stored in HDPE bottles acidified with nitric acid. Samples for pH, conductivity, alkalinity and total hardness were stored in unacidified HDPE bottles. All samples were stored on ice in coolers during transport from the site to the Water and Environmental Analysis Laboratory (WEAL) at the University of Wisconsin – Stevens Point. Samples were stored at 4°C until analysis.

Analytic Techniques

Conductivity and pH were measured in the laboratory immediately upon returning from the study site using a Mettler Toledo SevenEasy conductivity meter and a Corning Ion Analyzer 350. Alkalinity was performed by titration. Total hardness was calculated from calcium and magnesium results.

Nitrate and chloride were analyzed by a Lachat 8000 using flow injection analysis (FIA). Nitrate was measured as nitrate plus nitrite (NO_3^-/NO_2^-). A cadmium column reduced the nitrate to nitrite, and a sulfanilamide color reagent was used to determine the

concentration at 520 nm (Lachat Method 10-107-04-1-A). The WEAL has a method detection limit (MDL) of 0.1 mg/L NO_3^-/NO_2^- (N). Chloride analysis uses a mercuric color reagent to form ferric thiocyanate, which absorbs at 480 nm. This absorbance is proportional to the chloride concentration (Lachat Method 10-117-07-1-B). The WEAL MDL for chloride is 0.5 mg/L.

Phosphorus and other major ions were analyzed by Varian Vista inductively coupled plasma – optical emission spectroscopy (ICP-OES). The WEAL MDL for phosphorus was 0.005 mg/L.

Samples were extracted for chloroacetanilide herbicide metabolites according to the method USGS Open File Report #00-182 (Zimmerman et al., 2000). 125 mL of each sample was processed through the Dionex Autotrace 280 Solid Phase Extraction (SPE) system utilizing Waters SepPak C18 cartridges, which had been conditioned with methanol, ethyl acetate, again with methanol, and RO water. The C18 cartridge was first eluted with ethyl acetate, to remove the non-polar compounds. Methanol was used to elute the second fraction, containing the polar CAAMs, and was collected in 5 mL glass centrifuge tubes. Samples were concentrated using a Turbovap Concentration Work Station at 50ºC to take the samples to complete dryness. Extracts were reconstituted with 80:20 buffer:acetonitrile. Vials were fitted with a 200-µL insert and sample extracts were transferred to the appropriate vial for analysis. These samples were stored in a freezer until they were analyzed. Monitoring well sample extracts were analyzed by the Agilent 1100 HPLC, equipped with a UV photodiode array detector (PDA). Analytes were identified and quantified using a Betasil C18 250 x 5 mm column with 5 micron particles, and confirmed with an Aquasil C18 250 x 5 mm column with 5 micron particles. The

private well samples were analyzed using ESI-LC/MS/MS with a Hypersil Gold (150 x 2.1 mm; 1.9 u) column (Thermo Scientific).

Chemical indicators of human waste were identified. Samples were not filtered, but sediment in the monitoring well samples was allowed to settle out in the glass amber bottles, and sample lines for the Autotrace were kept off the bottom of the sample bottles. Solid phase extractions were performed on samples, a RO blank and a spike. The spike was prepared by adding 50 uL of human waste intermediate to 1000 mL of RO water to produce analyte concentrations of 5.0 – 50.0 ng/L. Waters Oasis HLB 6cc (200 mg) cartridges were conditioned with 5.0 mL of methanol and 5.0 mL of RO water at 5.0 mL/minute. The liquid handling syringe was rinsed with 5.0 mL of methanol, and then 100 mL of sample was loaded onto cartridges at 2.0 mL/minute. Cartridges were dried for 15 minutes. 2.5 mL of methanol was used to soak cartridges and then the eluent was collected in a sample tube at 2.0 mL/minute. Another 2.5 mL of methanol was used to soak cartridges, and this was collected in the same tube as the first fraction. Samples were dried down to approximately 50 uL with the Turbovap Concentration Work Station at 50ºC. Fifty uL of the human waste mix internal standard in methanol was added to each sample. Samples were brought to a final volume of 500 uL with 15 mM acetic acid in water. Analysis was performed by ESI-LC/MS/MS. Agilent MassHunter quantitation software was used to quantify analyte concentrations.

4. RESULTS AND DISCUSSION

Waste Tracer Method

Solid Phase Extraction Method Development

The development of a waste indicator suite requires a solid phase extraction method that will concentrate and then recover all analytes. Four solid phase extraction methods were evaluated. These methods varied the sample pH, dry down technique, and cartridge sorbent. The four methods are abbreviated with the two letter codes that were shown in Table 3.6. The results of the SPE trials are summarized in Table 4.1 and are shown in Figure 4.1 to Figure 4.5. Detailed results are shown in Appendix B.

Some analyte recoveries were quite inconsistent. With the NU method, cotinine recoveries ranged from 74.5 – 1290% and acesulfame recoveries ranged from 5.1 to 785.2%. Typically, no more than five extractions were performed within a single automated extraction; therefore, there is a possibility that errors could occur between extractions. For example, it is possible that the Autotrace unit had mechanical issues, e.g., pump failure or inaccurate loading volume. More likely, though, ion suppression in the mass spectrometer resulted in a lesser or greater internal standard area compared to standard areas. This would produce an artificially high or low quantified analyte concentration.

Recoveries also varied between compounds within a method. For example, most analytes had satisfactory recoveries within the HU method, but acesulfame had very poor recoveries. Because the recoveries were consistently low, this is likely due to an unsuitable extraction method for the analyte. Recoveries for acesulfame improved when

the sample was acidified prior to extraction in method HA. Similarly, recoveries for acetaminophen were lower with the NU method compared to HU, HA, and HD. This indicates that cartridge sorbent is important in the extraction process for this analyte.

Table 4.1 Range and median percent recoveries for solid phase extraction methods.

	COT	**ACE**	**AMN**	**PXN**	**CAF**	**SLF**	**SUC**	**CRB**	**TRI**
					NU				
N	12	12	6	12	12	12	12	12	12
Lowest % Rec	74.5	5.1	11.9	42.1	125.6	66.3	8.0	78.6	0.0
Highest %Rec	1290	785.2	22.6	253.0	543.0	94.2	55.5	94.0	115.0
Median %Rec	106.6	105.8	41.2	69.4	161.5	86.6	24.4	83.5	52.1
					HU				
N	9	9	9	9	9	9	9	9	9
Lowest % Rec	17.0	1.0	67.0	68.0	81.0	77.5	38.7	77.4	34.0
Highest %Rec	109.3	12.0	110.5	143.1	220.5	102.0	116.3	235.0	588.6
Median %Rec	56.7	3.4	94.1	102.3	95.6	89.1	66.0	82.7	155.0
					HA				
N	9	9	9	9	9	9	9	9	9
Lowest % Rec	0.0	20.4	0.4	49.0	75.5	58.7	0.0	9.3	3.3
Highest %Rec	5.0	59.0	85.6	133.0	142.0	86.5	58.2	96.9	31.6
Median %Rec	0.4	45.4	72.8	75.5	95.9	76.7	31.0	73.3	13.7
					HD				
N	15	15	10	15	15	15	15	15	15
Lowest % Rec	31.8	0.0	68.2	35.9	60.6	40.1	41.0	51.2	51.9
Highest %Rec	98.9	25.1	147.9	131.3	252.8	91.9	267.6	96.9	791.6
Median %Rec	68.7	15.0	91.4	61.1	152.7	81.6	147.9	70.3	103.8

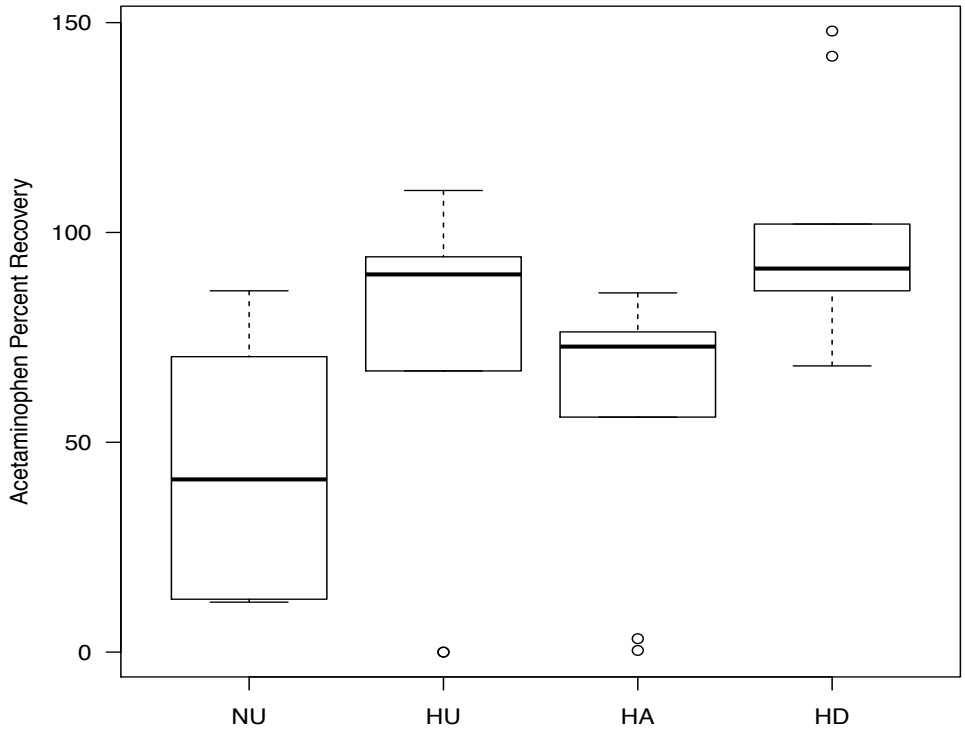


Figure 4.1 Box plot for acetaminophen recoveries from the four solid phase extraction methods.

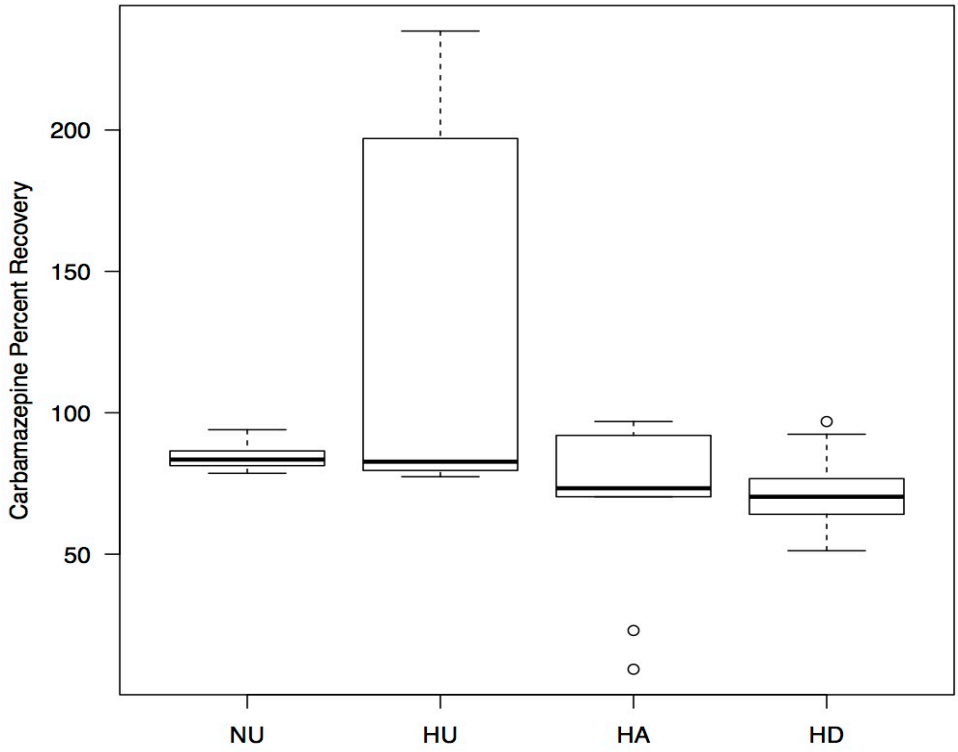


Figure 4.2 Box plot for carbamazepine recoveries from the four solid phase extraction methods.

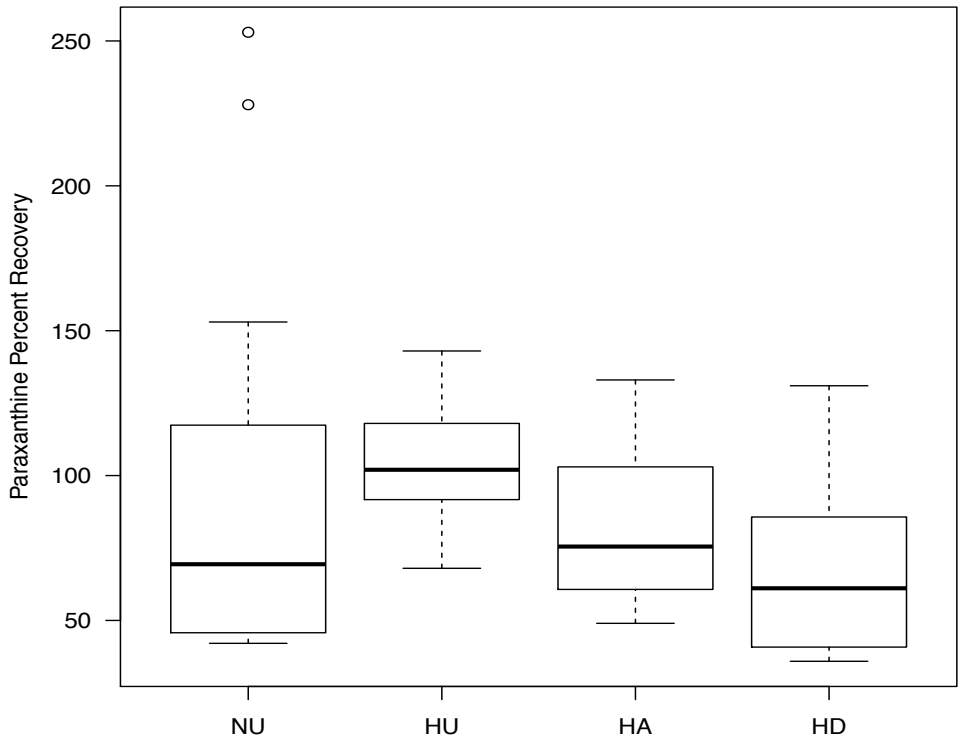


Figure 4.3 Box plot for paraxanthine recoveries from four solid phase extraction methods.

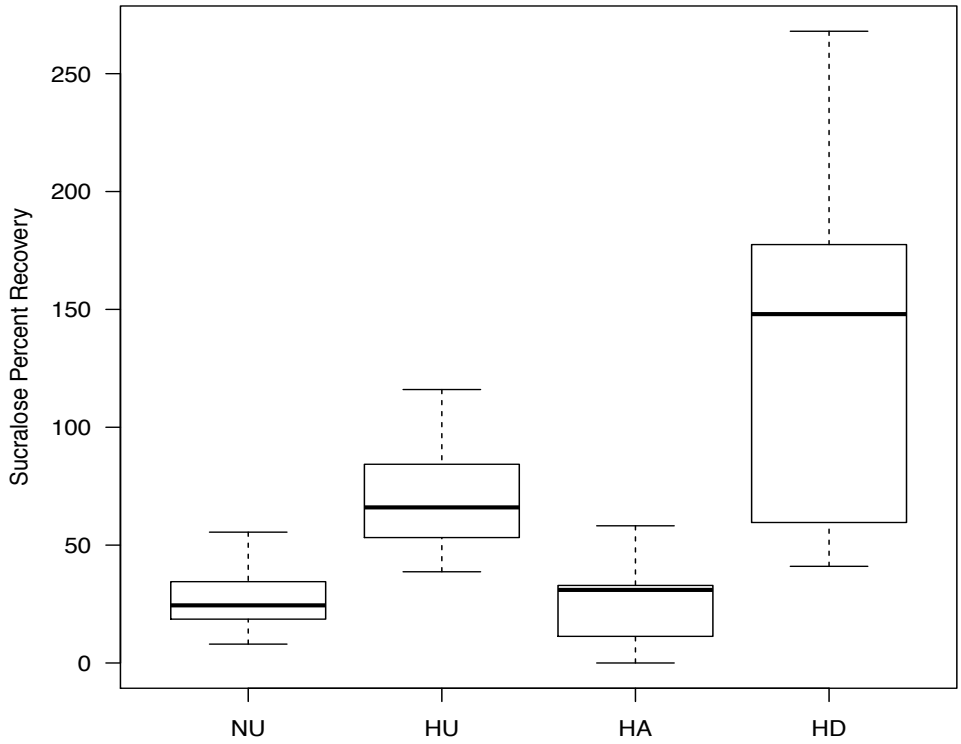


Figure 4.4 Box plot for sucralose recoveries from four solid phase extraction methods.

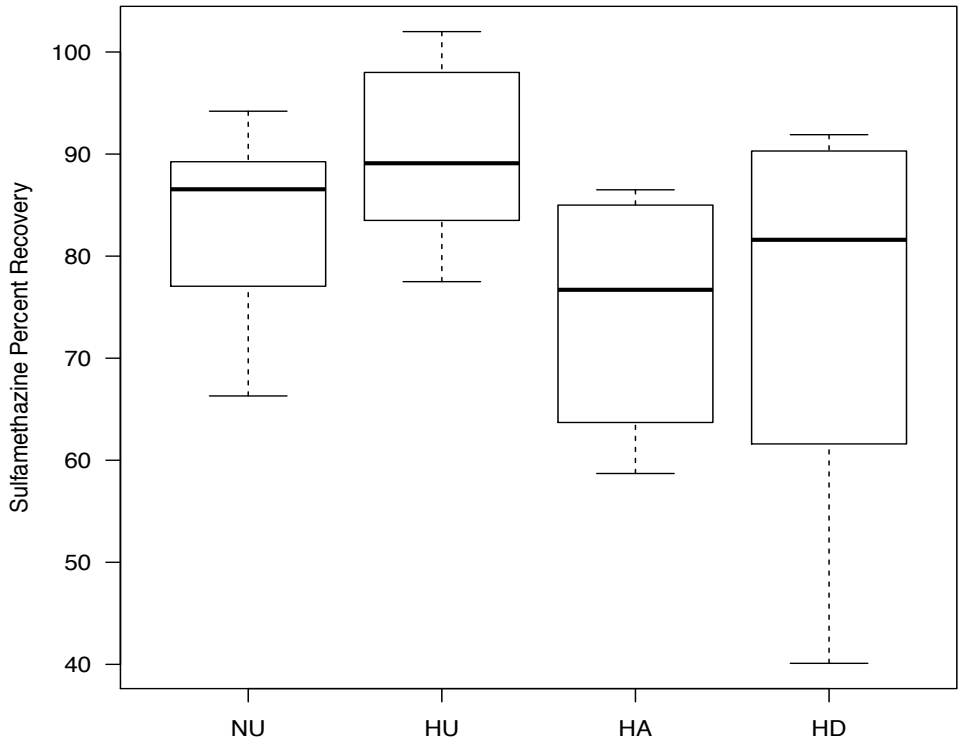


Figure 4.5 Box plot for sulfamethazine recoveries from four solid phase extraction methods.

Initial inspection of the recoveries suggested that method HU was superior to the others. A Mann-Whitney U-test was used to statistically compare the methods (Appendix C). The absolute value of the difference between the percent recovery and 100 for each trial of an analyte was used to calculate the distance from the desired recovery of 100% for each trial. A one-sided test was used to determine if analyte recoveries using the HU method were statistically closer to 100% when compared against another method (Table 4.2). p-values calculated from the test indicate that this method yields statistically better recoveries for seven analytes compared to at least one of the other methods. Paraxanthine, caffeine, and sulfamethzine had better recoveries with the HU method compared to two other methods. The HU method had better recoveries compared to all three of the other methods for sucralose.

The Mann-Whitney U-test was performed again with the NU, HA, and HD methods using the one-sided p-value. All resulting p-values were compared ($p < 0.01$). The HU method still exhibited statistically better recoveries for six analytes compared to at least one of the other methods. Caffeine and sucralose had better recoveries with the HU method compared to two other methods.

Acesulfame did not have statistically better recoveries with the HU method, nor with the NU or HD methods. The HA method, however, showed better recoveries when compared with the HU and HD methods. This was not surprising, as previous studies had indicated that acidifying a sample prior to extraction could produce better recoveries for acesulfame (Table 2.3).

Carbamazepine also did not have statistically better recoveries with the HU method, nor with the HA or HD methods. The NU method, however, showed better

recoveries when compared with the HU and HD methods. This would indicate that the C8+Aminopropyl CUNAX2 cartridge sorbent is more efficient for extracting carbamazepine than the HLB sorbent. Carbamazepine was the only analyte that had improved recovery with the NU method.

The HD method had statistically better recoveries for three analytes. Recoveries were better for cotinine and triclosan when compared to HA method. These two analytes also had better recoveries with the HU method compared to the HA method. This indicates that acidifying samples prior to extraction interferes with their recovery. Acetaminophen also had better recovery compared to the NU method. There was no statistical difference in recoveries for acetaminophen with any other method comparison. This would indicate that the HLB cartridge has a slight advantage over the NAX cartridge with this analyte.

The HA method also yielded statistically better recoveries for caffeine compared to the NU and HD methods. This would indicate that the HLB cartridges allow for better extraction of caffeine from water samples. It was somewhat surprising that the pH of the sample would not affect recovery, but drying the sample completely seemed to reduce recovery.

Table 4.2 p-values comparing the percent recoveries for analytes using the HU method with the other three methods. * = p <0.01

HU	NU	HA	HD
Cotinine	0.542	0.000*	0.845
Acesulfame	0.865	1.000	0.735
Acetaminophen	0.030	0.145	0.613
Paraxanthine	0.001*	0.093	0.014
Caffeine	0.005*	0.799	0.010*
Sulfamethazine	0.094	0.004*	0.037*
Sucralose	0.000*	0.001*	0.021*
Carbamazepine	0.994	0.892	0.571
Triclosan	0.840	0.007*	0.955

NU	HU	HA	HD
Cotinine	0.486	0.029	0.775
Acesulfame	0.151	0.793	0.158
Acetaminophen	0.975	0.954	0.998
Paraxanthine	0.999	0.987	0.841
Caffeine	0.996	1.000	0.696
Sulfamethazine	0.917	0.023	0.225
Sucralose	1.000	0.430	0.925
Carbamazepine	0.001*	0.273	0.002*
Triclosan	0.178	0.009*	0.760

HA	HU	NU	HD
Cotinine	1.000	0.975	1.000
Acesulfame	0.000*	0.228	0.000*
Acetaminophen	0.875	0.056	0.985
Paraxanthine	0.921	0.015	0.135
Caffeine	0.226	0.000	0.001*
Sulfamethazine	0.997	0.980	0.781
Sucralose	0.999	0.598	0.932
Carbamazepine	0.126	0.750	0.285
Triclosan	0.995	0.993	0.995

HD	HU	NU	HA
Cotinine	0.170	0.240	0.000*
Acesulfame	0.285	0.853	1.000
Acetaminophen	0.419	0.002*	0.019
Paraxanthine	0.988	0.171	0.878
Caffeine	0.992	0.322	0.999
Sulfamethazine	0.968	0.790	0.237
Sucralose	0.982	0.082	0.076
Carbamazepine	0.453	0.998	0.740
Triclosan	0.055	0.255	0.006

Instrument response

Seven calibration standards containing all of the target analytes were prepared. To qualify as a useful indicator, each analyte in the septic tracer mix should have an R^2 value, or correlation coefficient, of 0.990 or greater. This is the quality control value designated for organic compound analysis in the Wisconsin Administrative code NR 149.14. This also demonstrates that analyte concentrations can be reliably quantified within our calibrated range. R^2 values for each analyte were averaged from seven separate analytical runs. Table 4.2 shows that all of the analytes meet this criterion. Most correlations were linear; however, sucralose fit a quadratic equation.

Table 4.3 R^2 values based on an average of seven separate analytical runs and the calibrated range of standard concentrations.

	COT	ACE	AMN	PXN	CAF	SLF	SUC	CRB	TRI
R^2	0.9996	0.9995	0.9994	0.9991	0.9987	0.9998	0.9999	0.9989	0.9997
Range (ug/L)	0.1-40.0	0.2 – 80.0	0.2 – 80.0	0.1-40.0	0.1-40.0	0.1-40.0	0.5-100.0	0.05 – 10.0	0.5-100.0

Internal standard areas for each analyte were compared for reproducibility and reliability of results. Internal standard areas should be consistent across the seven calibration standards. While internal standard and analyte areas may vary between analytical runs, the ratio of internal standard to a calibration standard should be consistent. A calibration performed on 12/7/13 shows that the percent relative standard deviation for most internal standards was less than 5%. Cotinine-D4 had a %RSD of 5.6, and triclosan had a %RSD of 10.2, however, calibration curves were acceptable for these analytes. This variation is likely due to the poor chromatography of this compound.

Table 4.4 The percent relative standard deviation (%RSD) was calculated for internal standard areas in the calibration standards (12/7/13). The internal standard mix is added to all calibration levels at equal concentrations so their areas are expected to be similar.

	Internal Standard Areas						
Cal Level	**COT-D4** 20 ng/mL	**ACE-D4** 40ng/mL	**CAF-D9** 20 ng/mL	**SLF-D4** 20 ng/mL	**SUC-D6** 100 ng/mL	**CRB-D10** 10 ng/mL	**TRI-D3** 100 ng/mL
1	302703	102340	203628	180763	11829	1504787	44733
2	325389	102449	207464	184221	12653	1564159	45422
3	324258	98863	203324	182675	12488	1560660	43625
4	330189	105426	204243	185079	12991	1615332	46403
5	337694	106496	209339	182500	12859	1619382	49106
6	348318	108747	204993	181976	12957	1601561	52004
7	361134	110232	212319	182896	13811	N/A	57559
Avg	332812	104936	206473	182873	12798	1577647	48407
%RSD	**5.6**	**3.8**	**1.6**	**0.8**	**4.7**	**2.8**	**10.2**

Method Detection Limit

A method detection limit study was conducted using the HU method. These limits of detection were used for reporting the analytical results from the field study.

Table 4.5 Results for a method detection limit study conducted using the sample preparation method listed above (HU). Standard deviation (SD) and limits of detection (LOD) are reported as ng/L.

	COT	**ACE**	**AMN**	**PXN**	**CAF**	**SLF**	**BNZ**	**SUC**	**CRB**	**TRI**
N	6	6	4	6	6	6	6	6	6	6
Avg % Rec	75.5	4.8	102.1	112.3	140.7	85.2	76.4	84.8	88.7	205.1
Std Dev	1.6	0.4	1.4	1.4	4.0	0.4	10.3	0.6	0.8	72.9
LOD (ng/L)	**5.5**	**1.3**	**6.3**	**4.9**	**13.4**	**1.2**	**34.7**	**2.1**	**2.7**	**245**

Field study

Groundwater Chemistry comparison

Nitrate

Nitrate concentrations ranged from 4.4 mg/L to 50.5 mg/L in the five wells (Figure 4.6). Only W2 had a nitrate concentration below the drinking water standard of 10 mg/L. The shallowest well, W1, had a nitrate concentration of 17.7 mg/L. The three deepest wells, W3, W4, and W5, had nitrate concentrations greater than 20 mg/L, and the nitrate concentration increased with depth. Between 41 and 48.5 feet, the nitrate concentration almost doubled.

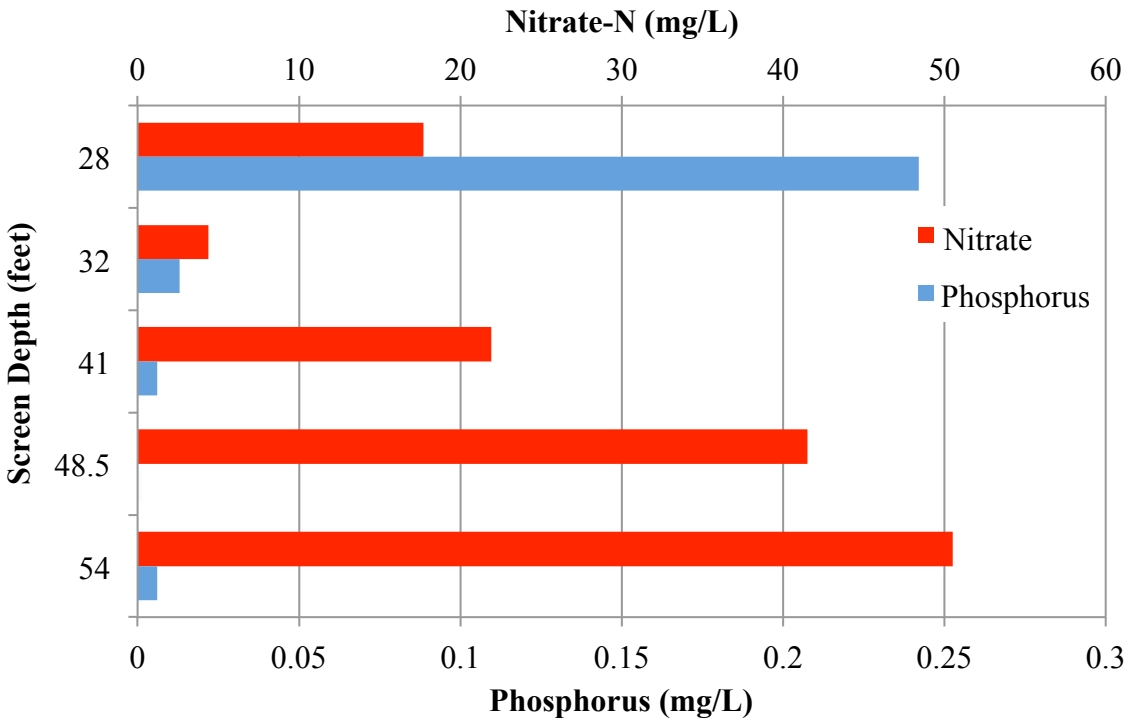


Figure 4.6 Graph of nitrate and phosphorus in wells. Well screen depths not to scale.

Phosphorus

Phosphorus concentrations ranged from less than the detection limit to 0.242 mg/L (Figure 4.6 and Table 4.8). The greatest concentration of phosphorus was found in the shallowest well. W2 had a concentration of 0.013 mg/L. In the three deepest wells, two had concentrations of 0.006 mg/L, and one was less than the limit of the detection.

Other Analyses

Analyses were performed for pH, conductivity, alkalinity, total hardness and chloride (Table 4.6). pH ranged between 7.31 and 7.96. Conductivity ranged from 340 to 857 umhos/cm (Figure 4.7). Conductivity was lowest in W2. Alkalinity ranged from 132 to 288 mg/L $CaCO_3$, and hardness ranged from 128 to 252 mg/L $CaCO_3$. There did not seem to be a relationship between alkalinity or hardness with depth or other analyte concentrations. Chloride ranged from 10.3 to 90.5 mg/L (Figure 4.8). Chloride was lowest in W2. Chloride was highest in the three deepest wells, which could be attributed to agricultural sources; however there was not a clear relationship with nitrate or chloracetanilde metabolite concentrations.

Table 4.6 Analytical results for pH, conductivity, alkalinity, total hardness and chloride from the five wells in the field study.

		W1	W2	W3	W4	W5
Nitrate	mg/L	17.7	4.4	21.9	41.5	50.5
pH	std units	7.31	7.96	7.81	7.92	7.94
Conductivity	umhos/cm	482	340	695	826	857
Alkalinity	mg/L $CaCO_3$	132	156	164	288	232
Total Hardness	mg/L $CaCO_3$	205	157	249	369	230
Chloride	mg/L	26.5	10.3	74.3	90.5	64.0

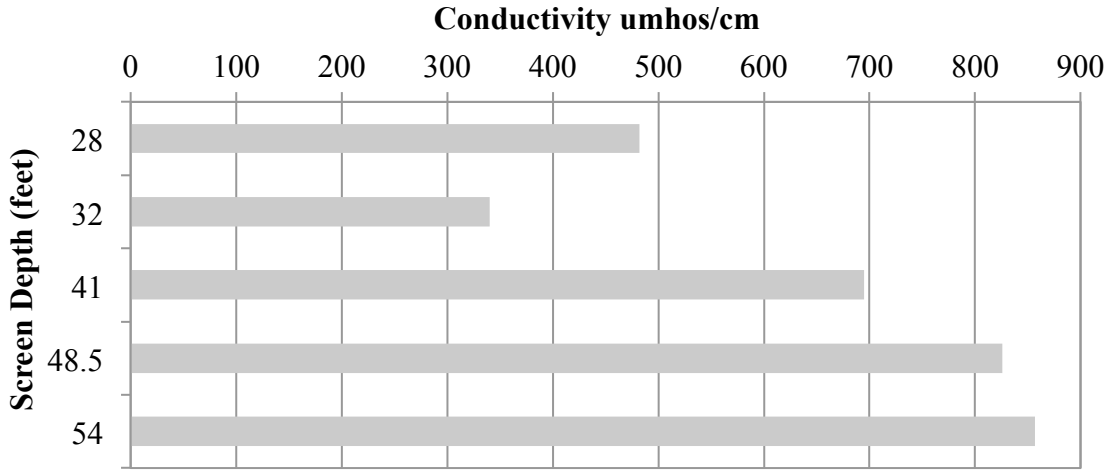


Figure 4.7 Comparison of conductivity by well depth.

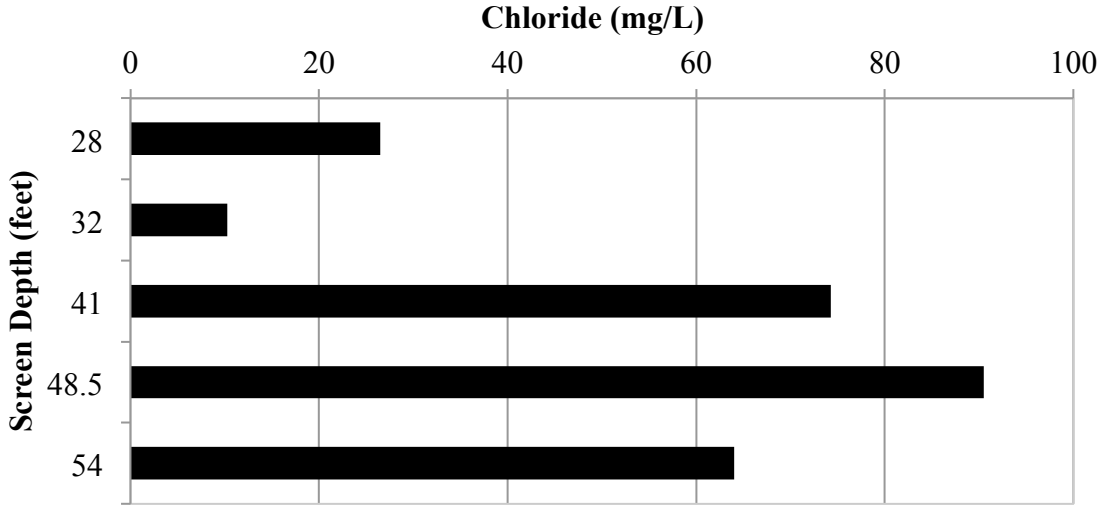


Figure 4.8 Chloride concentrations from five wells.

Samples were analyzed for other major ions, including: arsenic, calcium, copper, iron, lead, magnesium, manganese, potassium, sodium, sulfate, and zinc. None of the wells had concentrations of any of these analytes exceeding their respective drinking water standards. Calcium, iron, magnesium, and manganese concentrations were highest in W4. Potassium, sodium, and sulfate concentrations were highest in W5. W2 had the lowest concentrations of calcium, copper, magnesium, potassium, sodium, and sulfate.

Table 4.7 Analytical results for nitrate and metal concentrations for the five wells in the field study. The Environmental Protection Agency Drinking Water Standards (EPA DWS) are listed, if any. * Indicates a secondary, or aesthetic, standard.

		W1	W2	W3	W4	W5	EPA DWS
Nitrate	mg/L	17.7	4.4	21.9	41.5	50.5	10.0
Arsenic	mg/L	0.004	<LOD	0.004	<LOD	<LOD	0.010
Calcium	mg/L	46.6	35.3	57.9	89.5	57.5	none
Copper	mg/L	0.004	0.002	0.004	0.014	0.004	1.3
Iron	mg/L	0.048	0.071	0.031	0.365	0.116	0.3*
Lead	mg/L	<LOD	<LOD	0.002	<LOD	<LOD	0.015
Magnesium	mg/L	21.4	16.6	25.3	35.2	21.0	none
Manganese	mg/L	0.002	0.007	0.003	0.033	0.003	0.3 (0.05*)
Potassium	mg/L	1.4	0.9	1.4	1.1	2.2	none
Sodium	mg/L	15.7	13.1	49.0	18.6	77.7	none
Sulfate	mg/L	22.5	10.8	23.5	28.5	32.8	250*
Zinc	mg/L	0.022	0.019	0.138	0.014	0.026	5*

Pharmaceuticals, Personal Care Products, and Artificial Sweeteners

Of the ten pharmaceuticals, personal care products, and artificial sweeteners in the septic waste tracer suite, only the artificial sweeteners were found in concentrations above the detection limit (Appendix D). Acesulfame and sucralose concentrations in W1 were 136 and 397 ng/L, respectively. No septic waste tracer suite compounds were found in the other four wells.

Chloroacetanilide Herbicide Metabolites (CAAMs)

Samples were analyzed for six chloroacetanilide analyte metabolites analyzed. Alachlor ESA, metolachlor OA, and metolachlor ESA were detected in the three deepest wells. Metolachlor OA and ESA concentrations increased with well depth (Table 4.8 and Figure 4.9). None of the six CAAMs were detected in the two shallowest wells (Appendix D).

Table 4.8 Analytical results for nitrate and source indicator concentrations from the five wells in the field study.

		W1	W2	W3	W4	W5
Depth	feet	28	32	41	49	54
Nitrate	mg/L	17.7	4.4	21.9	41.5	50.5
Septic Indicators						
Phosphorus	mg/L	0.242	0.013	0.006	<LOD	0.006
Acesulfame	ng/L	136	<LOD	<LOD	<LOD	<LOD
Sucralose	ng/L	397	<LOD	<LOD	<LOD	<LOD
Agricultural Indicators						
Alachlor ESA	ug/L	<LOD	<LOD	0.58	0.30	0.39
Metolachlor OA	ug/L	<LOD	<LOD	0.46	0.59	0.92
Metolachlor ESA	ug/L	<LOD	<LOD	1.16	2.90	3.20

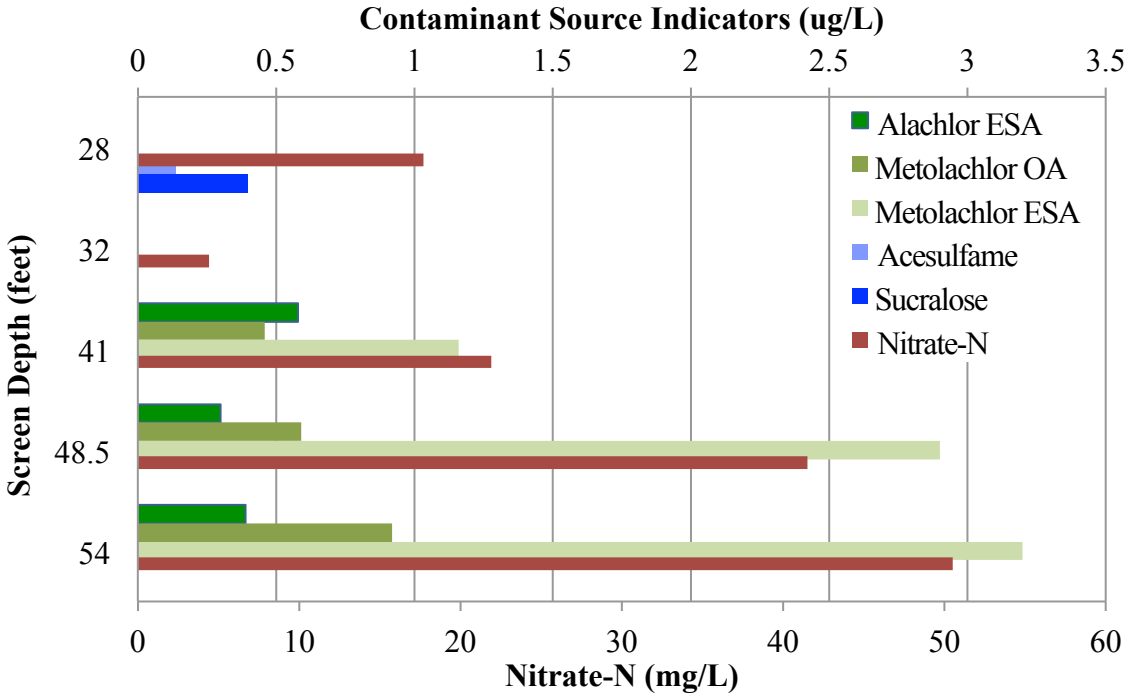


Figure 4.9 Graph of nitrate and source indicators in wells. Well screen depths not to scale.

Discussion

The concentration of nitrate in the wells ranged from below the drinking water standard to five times the standard. Nitrate concentrations were compared to source indicator concentrations. W2 had the lowest concentration of nitrate. It was the only well that did not have detectable concentrations of either chemical waste indicators or agricultural contaminant indicators. The nitrate concentration of W1 was 17.7 mg/L. This is the shallowest well and also the only well with human waste indicators detected. The three deepest wells, W3, W4, and W5, had nitrate concentrations two to five times the drinking water standard. No human waste indicators were detected these wells; however, chloroacetanilide metabolites were found in all three wells.

Nitrate and source indicator concentrations appeared to be consistent with groundwater flow. The groundwater in the area flows from a higher elevation at the groundwater divide east of the study site towards the lower elevation of the Plover River, located west of the study area (Figure 4.10). The wells are located approximately 7500 meters from the groundwater divide. The distance from the wells to the eastern edge of the subdivision is approximately 500 meters. There are some forested areas between the edge of the subdivision and the divide, but most of the area is agriculture. It is estimated that the agricultural area to the divide is 6500 meters. The water table is at an elevation of approximately 330 meters mean sea level (1080 feet) in the area around the wells. It is estimated that there is bedrock at 305 meters mean sea level (1000 feet); therefore, the thickness of the aquifer is approximately 25 meters (Holt, 1965). Using these estimated depths and distances, the source impacts on the aquifer can be calculated assuming a simple model for groundwater flow with distance from the divide (Table 4.9). The top

1.5 meters of the aquifer is recharge from the subdivision. The next 1.5 meters is recharge likely from forested or minimally used land. The groundwater flowing through the bottom 22 meters is recharge from the agricultural land.

The elevated concentration of nitrate in W1, as well as the presence of artificial sweeteners, indicates that the nitrate in this well is from septic waste. This fits with the groundwater estimates, as the bottom of this well is around 329 meters elevation. The lower concentration of nitrate and the lack of contaminant indicators in W2 also fits with the model. The bottom of this well is around 327 meters, so it is not likely being impacted by either septic or agricultural contaminants. The bottoms of the deepest three wells are below 327 meters, meaning they are receiving water recharged from farther away. This is supported by the presence of pesticide metabolites in these three wells.

Table 4.9 Table shows that the top 1.5 meters of the aquifer are susceptible to septic contamination , while the bottom 22 meters are impacted by agricultural practices.

Distance to edge of subdivision	Distance between subdivision and ag land	Agricultural land to the groundwater divide
500 meters	500 meters	6500 meters
Percent of aquifer in study area		
500 m /7500 m ~ 6.5%	500 m/7500 m ~ 6.5%	6500 m/7500 m ~ 87%
Depth of aquifer impacted		
6.5% of 25 m = 1.5 m	6.5% of 25 m = 1.5 m	87% of 25 m = 22 m

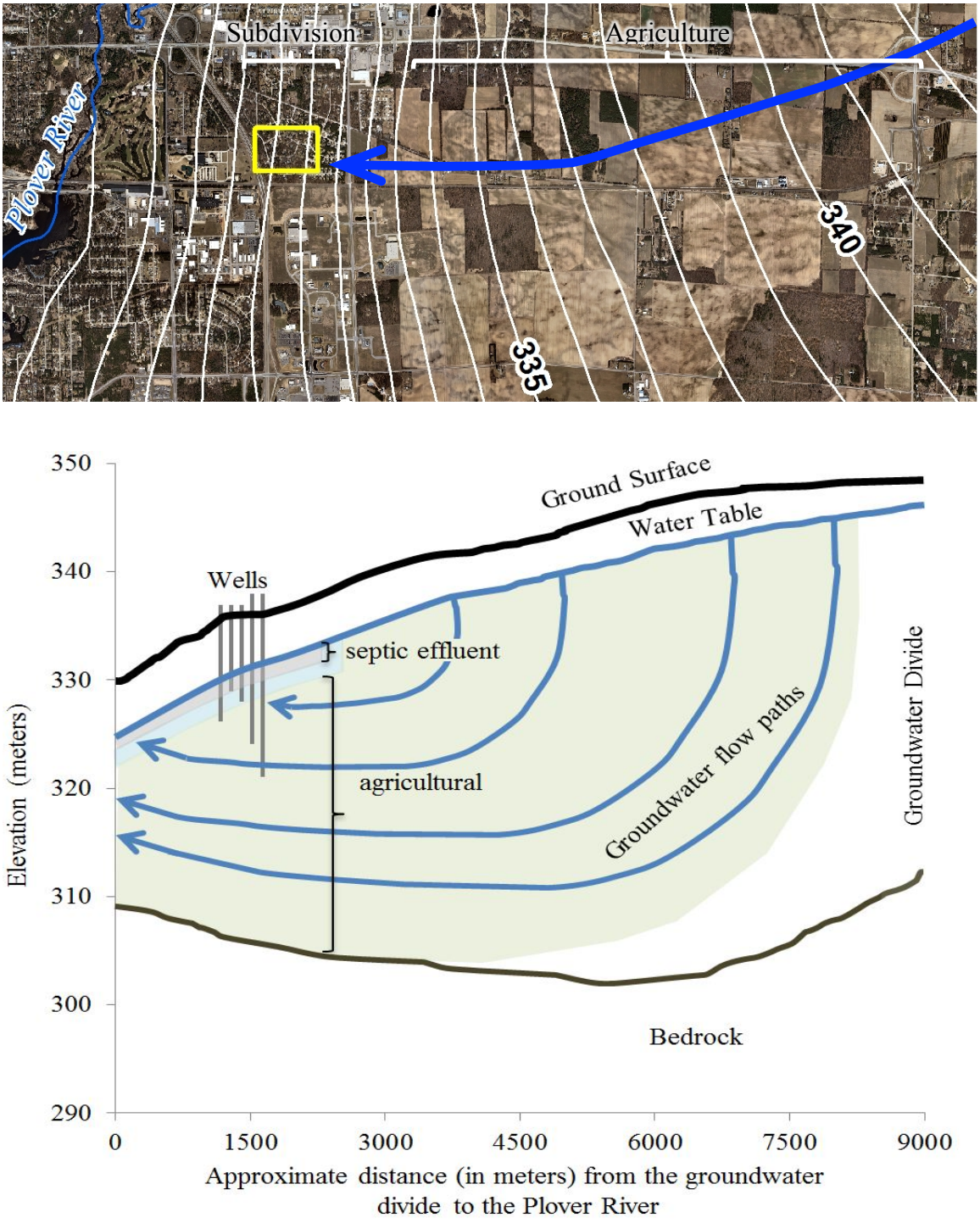


Figure 4.10 The upper figure is an aerial view (contour files from Dave Mechenich). The yellow box shows the subdivision in the study. West of the study area is agricultural land. The lower figure is an illustration of groundwater flow from higher elevation at the groundwater divide towards the Plover River. Shallow wells in the study are impacted by sources closer to the wells, e.g., septic systems. Deeper wells are affected by sources farther away (Holt, 1965; Kraft et al., 2004).

5. CONCLUSIONS/RECOMMENDATIONS

This study successfully identified and quantified a suite of septic waste contaminants. An analytical method was developed and improved upon for the detection and quantitation of these tracers by ESI-LC/MS/MS. The use of solid phase extraction increased the limit of quantitation of most analytes in the chemical waste marker suite nearly 200-fold. Manual optimization of the instrument conditions for the analysis of acesulfame and triclosan increased sensitivity for detection and quantitation of these compounds. Adding time segments to the method and increasing the methanol portion of the mobile phase shortened the analytical run time so samples can be analyzed more efficiently. The lower detection limits will improve identification of nitrate source contaminants that are present in groundwater at part per trillion (ng/L) concentrations.

This study has shown that analyzing for multiple indicators can help determine sources of nitrate contamination. In the shallow well, approximately 1.5 m below the water table, nitrate was found above the drinking water standard and only septic waste indicators were found. This indicates septic systems as the source of the excess nitrate in the groundwater at that depth and location. The three deepest wells also had nitrate at elevated concentrations, increasing with depth. These three wells all had detectable concentrations of alachlor ESA, metolachlor OA, and metolachlor ESA. None of the three deepest wells had detectable concentrations of any of the septic waste indicators. This signifies that agriculture practices are the source of nitrate in these wells. Knowing the source of contamination can help regulators identify potential health risks and the best approach to remedy the problem. This information will be beneficial for residents and developers when installing septic systems and private wells.

Recommendations

1) While most analytes had improved sensitivity and lower detection limits using the solid phase extraction process, acesulfame did not. It may be beneficial to explore other solid phase extraction techniques, particularly acidifying samples prior to analysis. This would be particularly important to consider as other analytes are added to the suite.
2) Adding a surrogate standard to samples prior to extraction would be useful in determining the efficiency of the extraction process in this method. Benzoylecgonine-D3 has good SPE recovery and detection by ESI-LC/MS. It has potential to be an effective surrogate standard.
3) It would be beneficial to identify additional compounds that could be analyzed with the same method extraction and analytical techniques. Other food additives, veterinary pharmaceuticals, and pesticides could identify/confirm contaminant sources.
4) While the results of this study demonstrate a clear connection between the concentrations of nitrate and nitrate contaminant source indicators, further evaluation is suggested. These results were obtained from one set of samples in one subdivision. It would be useful to compare nitrate and co-contaminant concentrations over several months to observe any seasonal variations. Concentrations of certain septic waste indicators, such as artificial sweeteners and caffeine, are not expected to vary much over time, as they are generally consumed on a regular basis. Concentrations of septic indicators such as antibiotics would be expected to fluctuate, as they are generally used for short periods of time.

Agricultural indicators would also be expected to fluctuate throughout the year, as they are applied seasonally.

5) It would be useful to consider other sampling sites. Wells in subdivisions with larger lot sizes would be expected to have less impact from septic systems than wells located near a higher density of septic systems. Use of pharmaceuticals may vary in a community, so a compound found in one subdivision may not be detected in another. This would further show the value of a multi-compound suite of chemical indicators.

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APPENDIX A – Standard Preparations

Human waste indicator intermediate standard mix prepared in 10.0 mL of methanol.

Compound	Stock Standard Concentration (ug/mL)	Stock Standard Volume (uL)	Spike Mix Conc. (ng/mL)
Acesulfame	10.4	577	600
Acetaminophen	10.0	300	300
Benzoylecgonine	10.0	100	100
Caffeine	10.0	150	150
Carbamazepine	10.0	100	100
Cotinine	10.0	150	150
Paraxanthine	10.0	150	150
Sucralose	20.0	500	1000
Sulfamethazine	10.0	100	100
Triclosan	10.0	1000	1000

Human waste indicator internal standard mix in 10.0 mL methanol.

Internal Standard	Stock Concentration (ug/mL)	Aliquot (uL)	Final Concentration (ng/mL)
Acesulfame-D4	10.0	400	400
Caffeine-D9	20.0	100	200
Carbamazepine-D10	100.0	10	100
Cotinine-D4	10.0	200	200
Sucralose-D6	10.0	1000	1000
Sulfamethazine-D4	10.0	200	200
Triclosan-D3	100.0	100	1000

APPENDIX B – Well Construction Reports

Identifying information blacked out for the privacy of the property owner.

W4

State of WI - Private Water Systems - DG/2
Department of Natural Resources, Box 7921
Madison, WI 53707
Please type or Print using a black Pen
Please Use Decimals Instead of Fractions.

Form 3300-77A
(R 8/00)

[redacted]

1. Well Location: [X] Town [ ] City [ ] Village — of **HULL**

Fire # (if available): **5488**

[redacted]

Subdivision Name: **CONIFER** — Lot # — Block #

Well Constructor (Business Name): **HAUPT WELL & PUMP CO INC** — License #: **529** — Facility ID Number (Public Wells)

Address: **DAVID HAUPT** — Public Well Plan Approval # W--

City: **AUBURNDALE** — State: **WI** — Zip Code: **54412** — Date of Approval (mm/dd/yyyy)

Hicap Permanent well # — Common Well # — Specific Capacity: **6** gpm/ft

Gov't Lot # — or **SW** 1/4 of **SE** 1/4 of

Section **35** T **24** N; R **8** [X] E [ ] W

Latitude Deg. **44** Min. **30.884**

Longitude Deg **89** Min. **30.945**

2. Well Type: [X] Replacement [ ] New [ ] Reconstruction — Lat/Long Method: **GPS006**

of previous unique well # — constructed in

Reason for replaced or Reconstructed Well? **QUANTITY**

3. Well serves **1** # of homes and or (e.g. barn, restaurant, church, school, industry, etc.)

High capacity Well? [ ] Yes [X] No

Property? [ ] Yes [X] No

[X] Drilled [ ] Driven Point [ ] Jetted [ ] Other:

4. Is the well located upslope or sideslope and not downslope from any contamination source, including those on neighboring properties? [ ] Yes [ ] No

Well located within 1,200 feet of a quarry? [ ] Yes [X] No If yes, distance in feet from quarry:

Well located in floodplain? [ ] Yes [X] No

Distance in Feet from Well to Nearest:

1. Landfill
7 2. Building Overhang
34 3. Septic [X] Holding Tank [ ]
55 4. Sewage Absorption Unit
5. Nonconforming Pit
6. Buried Home Heating Oil Tank
7. Buried Petroleum Tank
8. Shoreline [ ] Swimming Pool [ ]
9. Downspout/Yard Hydrant
10. Privy
11. Foundation Drain to Clearwater
12. Foundation Drain to Sewer
13. Building Drain [ ] Cast Iron or Plastic [ ] Other
14. Building Sewer [ ] Gravity [ ] Pressure [ ] Cast Iron or Plastic [ ] Other
15. Collector or Street Sewer: [ ] Sanitary units in. diam. [ ] Storm [ ] =< 6 [ ] > 6
16. Clearwater Sump
17. Wastewater Sump
18. Paved Animal Barn Pen
19. Animal Yard or Shelter
20. Silo
21. Barn Gutter
22. Manure Pipe [ ] Gravity [ ] Pressure [ ] Cast Iron or Plastic [ ] Other
23. Other Manure Storage
24. Ditch
25. Other NR 812 Waste Storage

5. Drillhole Dimensions and Construction Method

Dia (in.)	From (ft.)	To (ft.)
6	**0**	**48.5**

Upper Enlarged Drillhole / Lower Open Bedrock

[ ] ---1. Rotary - Mud Circulation----------
[ ] ---2. Rotary - Air----------
[ ] ---3. Rotary - Air and Foam----------
[ ] ---4.Drill-Through Casing Hammer
[ ] ---5. Reverse Rotary
[ ] ---6. Cable-tool Bit in. dia------
[ ] 7. Dual Rotary
[ ] 8. Temp. Outer Casing in. dia. depth (ft)
Removed? [ ] Yes [ ] No
If no, why not?

8. Geology — Type, Caving/Noncaving, Color, Hardness, etc

From (ft.)	To (ft.)	
0	**48.5**	**--S- SAND**

6. Casing, Liner, Screen — Material, Weight, Specification

Dia. (in.)	From (ft.)	To (ft.)
6 STEEL 18.97 A53 EXLTUBE WELD	**0**	**45.5**

Dia. (in.)	Screen type, material & slot size	From (ft.)	To (ft.)
5	**TELE SS #10 UOP**	**45.5**	**48.5**

9. Static Water Level
ft. above ground surface
26 ft. below ground surface

10. Pump Test
Pumping Level **29** ft. below surface
Pumping at **18** GPM for **2** hours

11. Well is: [X] Above Grade **18** in. [ ] Below Grade
Developed? [X] Yes [ ] No
Disinfected? [X] Yes [ ] No
Capped? [X] Yes [ ] No

7. Grout or Other Sealing Material. Method
Method: **MOUND**
Kind of Sealing Material

From (ft.)	To (ft.)	# Sacks Cement
0		**1.5**

#8

12. Did you notify the owner of the need to permanently abandon and fill all unused wells on this property?
[ ] Yes [ ] No If no, explain: **PLUMBER TO DO**

13. Signature of the Well Constructor or Supervisory Driller: **AH** — Date signed: **08/14/2013**

Signature of Drill Rig Operator (Mandatory unless same as above) — Date signed

Make additional comments on reverse side about geology, additional screens, water quality, etc. **Variance issued** [ ] Yes [X] No

WI

Property Owner [redacted]

Mailing Address

City

County o
Portag

State of WI - Private Water Systems - DG/2
Department of Natural Resources, Box 7921
Madison, WI 53707
Please type or Print using a black Pen
Please Use Decimals Instead of Fractions.

Form 3300-77A
(R 8/00)

1. Well Location [X] Town [ ] City [ ] Village
of **HULL**

Fire # (if available)

Subdivision Name [redacted] Lot # Block #

Well Constructor (Business Name)
DONALD J FIRKUS SR

License #
222

Facility ID Number (Public Wells)

Address
6522 OAK DR

Public Well Plan Approval #
W--

City
AMHERST

State
WI

Zip Code
54406-9189

Date of Approval (mm/dd/yyyy)

Hicap Permanent well #

Common Well #

Specific Capacity
2.5 gpm/ft

Gov't Lot # or **SE** 1/4 of **SE** 1/4 of
Section **35** T **24** N; R **8** [X] E [ ] W
Latitude Deg. Min.
Longitude Deg Min.

2. Well Type [X] New [ ] Reconstruction [ ] Replacement
of previous unique well # constructed in
Reason for replaced or Reconstructed Well?

Lat/Long Method
GPS008

3. Well serves **1** # of homes and or
(e.g. barn, restaurant, church, school, industry, etc.)

High capacity Well? [ ] Yes [X] No
Property? [ ] Yes [X] No

[X] Drilled [ ] Driven Point [ ] Jetted [ ] Other:

4. Is the well located upslope or sideslope and not downslope from any contamination source, including those on neighboring properties? [X] Yes [ ] No
Well located within 1,200 feet of a quarry? [ ] Yes [ ] No If yes, distance in feet from quarry:
Well located in floodplain? [ ] Yes [X] No
Distance in Feet from Well to Nearest:
1. Landfill
3 2. Building Overhang
42 3. Septic [ ] Holding Tank [ ]
56 4. Sewage Absorption Unit
5. Nonconforming Pit
6. Buried Home Heating Oil Tank
7. Buried Petroleum Tank
8. Shoreline [ ] Swimming Pool [ ]
9. Downspout/Yard Hydrant
10. Privy
11. Foundation Drain to Clearwater
12. Foundation Drain to Sewer
13. Building Drain [ ] Cast Iron or Plastic [ ] Other
14. Building Sewer [ ] Gravity [ ] Pressure [ ] Cast Iron or Plastic [ ] Other
15. Collector or Street Sewer: [ ] Sanitary [ ] Storm units [ ] =< 6 [ ] > 6 in. diam.
16. Clearwater Sump
17. Wastewater Sump
18. Paved Animal Barn Pen
19. Animal Yard or Shelter
20. Silo
21. Barn Gutter
22. Manure Pipe [ ] Gravity [ ] Pressure [ ] Cast Iron or Plastic [ ] Other
23. Other Manure Storage
24. Ditch
25. Other NR 812 Waste Storage

5. Drillhole Dimensions and Construction Method

Dia (in.)	From (ft.)	To (ft.)
6	**0**	**54**

Upper Enlarged Drillhole
Lower Open Bedrock
[ ] ---1. Rotary - Mud Circulation----------
[ ] ---2. Rotary - Air----------
[ ] ---3. Rotary - Air and Foam----------
[ ] ---4.Drill-Through Casing Hammer
[ ] ---5. Reverse Rotary
[ ] ---6. Cable-tool Bit in. dia-----
[ ] 7. Dual Rotary
[ ] 8. Temp. Outer Casing in. dia. depth (ft)
Removed? [ ] Yes [ ] No
If no, why not?

8. Geology

Type, Caving/Noncaving, Color, Hardness, etc	From (ft.)	To (ft.)
--S- SAND	**0**	**54**

6. Casing, Liner, Screen

Dia. (in.)	Material, Weight, Specification	From (ft.)	To (ft.)
6	**A53B IPSCO 18.97 WELD**	**0**	**50**

Dia. (in.)	Screen type, material & slot size	From (ft.)	To (ft.)
6	**STAINLESS STEEL COOK 12#**	**50**	**54**

9. Static Water Level
ft. above ground surface
30 ft. below ground surface

10. Pump Test
Pumping Level **36** ft. below surface
Pumping at **15** GPM for **2** hours

11. Well is: [X] Above Grade
12 in. [ ] Below Grade
Developed? [X] Yes [ ] No
Disinfected? [X] Yes [ ] No
Capped? [X] Yes [ ] No

7. Grout or Other Sealing Material. Method
Method:
Kind of Sealing Material

From (ft.)	To (ft.)	# Sacks Cement
0		

12. Did you notify the owner of the need to permanently abandon and fill all unused wells on this property?
[X] Yes [ ] No If no, explain:

13. Signature of the Well Constructor or Supervisory Driller
DF
Date signed
12/20/2000

Signature of Drill Rig Operator (Mandatory unless same as above) Date signed

Make additional comments on reverse side about geology, additional screens, water quality, etc.

Variance issued [ ] Yes [X] No

APPENDIX C – Solid phase extraction method trial results

Analytical results for the NU solid phase extraction method trials.

NU	COT		ACE		AMN		PXN		CAF	
Lab Number	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**
Spiked conc	**15.0**		**15.0**		**7.5**		**15.0**		**7.5**	
A030413	13.0	86.2	1.0	260	6.5	86.1	6.5	43.8	17.5	236
B030413	12.5	81.8	6.5	107	4.5	59.7	12.0	79.8	17.0	230
C030413	12.0	81.5	5.0	83.7	5.5	70.4	10.0	66.7	14.0	188
Spiked conc	**7.5**		**15.0**		*		**7.5**		**7.5**	
A031513	25.5	340	44.5	298	--	--	2.0	134	40.0	533
B031513	26.5	353	15.0	101	--	--	2.2	147	13.5	180
C031513	96.5	1290	10.5	71.6	--	--	1.4	93.4	16.5	221
A031813	9.5	127	15.5	105	--	--	0.7	43.9	9.5	127
B031813	12.0	162	16.5	110	--	--	0.7	47.6	9.5	128
C031813	11.0	148	3.5	24.0	--	--	1.2	81.8	9.5	126
A032013	5.5	74.5	118	785	--	--	0.8	50.3	11.0	147
B032013	6.0	79.3	111	736	--	--	1.1	72.1	10.5	142
C032013	6.5	86.5	43.5	290	--	--	0.6	42.1	11.5	155

NU	SLF		SUC		BNZ		CRB		TRI	
Lab Number	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**
Spiked conc	**7.5**		**150**		**7.5**		**7.5**		**75.0**	
A030413	5.0	66.3	82.0	54.6	4.0	55.4	6.5	83.4	38.0	50.5
B030413	5.0	69.6	45.5	30.3	4.5	62.6	6.5	83.5	73.0	97.6
C030413	5.5	72,3	83.5	55.5	4.0	53.2	6.0	79.8	59.5	79.4
Spiked conc	**7.5**		**37.5**		**3.75**		**3.75**		**37.5**	
A031513	6.5	87.0	19.5	52.0	3.5	94.2	3.5	88.9	36.5	97.6
B031513	6.5	90.0	8.5	22.7	3.5	93.9	3.0	85.4	22.0	59.2
C031513	7.0	94.5	14.5	39.1	3.5	94.9	3.5	91.7	20.0	52.7
A031813	6.5	86.0	9.5	25.1	3.5	95.7	3.5	88.6	1.5	4.5
B031813	6.5	89.3	8.5	22.8	3.5	94.0	3.5	94.0	17.0	44.9
C031813	6.5	87.1	4.0	11.1	3.0	85.7	3.0	85.6	0.0	0.0
A032013	6.5	89.2	9.0	23.8	3.5	93.6	3.0	83.2	20.0	53.7
B032013	7.0	94.2	12.5	33.4	4.0	100	3.5	87.4	11.5	30.4
C032013	6.5	88.7	5.5	14.4	3.5	91.0	3.0	80.5	8.5	22.6

Analytical results for the HU solid phase extraction method trials.

HU	COT		ACE		AMN		PXN		CAF	
Lab Number	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**
Spiked conc	**7.5**		**15**		**15**		**7.5**		**7.5**	
A032513	7.1	94.6	0.5	3.4	--	--	9.2	123	8.9	119
C032513	8.2	109	1.0	6.4	--	--	10.7	143	2.9	193
B040313	5.3	70.5	1.4	9.0	16.6	110	8.8	118	3.3	220
C040313	4.9	65.9	0.7	4.7	16.4	110	7.7	102	1.8	122
B042213	4.3	56.7	0.4	2.6	14.1	94.2	6.9	91.7	7.2	95.6
C042213	4.2	55.8	0.4	2.7	14.1	94.1	7.3	96.7	7.0	93.4
Spiked conc	**7.5**		**30.0**		**15.0**		**7.5**		**7.5**	
A061313	2.3	31	1.8	12	13.5	90	8.0	107	7.1	95
B061313	1.3	17	0.5	3	10.4	69	5.8	77	6.3	84
C061313	2.6	35	0.2	1	10.1	67	5.1	68	6.1	81

HU	SLF		SUC		BNZ		CRB		TRI	
Lab Number	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**
Spiked conc	**7.5**		**37.5**		**3.75**		**3.75**		**37.5**	
A032513	6.7	89.1	14.5	38.7	3.7	99.3	9.2	77.4	67.4	180
C032513	6.8	90.5	20.0	53.2	4.1	108	10.7	78.8	220.7	589
B040313	6.6	87.5	28.6	76.3	3.2	84.7	8.8	79.6	13.9	37.0
C040313	5.8	77.5	43.6	116	2.3	62.0	7.7	132	58.2	155
B042213	6.2	83.0	31.6	84.3	2.8	75.7	3.1	82.7	43.1	115
C042213	6.3	83.5	33.6	89.6	3.0	79.1	3.1	81.4	58.1	155
Spiked conc	**5.0**		**50.0**		**5.0**		**5.0**		**50.0**	
A061313	4.9	98	33.0	66	--	--	9.9	197	71.5	143
B061313	5.1	102	21.5	43	--	--	21.9	222	24.3	34
C061313	5.1	101	27.5	55	--	--	51.4	235	38.7	159

Analytical results for the HA solid phase extraction method trials.

HA	COT		ACE		AMN		PXN		CAF	
Lab Number	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**
Spiked conc	**500**		**500**		**500**		**500**		**500**	
A012413	0	0.0	252	50.4	428	85.6	665	133	710	142
B012413	0	0.0	196	39.1	382	76.3	540	108	610	122
A013013	8	1.6	221	44.2	391	78.2	515	103	492	98.3
B013013	3.5	0.7	227	45.4	364	72.8	464	92.7	480	95.9
C020413	25	5.0	102	20.4	16	3.2	304	60.7	378	75.5
D020413	2	0.4	110	21.9	2	0.4	308	61.6	429	85.8
E020413	9	1.8	243	48.5	376	75.2	378	75.5	531	106.2
C021113	0	0.0	265	53.0	305	61.0	245	49.0	420	84.0
D021113	0	0.0	295	59.0	280	56.0	245	49.0	460	92.0

HA	SLF		SUC		BNZ		CRB		TRI	
Lab Number	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**	**Conc (ppt)**	**% Rec**
Spiked conc	**500**		**1000**		**500**		**500**		**1000**	
A012413	433	86.5	113	11.3	500	99.9	462	92.4	47	4.7
B012413	413	82.5	227	22.7	458	91.6	485	96.9	93	9.3
A013013	319	63.7	329	32.9	412	82.3	367	73.3	137	13.7
B013013	304	60.7	418	41.8	414	82.7	352	70.3	221	22.1
C020413	350	69.9	0	0	274	54.8	115	23	33	3.3
D020413	294	58.7	0	0	126	25.1	46.5	9.3	138	13.8
E020413	384	76.7	582	58.2	414	82.7	353	70.5	316	31.6
C021113	425	85.0	315	31.5	500	100	460	92.0	245	24.5
D021113	430	86.0	310	31.0	520	104	450	90.0	85	8.5

Analytical results for the HD solid phase extraction method trials.

HD	COT		ACE		AMN		PXN		CAF	
Lab Number	Conc (ppt)	% Rec	Conc (ppt)	% Rec	Conc (ppt)	% Rec	Conc (ppt)	% Rec	Conc (ppt)	% Rec
Spk Conc	**15**		**15**		**7.5**		**15**		**7.5**	
A022813	8.5	56.4	3.1	20.9	5.1	68.2	5.6	37.4	11.5	153
B022813	9.0	59.9	3.2	21.2	6.5	86.1	7.7	51.2	11.8	157
C022813	12.9	85.7	2.3	15	11.1	148	12.8	85.2	15.2	203
D022813	11.7	78.1	3.3	22.2	10.7	142	9.2	61.1	13.7	182
E022813	8.3	55.1	3.8	25.1	6.7	88.8	6.2	41	9.8	130
F022813	8.0	53.5	3.3	22.2	6.5	86.9	5.4	35.9	7.4	98.6
G022813	8.2	54.8	3.2	21.5	7.1	94	7.5	50.2	10.7	142
H022813	7.7	51.5	2.4	16.1	6.1	81.3	5.5	36.8	10.5	140
Spk Conc	**7.5**		**15**		**15**		**7.5**		**7.5**	
A032113	6.4	84.7	0.0	0	0.0	0	6.5	86.2	18.6	248
B032113	5.2	68.7	0.2	1.6	0.0	0	7.7	103	19.0	253
C032113	7.2	96.1	0.3	2.3	0.0	0	5.4	72.6	18.4	245
D032113	7.3	97.3	0.0	0	0.0	0	6.8	90.8	16.6	221
B032513	7.4	98.9	0.5	3	0.0	0	9.8	131	9.4	125
B040213	5.3	70.9	0.0	0	15.0	100	5.6	74.4	9.4	125
A040113	2.4	31.8	0.0	0	15.3	102	3.0	40.6	4.5	60.6

HD	SLF		SUC		BNZ		CRB		TRI	
Lab Number	Conc (ppt)	% Rec	Conc (ppt)	% Rec	Conc (ppt)	% Rec	Conc (ppt)	% Rec	Conc (ppt)	% Rec
Spk Conc	**7.5**		**150**		**7.5**		**7.5**		**75**	
A022813	3.0	40.1	281	187	6.7	89.2	4.1	54.8	72.2	96.3
B022813	4.4	58.9	285	190	6.7	89.2	4.3	57.9	78.0	104
C022813	6.8	90.5	242	161	10.5	140	6.9	92.4	112	149
D022813	6.8	91.2	252	168	9.8	131	7.3	96.9	77.3	103
E022813	4.3	57.2	222	148	5.8	76.7	3.8	51.2	71.9	95.8
F022813	4.5	60.1	237	158	6.3	84.2	4.8	64.5	38.9	51.9
G022813	4.7	63.1	402	268	6.7	89.9	4.8	63.7	41.9	55.9
H022813	4.8	64	341	227	6.8	90.8	5.2	68.8	67.9	90.5
Spk Conc	**7.5**		**37.5**		**3.75**		**3.75**		**37.5**	
A032113	6.6	88.1	86.6	57.7	8.1	108	5.7	76.5	146	194
B032113	6.5	87	78.3	52.2	7.8	104	5.6	74.8	42.0	56
C032113	6.1	81.1	80.7	53.8	6.2	83.1	5.2	69.1	110	147
D032113	6.9	91.9	61.5	41	7.3	97.7	5.6	74.6	594	792
B032513	6.8	90.1	92.3	61.5	7.4	99.1	6.3	84.5	82.5	110
B040213	6.8	90.5	159	106	6.7	89.5	5.8	76.9	119	159
A040113	6.1	81.6	115	76.6	4.0	53.3	5.3	70.3	335	446

Analytical results for method blanks in solid phase extraction method trials.
* indicates analyte was not quantified

NU	COT	ACE	AMN	PXN	CAF	SLF	SUC	BNZ	CRB	TRI
Lab Number	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb
D030413	0.1	0.1	0.3	0.1	0.0	0.2	0.0	0.0	0.4	0.5
D031513	5.1	1.0	0.7	0.5	0.9	0.2	0.0	0.1	0.1	0.3
D031813	0.2	0.1	0.0	0.0	0.4	0.2	0.3	0.2	0.1	0.0
D032013	0.0	0.5	0.0	0.0	0.7	0.2	0.4	0.2	0.1	0.0

HU	COT	ACE	AMN	PXN	CAF	SLF	SUC	BNZ	CRB	TRI
Lab Number	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb
A040313	0.0	0.1	0.1	0.1	0.1	0.2	0.6	0.0	0.1	0.0
A042213	0.0	0.1	0.0	0.0	0.2	0.2	0.0	0.1	0.1	1.9
D061313	0.0	0.1	0.0	0.0	0.5	0.3	0.0	*	0.1	0.0

HA	COT	ACE	AMN	PXN	CAF	SLF	SUC	BNZ	CRB	TRI
Lab Number	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb
F020413	0.0	0.9	0.0	0.0	0.1	0.4	0.0	0.0	0.2	12.8
E021113	0.0	0.5	0.4	0.0	0.0	0.7	3.7	0.8	0.8	5.3

HD	COT	ACE	AMN	PXN	CAF	SLF	SUC	BNZ	CRB	TRI
Lab Number	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb	Conc ppb
I022813	0.0	0.2	0.3	0.0	0.0	0.1	0.0	0.2	0.0	2.2
E032113	0.0	0.0	0.0	0.1	4.3	0.3	0.7	0.2	0.2	4.2
D032513	0.0	0.1	0.0	0.4	0.4	0.2	1.1	0.2	0.1	2.7
B040113	0.0	0.0	0.0	0.0	0.0	0.1	1.3	0.0	0.0	8.8
A040213	0.0	0.0	0.0	0.0	0.2	0.1	0.0	0.0	0.0	1.1

APPENDIX D – Mann-Whitney U-Test

The Mann-Whitney U-test is a non-parametric statistical test that was used to evaluate which solid phase extraction method would yield analyte recoveries closest to the desired recovery of 100%. If there is statistically no difference between two methods, the sum of ranks for each method should be similar. The probability was calculated to determine if there was a difference in the rank sums, and if one method yielded recoveries significantly closer to 100% than another method. This test was useful, as methods were not run with an equal number of trials. This test also minimized the effects of outliers. The statistical program R (x62 3.0.1) was used to perform the test.

Procedure

1. The absolute value of difference between the percent recovery for each analyte and 100% was calculated.
Example:
Method: HU
Analyte: Cotinine
Sample number: A032513
Percent recovery: 94.6
Formula: |94.6 – 100| = 5.4

HU Method			NU Method		
Lab number	Percent Recovery	|% Recovery – 100|	Lab number	Percent Recovery	|% Recovery – 100|
A032513	94.6	5.4	A030413	86.2	13.8
C032513	109	9	B030413	81.8	18.2
B040313	70.5	29.5	C030413	81.5	18.5
C040313	65.9	34.1	A031513	340	240
B042213	56.7	43.3	B031513	353	253
C042213	55.8	44.2	C031513	1290	1190
A061313	31	69	A031813	127	27
B061313	17	83	B031813	162	62
C061313	35	65	C031813	148	48
			C031813	148	48
			A032013	74.5	25.5
			B032013	79.3	20.7
			C032013	86.5	13.5

2. The resulting data was entered into an Excel spreadsheet as follows. The first two letters indicate the method name. The next three letters are abbreviations for the analyte. The method-analyte combinations were listed in row one of the spreadsheet. The difference of recovery from 100% data was listed in rows below for each.

HU_COT	HU_ACE	HU_AMN	HU_PXN	HU_CAF	HU_SLF	HU_SUC	HU_CRB	HU_TRI
83.0	99.0	100.0	32.0	19.0	22.5	61.3	22.6	66.0
69.0	97.4	100.0	23.0	16.0	17.0	57.0	21.2	63.0
65.0	97.3	33.0	8.3	6.6	16.5	46.8	20.4	15.0
44.2	97.0	31.0	3.3	5.0	12.5	45.0	18.6	43.0
43.3	96.6	10.0	2.3	4.4	10.9	34.0	17.3	55.0
34.1	95.3	5.9	7.0	19.2	9.5	23.7	32.1	55.2
29.5	93.6	5.8	17.7	22.4	2.0	15.7	97.0	59.0
5.4	91.0	9.6	22.5	93.2	1.0	10.4	122.0	79.8
9.3	88.0	10.5	43.1	120.5	2.0	16.3	135.0	488.6

HA_COT	HA_ACE	HA_AMN	HA_PXN	HA_CAF	HA_SLF	HA_SUC	HA_CRB	HA_TRI
100	79.6	99.6	51	24.5	41.3	100	90.7	96.8
100	78.1	96.8	51	16	39.3	100	77.0	95.4
100	60.9	44	39.3	14.2	36.3	88.7	29.7	91.5
100	55.8	39	38.4	8	30.1	77.4	29.5	90.8
99.6	54.6	27.2	24.5	4.1	23.3	69	26.7	86.4
99.3	51.5	24.8	7.3	1.7	17.5	68.5	10.0	86.2
98.4	49.6	23.7	3	6.2	15	67.2	8.0	77.9
98.2	47	21.8	8	22	14	58.2	7.6	75.5
95	41	14.4	33	42	13.5	41.8	3.1	68.5

HD_COT	HD_ACE	HD_AMN	HD_PXN	HD_CAF	HD_SLF	HD_SUC	HD_CRB	HD_TRI
68.2	100.0	31.8	64.1	39.4	59.9	59.0	48.8	48.1
48.5	100.0	18.7	63.2	1.4	42.8	47.8	45.2	44.1
46.5	100.0	13.9	62.6	25.0	41.1	46.2	42.1	44.0
45.2	100.0	13.1	59.4	25.4	39.9	42.3	36.3	9.5
44.9	98.4	11.2	59.0	29.8	36.9	38.5	35.5	4.2
43.6	97.7	6.0	49.8	39.8	36.0	23.4	31.2	3.7
40.1	97.0	0.0	48.8	41.5	18.9	6.0	30.9	2.7
31.3	85.0	2.0	38.9	52.7	18.4	47.9	29.7	3.8
29.1	83.9	41.9	27.4	56.7	13.0	58.0	25.4	9.7
21.9	79.1	47.9	25.6	82.4	11.9	60.8	25.2	47.4
15.3	78.8		14.8	103.3	9.9	68.2	23.5	48.6
14.3	78.5		13.8	120.6	9.5	87.0	23.1	59.0
3.9	77.8		9.2	144.6	9.5	90.4	15.5	94.2
2.7	77.8		3.0	147.6	8.8	127.3	7.6	346.0
1.1	74.9		31.3	152.8	8.1	167.6	3.1	691.6

NU_COT	NU_ACE	NU_AMN	NU_PXN	NU_CAF	NU_SLF	NU_SUC	NU_CRB	NU_TRI
13.8	94.9	13.9	56.2	135.6	33.7	45.4	16.6	49.5
18.2	57.5	40.3	20.2	129.5	30.4	69.7	16.5	2.4
18.5	66.8	29.6	33.3	87.5	27.7	44.5	20.2	20.6
240	160	87.4	128.0	443.0	18.2	64.4	17.9	15.0
253	7.0	88.1	153.0	68.0	15.1	92.0	21.4	19.9
1190	16.3	77.4	53.0	112.0	10.4	76.6	15.1	25.9
26.8	4.6		56.1	26.9	14.0	74.9	11.4	95.5
62.2	9.8		52.4	27.9	10.7	77.2	6.0	55.1
48.0	76.0		18.2	25.6	12.9	88.9	14.4	100.0
25.5	685.2		49.7	47.2	10.8	76.2	16.8	46.3
20.7	636.1		27.9	42.2	5.8	66.6	12.6	69.6
13.5	189.8		57.9	54.9	11.3	85.6	19.5	77.4

3. The Mann-Whitney U-test ranks the resulting differences from smallest to greatest. The sum of ranks and resulted p-value are calculated. Below is an example of calculating the sum of ranks. The statistical program R was used to perform this test.

Results	**Ranks**	**Method**	**HU Ranks**	**NU Ranks**
5.4	1	HU	1	3
9	2	HU	2	4
13.5	3	NU	10	5
13.8	4	NU	11	6
18.2	5	NU	12	7
18.5	6	NU	13	8
20.7	7	NU	16	9
25.5	8	NU	17	14
27	9	NU	18	15
29.5	10	HU		19
34.1	11	HU		20
43.3	12	HU		21
44.2	13	HU		
48	14	NU		
62	15	NU		
65	16	HU		
69	17	HU		
83	18	HU		
240	19	NU		
253	20	NU	**Sum of Ranks**	**Sum of Ranks**
1190	21	NU	100	131

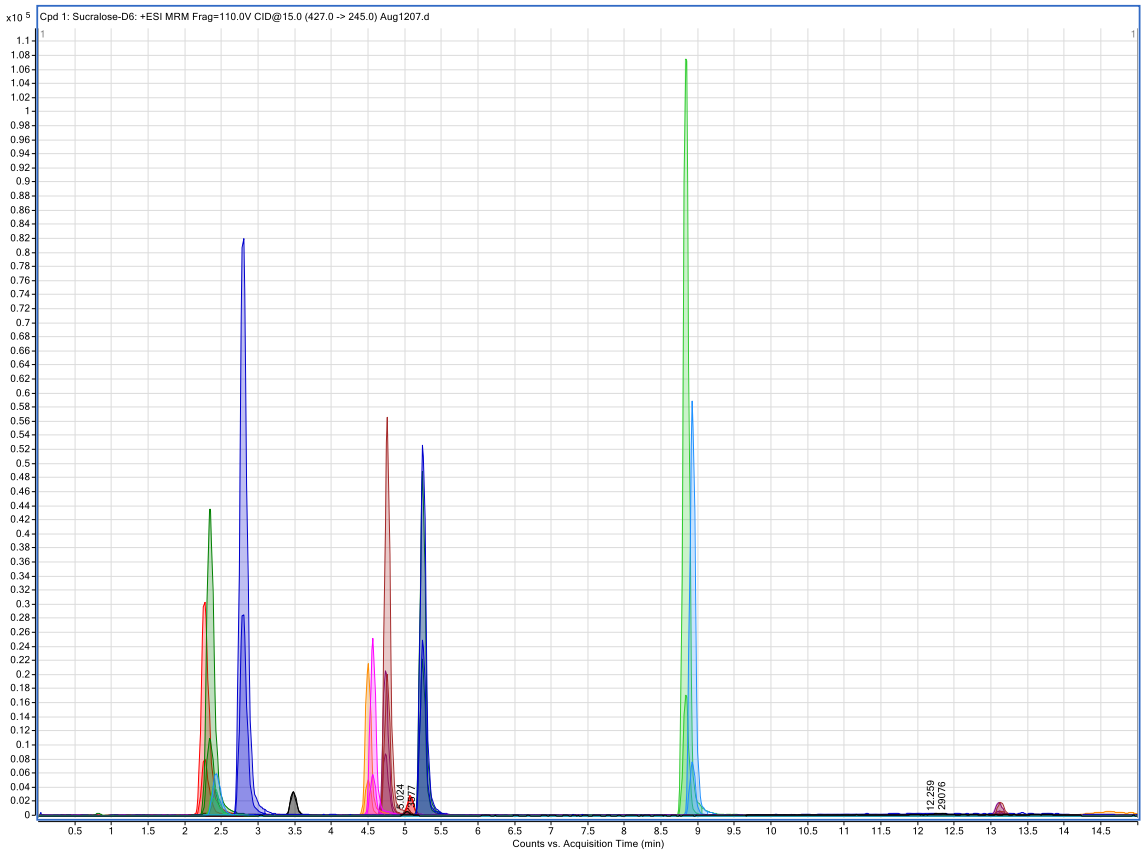
Example:

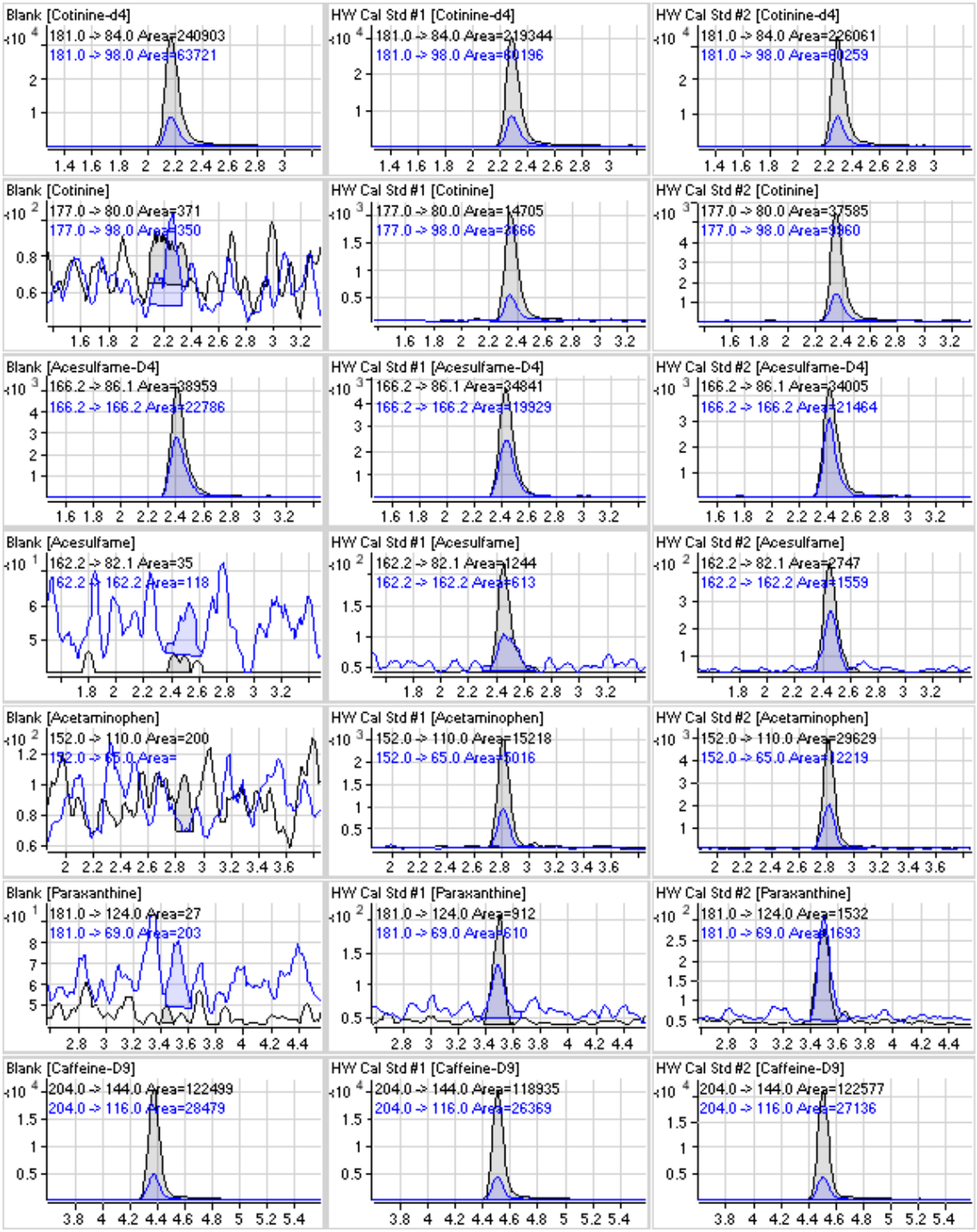
A one-sided test was performed to determine if the HU method would statistically yield recoveries closer to 100% than the NU method. For the analyte cotinine, the p-value was 0.542, indicating that the HU method does not produce recoveries closer to 100% than the NU method. The same procedure was performed to determine if the NU method would yield recoveries closer to 100% than the HU method. The p-value for this comparison was 0.486.
When the test was performed for the analyte paraxanthine, the p-value was 0.001, indicating that the HU method yields recoveries closer to 100% than the NU method. When the NU method was compared to the HU method, the p-value was 0.999. Below is an example of a Mann-Whitney U-test calculated in R.

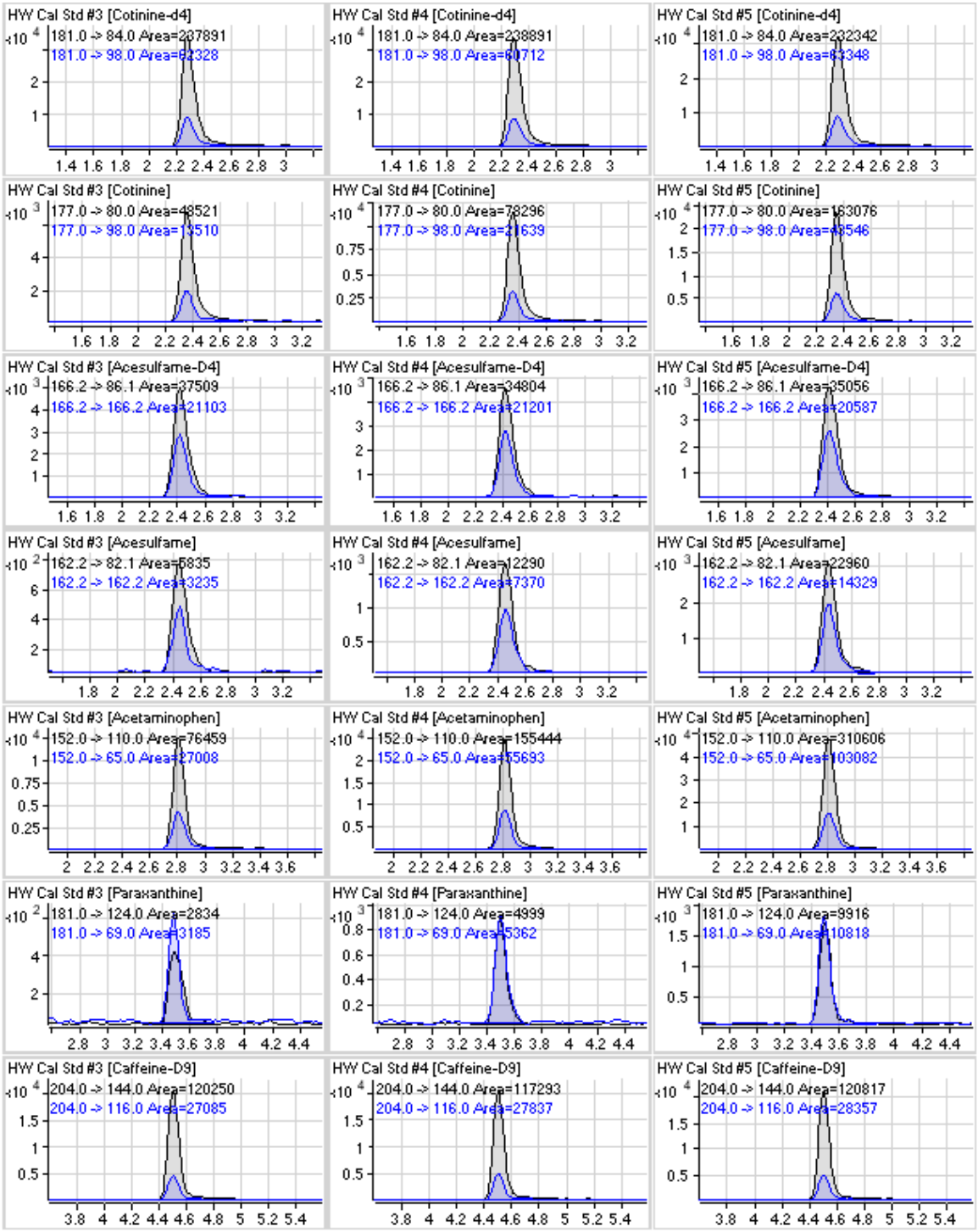
```
> setwd("C:/test")
> mydata<-read.csv("mwrr.csv",header=TRUE)
> names(mydata)
 [1] "NU_COT" "NU_ACE" "NU_AMN" "NU_PXN" "NU_CAF" "NU_SLF"
"NU_SUC" "NU_BNZ"
 [9] "NU_CRB" "NU_TRI" "HU_COT" "HU_ACE" "HU_AMN" "HU_PXN"
"HU_CAF" "HU_SLF"
[17] "HU_SUC" "HU_CRB" "HU_TRI" "HA_COT" "HA_ACE" "HA_AMN"
"HA_PXN" "HA_CAF"
[25] "HA_SLF" "HA_SUC" "HA_CRB" "HA_TRI" "HD_COT" "HD_ACE"
"HD_AMN" "HD_PXN"
[33] "HD_CAF" "HD_SLF" "HD_SUC" "HD_CRB" "HD_TRI"
> wilcox.test(mydata$HU_COT,mydata$NU_COT, alternative="l", mu=0,
exact=FALSE, paired=FALSE)

        Wilcoxon rank sum test with continuity correction

data:  mydata$HU_COT and mydata$NU_COT
W = 55, p-value = 0.5424
alternative hypothesis: true location shift is less than 0
```

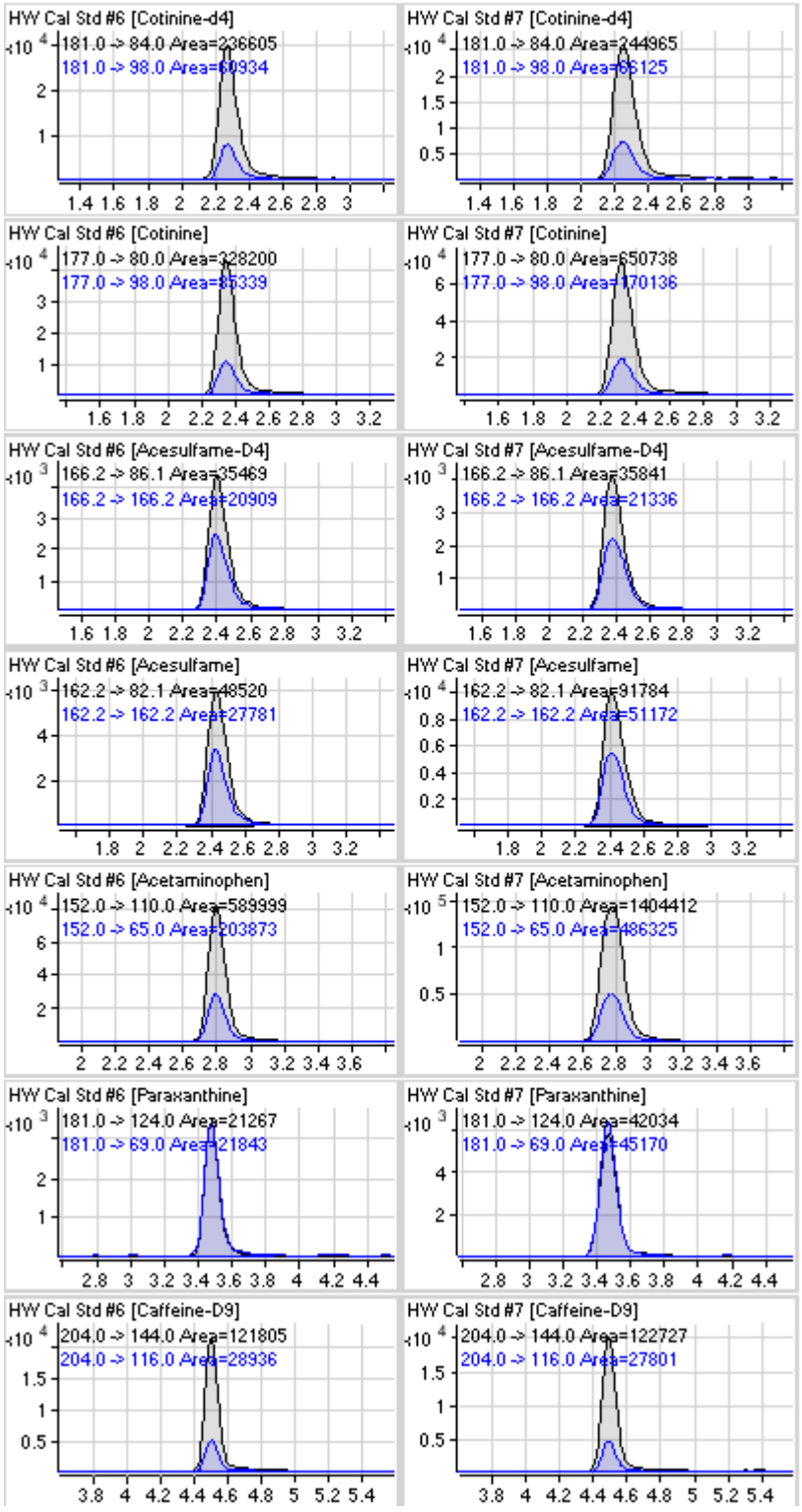
APPENDIX E – Chromatograms

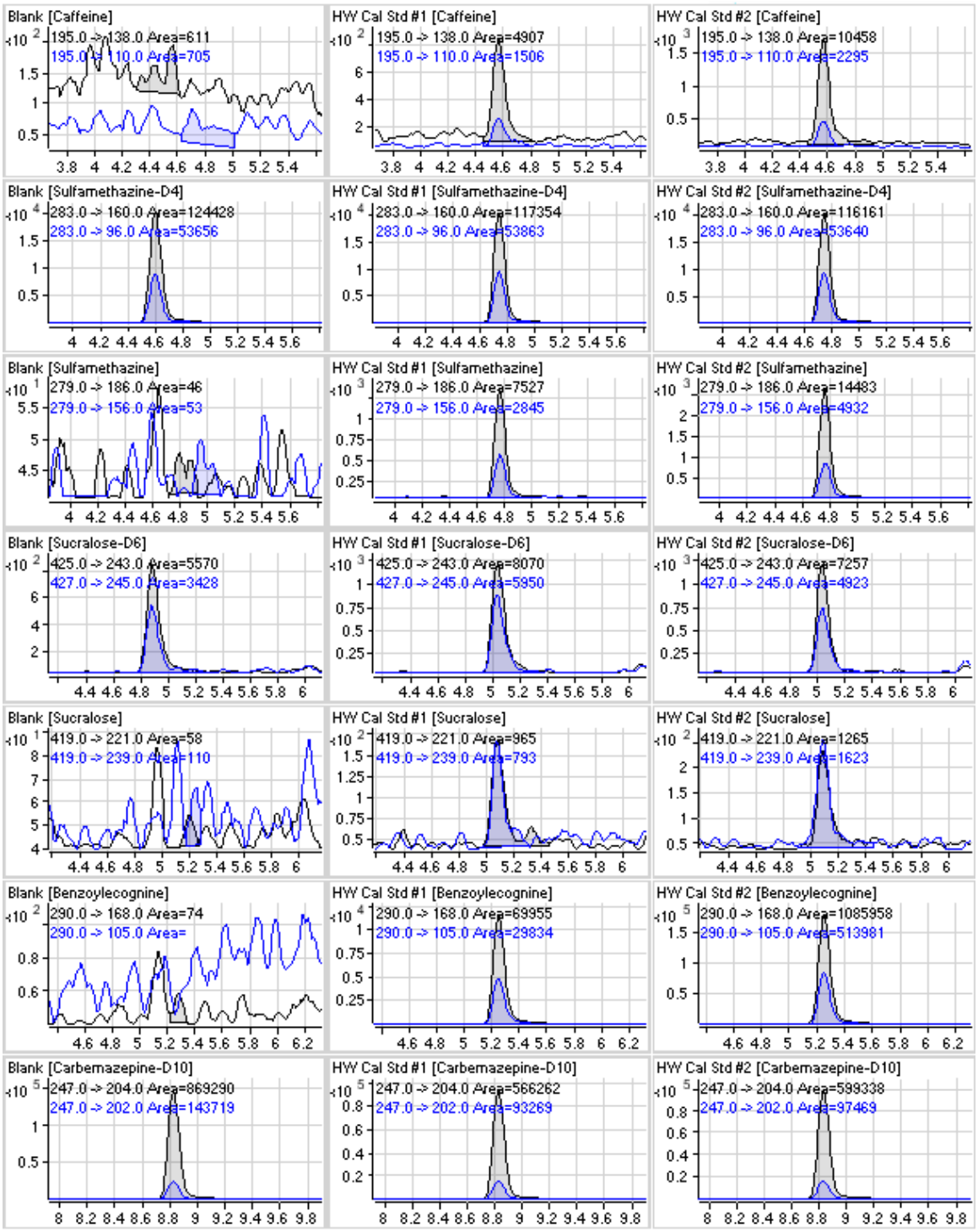
This section shows the chromatograms for the analytes in the human waste marker suite. The first chromatogram shows the peaks of all of the analytes and their respective deuterated internal standards in the level six standard. Following that are the individual chromatograms for each analyte and deuterated internal standard for each calibration level and an instrument blank. The instrument blank contained the mobile phase and the internal standards. The individual chromatograms show the peak areas for each product ion.

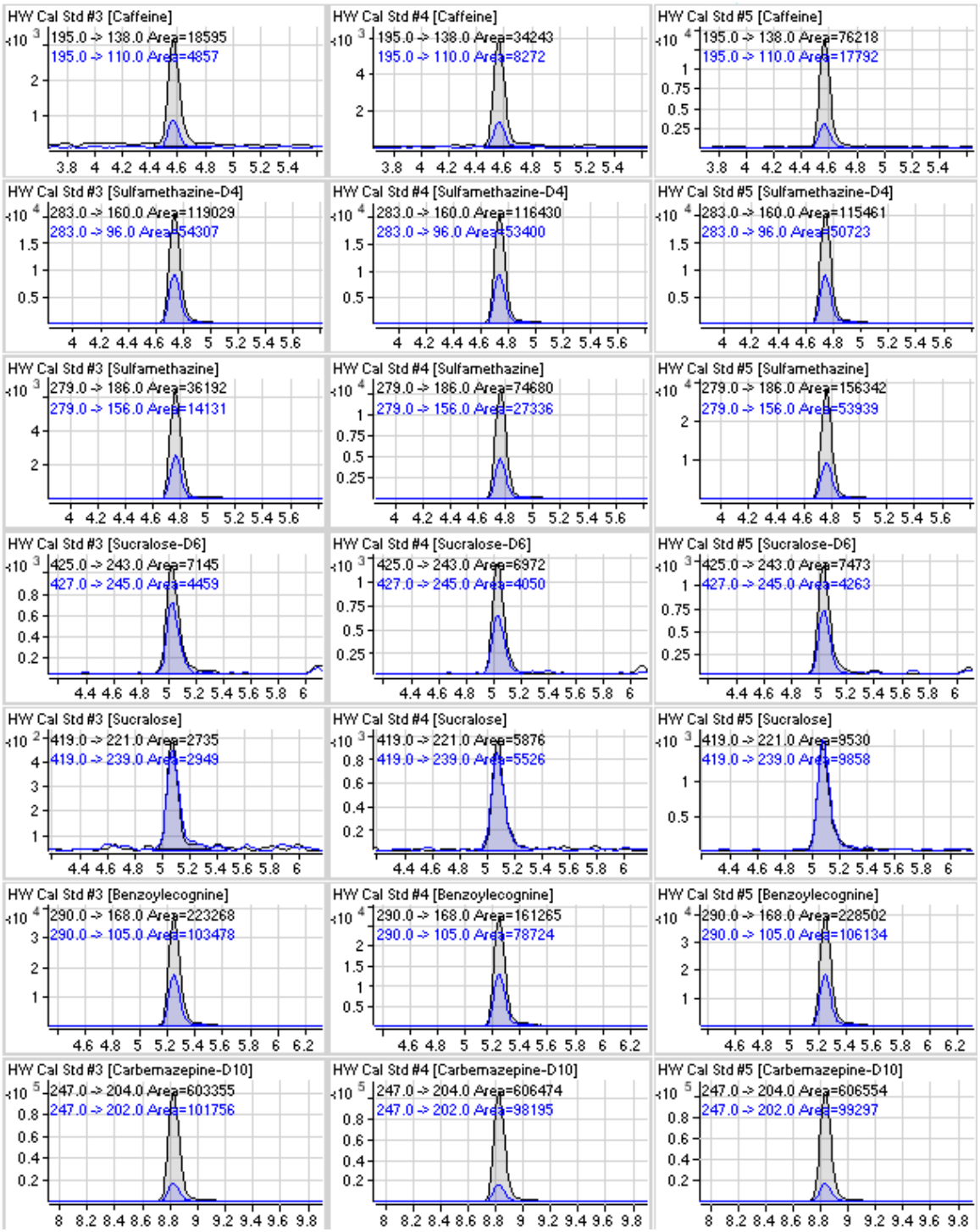



Blank [Cotinine-d4]
181.0 -> 84.0 Area=240903
181.0 -> 98.0 Area=63721
HW Cal Std #1 [Cotinine-d4]
181.0 -> 84.0 Area=219344
181.0 -> 98.0 Area=60196
HW Cal Std #2 [Cotinine-d4]
181.0 -> 84.0 Area=226061
181.0 -> 98.0 Area=60259
Blank [Cotinine]
177.0 -> 80.0 Area=371
177.0 -> 98.0 Area=350
HW Cal Std #1 [Cotinine]
177.0 -> 80.0 Area=14705
177.0 -> 98.0 Area=3666
HW Cal Std #2 [Cotinine]
177.0 -> 80.0 Area=37585
177.0 -> 98.0 Area=9360
Blank [Acesulfame-D4]
166.2 -> 86.1 Area=38959
166.2 -> 166.2 Area=22786
HW Cal Std #1 [Acesulfame-D4]
166.2 -> 86.1 Area=34841
166.2 -> 166.2 Area=19929
HW Cal Std #2 [Acesulfame-D4]
166.2 -> 86.1 Area=34005
166.2 -> 166.2 Area=21464
Blank [Acesulfame]
162.2 -> 82.1 Area=35
162.2 -> 162.2 Area=118
HW Cal Std #1 [Acesulfame]
162.2 -> 82.1 Area=1244
162.2 -> 162.2 Area=613
HW Cal Std #2 [Acesulfame]
162.2 -> 82.1 Area=2747
162.2 -> 162.2 Area=1559
Blank [Acetaminophen]
152.0 -> 110.0 Area=200
152.0 -> 65.0 Area=
HW Cal Std #1 [Acetaminophen]
152.0 -> 110.0 Area=15218
152.0 -> 65.0 Area=5016
HW Cal Std #2 [Acetaminophen]
152.0 -> 110.0 Area=29629
152.0 -> 65.0 Area=12219
Blank [Paraxanthine]
181.0 -> 124.0 Area=27
181.0 -> 69.0 Area=203
HW Cal Std #1 [Paraxanthine]
181.0 -> 124.0 Area=912
181.0 -> 69.0 Area=610
HW Cal Std #2 [Paraxanthine]
181.0 -> 124.0 Area=1532
181.0 -> 69.0 Area=1693
Blank [Caffeine-D9]
204.0 -> 144.0 Area=122499
204.0 -> 116.0 Area=28479
HW Cal Std #1 [Caffeine-D9]
204.0 -> 144.0 Area=118935
204.0 -> 116.0 Area=26369
HW Cal Std #2 [Caffeine-D9]
204.0 -> 144.0 Area=122577
204.0 -> 116.0 Area=27136


HW Cal Std #3 [Cotinine-d4]
181.0 -> 84.0 Area=237891
181.0 -> 98.0 Area=62328
HW Cal Std #4 [Cotinine-d4]
181.0 -> 84.0 Area=238891
181.0 -> 98.0 Area=60712
HW Cal Std #5 [Cotinine-d4]
181.0 -> 84.0 Area=232342
181.0 -> 98.0 Area=63348
HW Cal Std #3 [Cotinine]
177.0 -> 80.0 Area=48521
177.0 -> 98.0 Area=13510
HW Cal Std #4 [Cotinine]
177.0 -> 80.0 Area=78296
177.0 -> 98.0 Area=21639
HW Cal Std #5 [Cotinine]
177.0 -> 80.0 Area=163076
177.0 -> 98.0 Area=43546
HW Cal Std #3 [Acesulfame-D4]
166.2 -> 86.1 Area=37509
166.2 -> 166.2 Area=21103
HW Cal Std #4 [Acesulfame-D4]
166.2 -> 86.1 Area=34804
166.2 -> 166.2 Area=21201
HW Cal Std #5 [Acesulfame-D4]
166.2 -> 86.1 Area=35056
166.2 -> 166.2 Area=20587
HW Cal Std #3 [Acesulfame]
162.2 -> 82.1 Area=5835
162.2 -> 162.2 Area=3235
HW Cal Std #4 [Acesulfame]
162.2 -> 82.1 Area=12290
162.2 -> 162.2 Area=7370
HW Cal Std #5 [Acesulfame]
162.2 -> 82.1 Area=22960
162.2 -> 162.2 Area=14329
HW Cal Std #3 [Acetaminophen]
152.0 -> 110.0 Area=76459
152.0 -> 65.0 Area=27008
HW Cal Std #4 [Acetaminophen]
152.0 -> 110.0 Area=155444
152.0 -> 65.0 Area=55693
HW Cal Std #5 [Acetaminophen]
152.0 -> 110.0 Area=310606
152.0 -> 65.0 Area=103082
HW Cal Std #3 [Paraxanthine]
181.0 -> 124.0 Area=2834
181.0 -> 69.0 Area=3185
HW Cal Std #4 [Paraxanthine]
181.0 -> 124.0 Area=4999
181.0 -> 69.0 Area=5362
HW Cal Std #5 [Paraxanthine]
181.0 -> 124.0 Area=9916
181.0 -> 69.0 Area=10818
HW Cal Std #3 [Caffeine-D9]
204.0 -> 144.0 Area=120250
204.0 -> 116.0 Area=27085
HW Cal Std #4 [Caffeine-D9]
204.0 -> 144.0 Area=117293
204.0 -> 116.0 Area=27837
HW Cal Std #5 [Caffeine-D9]
204.0 -> 144.0 Area=120817
204.0 -> 116.0 Area=28357


HW Cal Std #6 [Cotinine-d4]
181.0 -> 84.0 Area=236605
181.0 -> 98.0 Area=60934
HW Cal Std #7 [Cotinine-d4]
181.0 -> 84.0 Area=244965
181.0 -> 98.0 Area=66125
HW Cal Std #6 [Cotinine]
177.0 -> 80.0 Area=328200
177.0 -> 98.0 Area=85339
HW Cal Std #7 [Cotinine]
177.0 -> 80.0 Area=650738
177.0 -> 98.0 Area=170136
HW Cal Std #6 [Acesulfame-D4]
166.2 -> 86.1 Area=35469
166.2 -> 166.2 Area=20909
HW Cal Std #7 [Acesulfame-D4]
166.2 -> 86.1 Area=35841
166.2 -> 166.2 Area=21336
HW Cal Std #6 [Acesulfame]
162.2 -> 82.1 Area=48520
162.2 -> 162.2 Area=27781
HW Cal Std #7 [Acesulfame]
162.2 -> 82.1 Area=91784
162.2 -> 162.2 Area=51172
HW Cal Std #6 [Acetaminophen]
152.0 -> 110.0 Area=589999
152.0 -> 65.0 Area=203873
HW Cal Std #7 [Acetaminophen]
152.0 -> 110.0 Area=1404412
152.0 -> 65.0 Area=486325
HW Cal Std #6 [Paraxanthine]
181.0 -> 124.0 Area=21267
181.0 -> 69.0 Area=21843
HW Cal Std #7 [Paraxanthine]
181.0 -> 124.0 Area=42034
181.0 -> 69.0 Area=45170
HW Cal Std #6 [Caffeine-D9]
204.0 -> 144.0 Area=121805
204.0 -> 116.0 Area=28936
HW Cal Std #7 [Caffeine-D9]
204.0 -> 144.0 Area=122727
204.0 -> 116.0 Area=27801


Blank [Caffeine]
195.0 -> 138.0 Area=611
195.0 -> 110.0 Area=705
HW Cal Std #1 [Caffeine]
195.0 -> 138.0 Area=4907
195.0 -> 110.0 Area=1506
HW Cal Std #2 [Caffeine]
195.0 -> 138.0 Area=10458
195.0 -> 110.0 Area=2295
Blank [Sulfamethazine-D4]
283.0 -> 160.0 Area=124428
283.0 -> 96.0 Area=53656
HW Cal Std #1 [Sulfamethazine-D4]
283.0 -> 160.0 Area=117354
283.0 -> 96.0 Area=53863
HW Cal Std #2 [Sulfamethazine-D4]
283.0 -> 160.0 Area=116161
283.0 -> 96.0 Area=53640
Blank [Sulfamethazine]
279.0 -> 186.0 Area=46
279.0 -> 156.0 Area=53
HW Cal Std #1 [Sulfamethazine]
279.0 -> 186.0 Area=7527
279.0 -> 156.0 Area=2845
HW Cal Std #2 [Sulfamethazine]
279.0 -> 186.0 Area=14483
279.0 -> 156.0 Area=4932
Blank [Sucralose-D6]
425.0 -> 243.0 Area=5570
427.0 -> 245.0 Area=3428
HW Cal Std #1 [Sucralose-D6]
425.0 -> 243.0 Area=8070
427.0 -> 245.0 Area=5950
HW Cal Std #2 [Sucralose-D6]
425.0 -> 243.0 Area=7257
427.0 -> 245.0 Area=4923
Blank [Sucralose]
419.0 -> 221.0 Area=58
419.0 -> 239.0 Area=110
HW Cal Std #1 [Sucralose]
419.0 -> 221.0 Area=965
419.0 -> 239.0 Area=793
HW Cal Std #2 [Sucralose]
419.0 -> 221.0 Area=1265
419.0 -> 239.0 Area=1623
Blank [Benzoylecognine]
290.0 -> 168.0 Area=74
290.0 -> 105.0 Area=
HW Cal Std #1 [Benzoylecognine]
290.0 -> 168.0 Area=69955
290.0 -> 105.0 Area=29834
HW Cal Std #2 [Benzoylecognine]
290.0 -> 168.0 Area=1085958
290.0 -> 105.0 Area=513981
Blank [Carbemazepine-D10]
247.0 -> 204.0 Area=869290
247.0 -> 202.0 Area=143719
HW Cal Std #1 [Carbemazepine-D10]
247.0 -> 204.0 Area=566262
247.0 -> 202.0 Area=93269
HW Cal Std #2 [Carbemazepine-D10]
247.0 -> 204.0 Area=599338
247.0 -> 202.0 Area=97469


HW Cal Std #3 [Caffeine]
195.0 -> 138.0 Area=18595
195.0 -> 110.0 Area=4857
HW Cal Std #4 [Caffeine]
195.0 -> 138.0 Area=34243
195.0 -> 110.0 Area=8272
HW Cal Std #5 [Caffeine]
195.0 -> 138.0 Area=76218
195.0 -> 110.0 Area=17792
HW Cal Std #3 [Sulfamethazine-D4]
283.0 -> 160.0 Area=119029
283.0 -> 96.0 Area=54307
HW Cal Std #4 [Sulfamethazine-D4]
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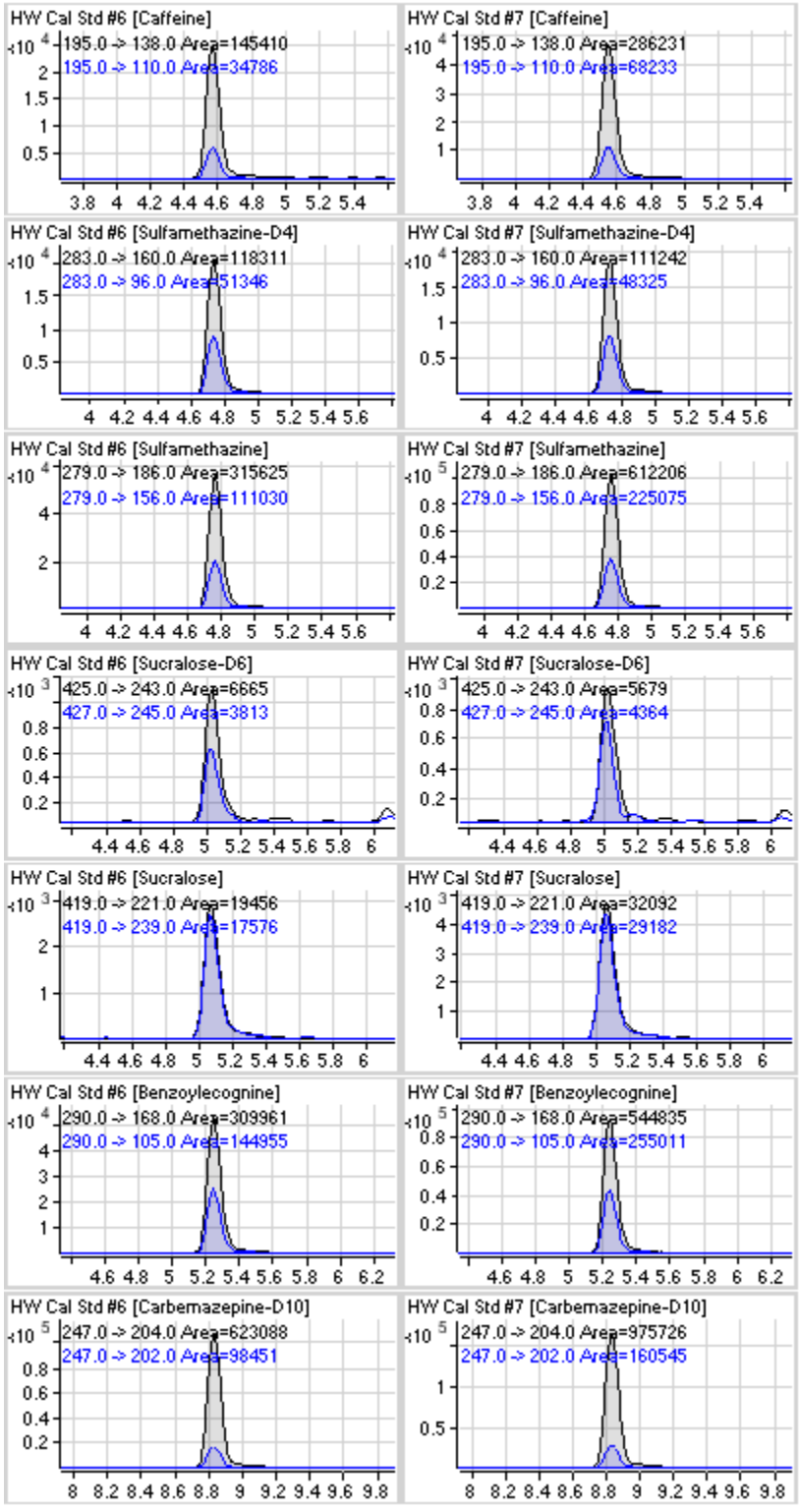
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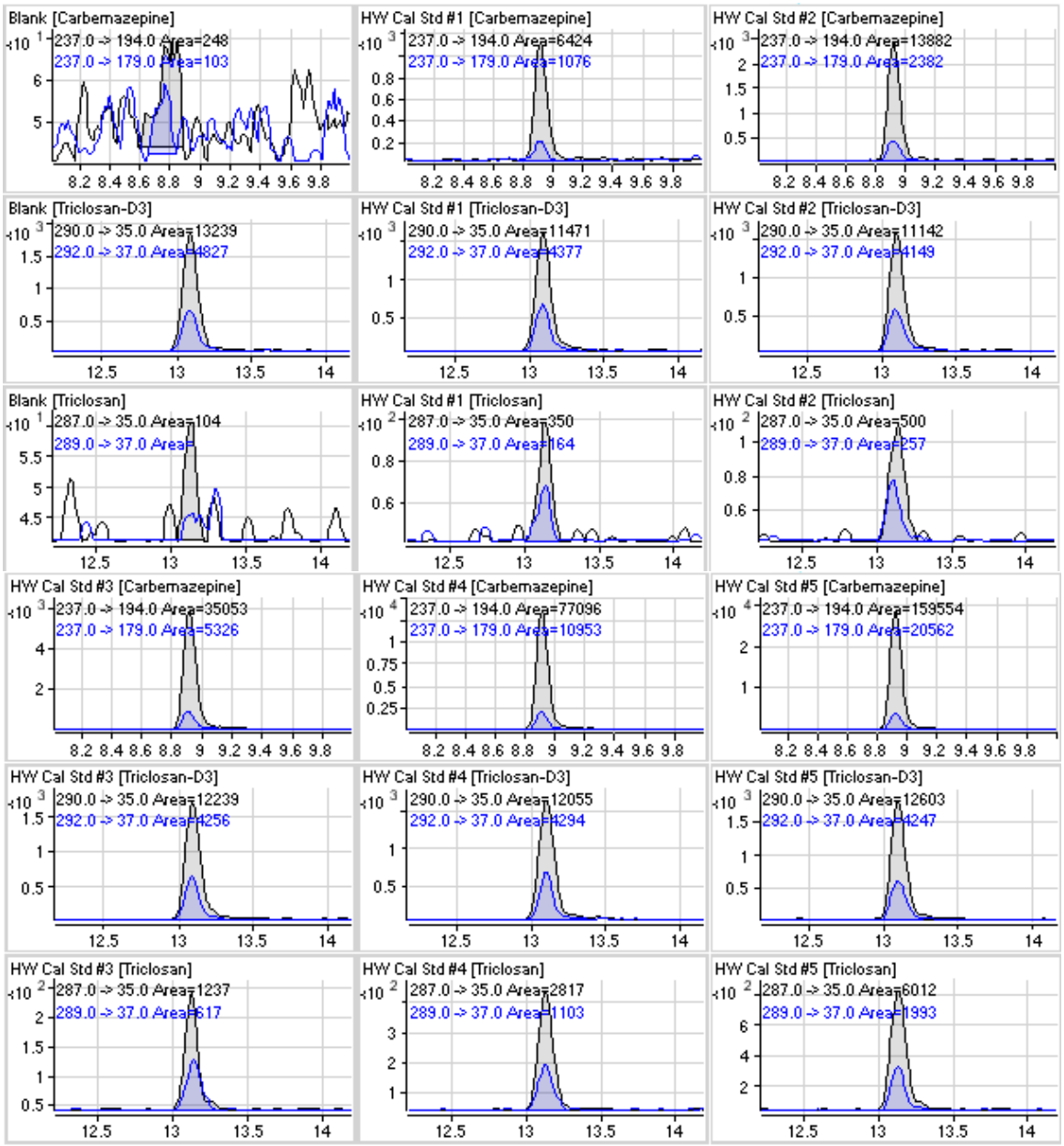
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237.0 -> 179.0 Area=2382
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237.0 -> 194.0 Area=35053
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292.0 -> 37.0 Area=4294
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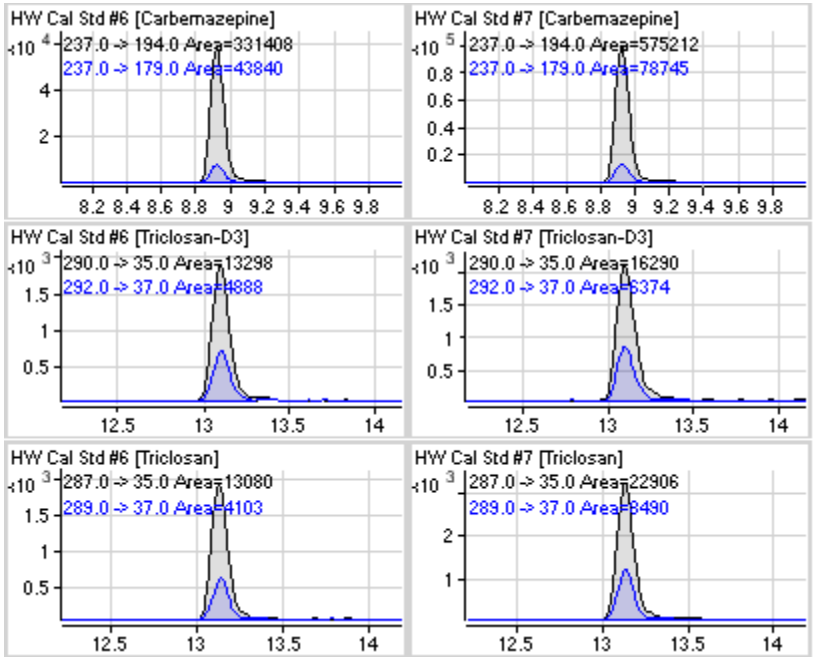
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x10 5
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237.0 -> 179.0 Area=78745
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x10 3
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x10 3
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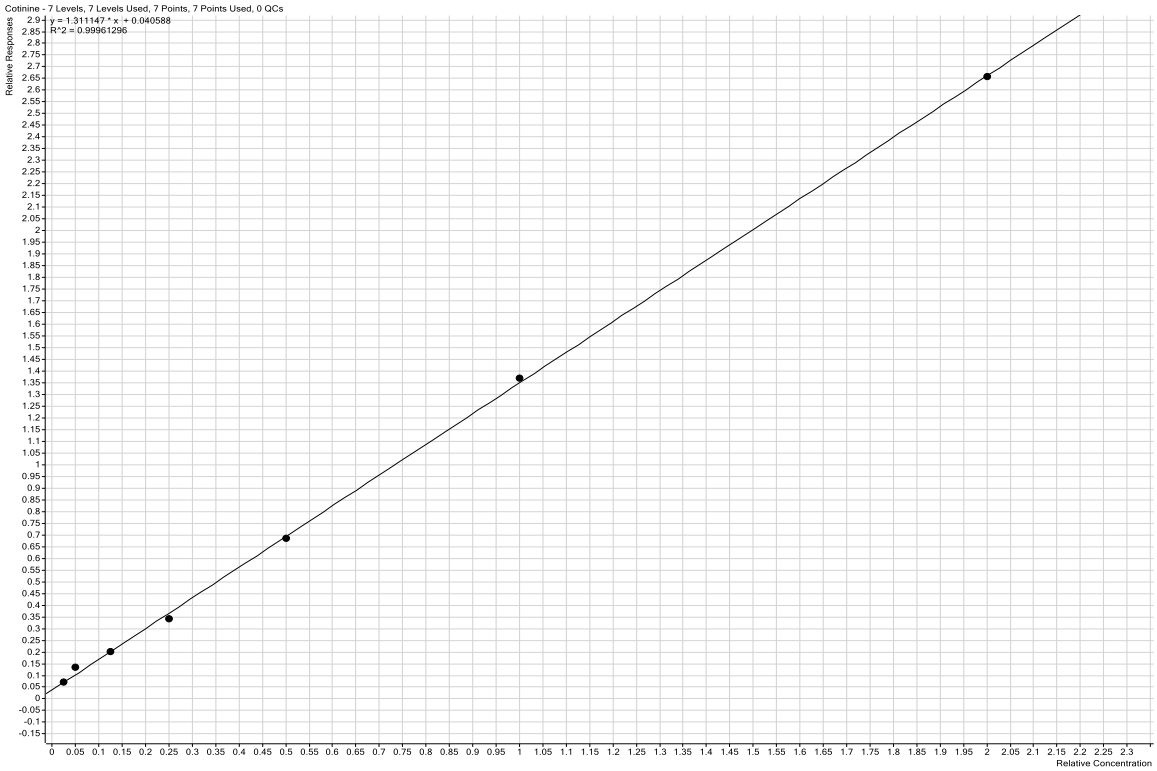
APPENDIX F – Analytical results

This section details the information from the analysis of the field study samples. Seven calibration standards were used to determine the goodness of fit, or R^2, for each target analyte in the human waste suite. The percent accuracy, analyte peak area, and corresponding internal standard area are listed for each standard level. Analyte concentrations for sample extracts and calculated sample concentrations are listed, along with extract blank concentrations and spike recoveries. A duplicate was extracted and analyzed for W1 to show reproducibility of the method results.

Analyte Detection Limits (ng/L)

COT	ACE	AMN	PXN	CAF	SLF	BNZ	SUC	CRB	TRI
5.5	1.3	6.3	4.9	13.4	1.2	34.7	2.1	2.7	245

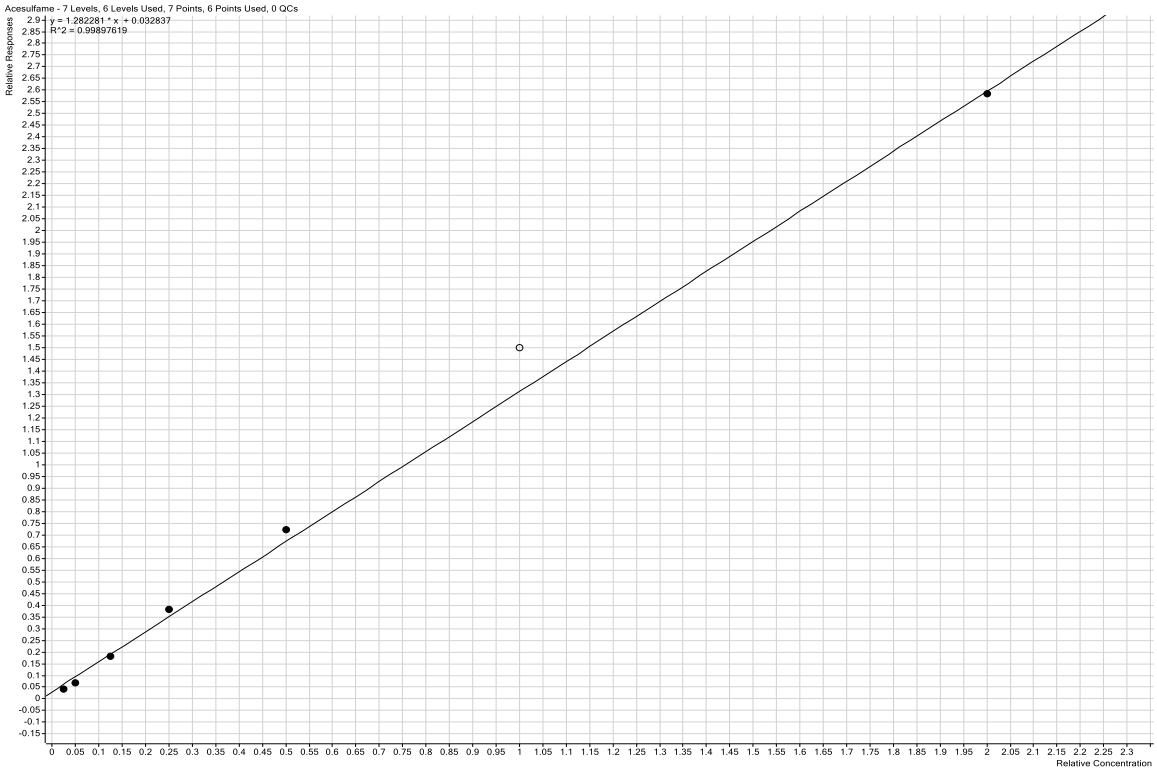
Cotinine



Standard Level	Expected concentration ug/L	Actual Concentration ug/L	Percent Accuracy
1	0.5	0.3	55.4
2	1.0	1.8	179
3	2.5	2.4	94.8
4	5.0	4.3	85.2
5	10.0	10.0	99.8
6	20.0	20.5	102
7	40.0	39.9	99.6

Sample Description	Extract Concentration ug/L	Sample Concentration ng/L	Spike % Recovery
W3	0.0	<LOD	--
W2	0.0	<LOD	--
W1	0.0	<LOD	--
W1 duplicate	0.0	<LOD	--
Blank	0.0	<LOD	--
Spike	3.7	18.4	61%
Spike	3.6	17.9	60%
Spike	2.7	13.3	44%

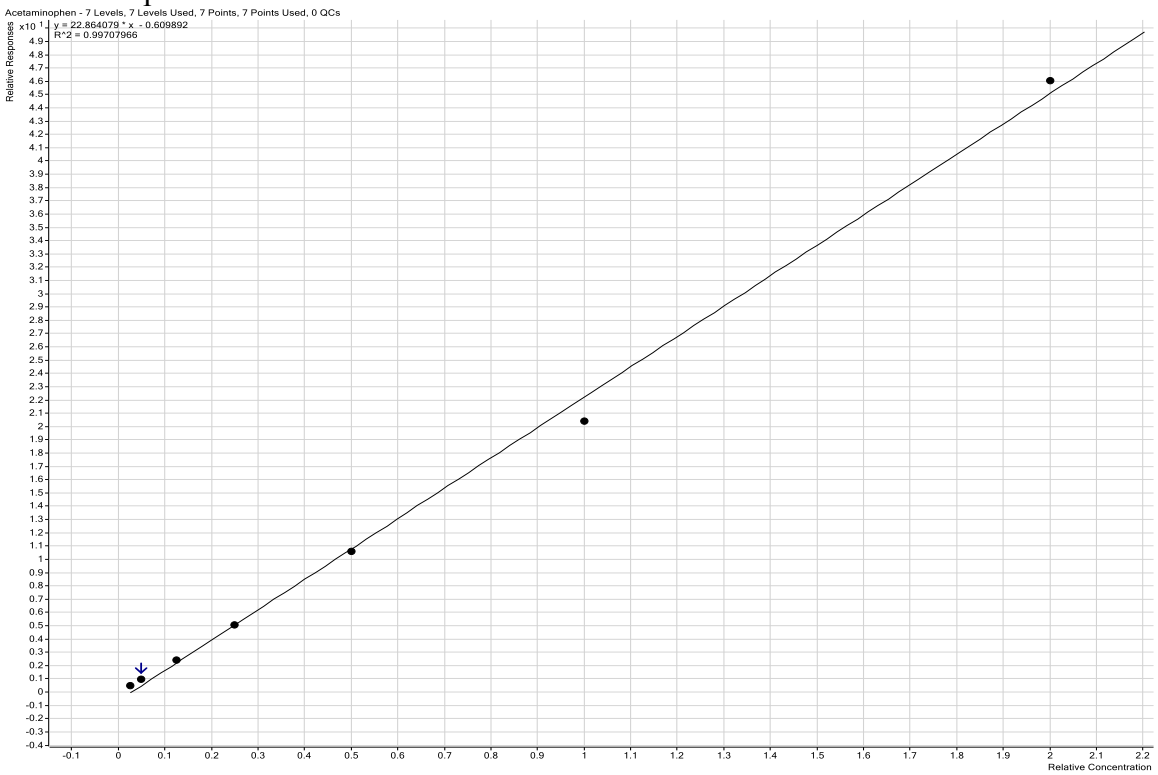
Acesulfame



Standard Level	Expected concentration ug/L	Actual Concentration ug/L	Percent Accuracy
1	1.0	0.5	54.1
2	2.0	1.9	97.1
3	5.0	4.3	85.4
4	10.0	10.4	104
5	20.0	19.8	99.0
6	40.0	42.0	105
7	80.0	79.1	98.8

Sample Description	Extract Concentration ug/L	Sample Concentration ng/L	Spike % Recovery
W3	0.0	<LOD	--
W2	0.0	<LOD	--
W1	27.2	136	--
W1 duplicate	26.7	133	--
Blank	0.0	<LOD	--
Spike	0.7	3.7	3%
Spike	0.3	1.7	1%
Spike	0.4	2.2	2%

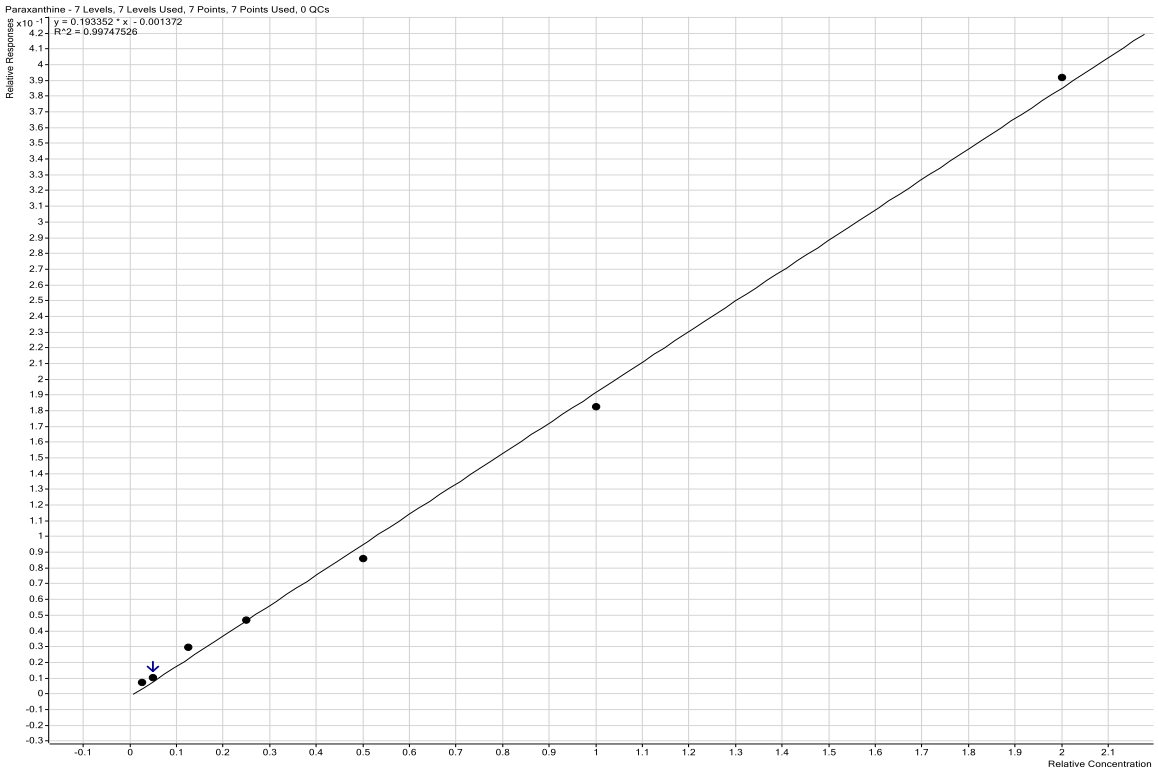
Acetaminophen



Standard Level	Expected concentration ug/L	Actual Concentration ug/L	Percent Accuracy
1	1.0	2.0	204
2	2.0	2.9	147
3	5.0	5.4	107
4	10.0	10.4	104
5	20.0	19.5	97.4
6	40.0	35.6	88.9
7	80.0	82.2	103

Sample Description	Extract Concentration ug/L	Sample Concentration ng/L	Spike % Recovery
W3	1.2	<LOD	--
W2	1.2	<LOD	--
W1	1.2	<LOD	--
W1 duplicate	1.2	<LOD	--
Blank	1.2	<LOD	--
Spike	13.1	65.6	109%
Spike	10.6	52.9	88%
Spike	18.4	91.9	153%

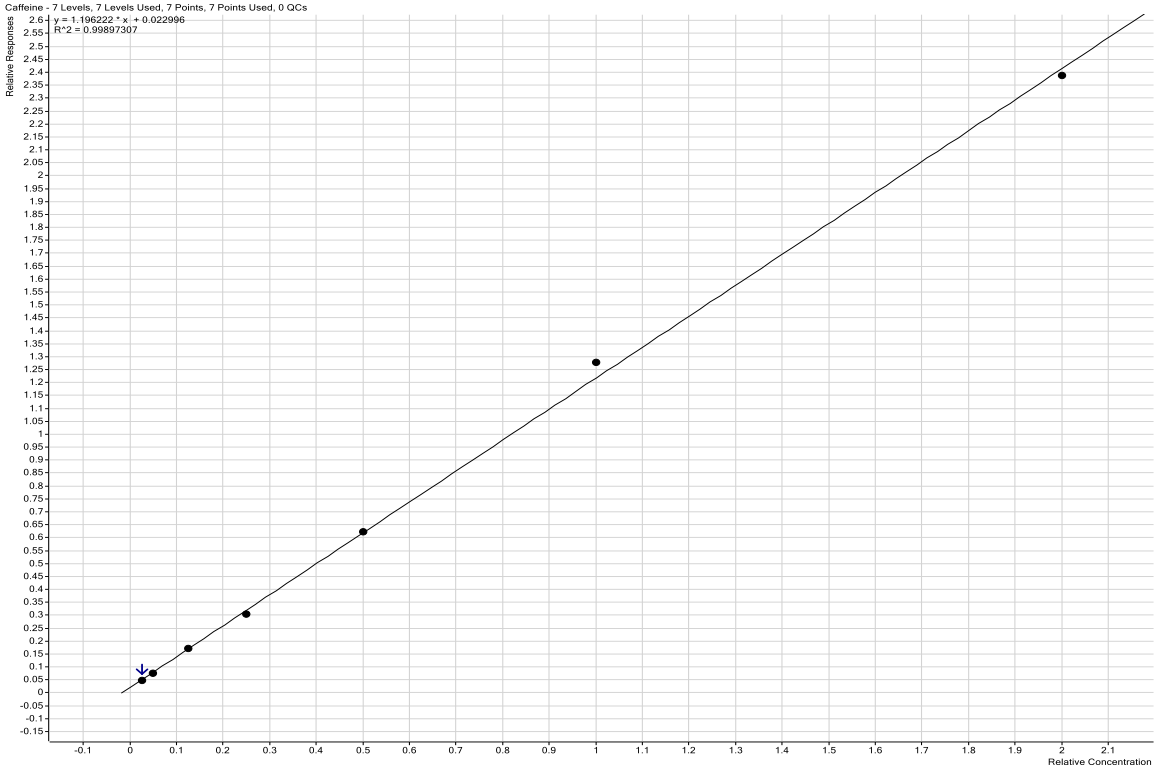
Paraxanthine



Standard Level	Expected concentration ug/L	Actual Concentration ug/L	Percent Accuracy
1	0.5	0.7	137
2	1.0	1.3	125
3	2.5	2.6	102
4	5.0	4.8	95.8
5	10.0	9.4	94.2
6	20.0	20.3	101
7	40.0	40.0	100

Sample Description	Extract Concentration ug/L	Sample Concentration ng/L	Spike % Recovery
W3	0.4	<LOD	--
W2	0.2	<LOD	--
W1	0.2	<LOD	--
W1 duplicate	0.2	<LOD	--
Blank	0.0	<LOD	--
Spike	5.2	26.0	87%
Spike	3.4	16.9	56%
Spike	2.0	10.1	34%

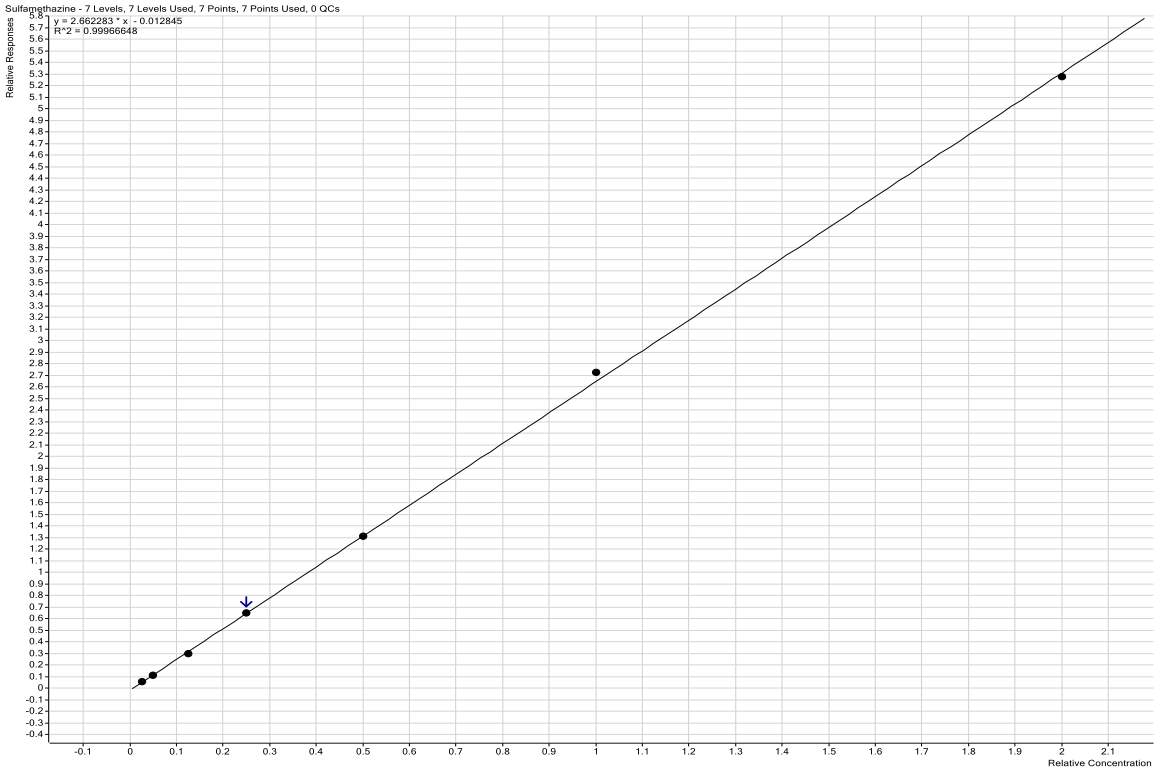
Caffeine



Standard Level	Expected concentration ug/L	Actual Concentration ug/L	Percent Accuracy
1	0.5	0.4	71.5
2	1.0	1.1	112
3	2.5	2.3	92.4
4	5.0	4.7	93.5
5	10.0	10.5	105
6	20.0	20.2	101
7	40.0	39.8	99.5

Sample Description	Extract Concentration ug/L	Sample Concentration ng/L	Spike % Recovery
W3	0.2	<LOD	--
W2	1.4	<LOD	--
W1	0.5	<LOD	--
W1 duplicate	0.5	<LOD	--
Blank	0.1	<LOD	--
Spike	5.2	25.9	86%
Spike	4.6	23.2	77%
Spike	3.9	19.4	65%

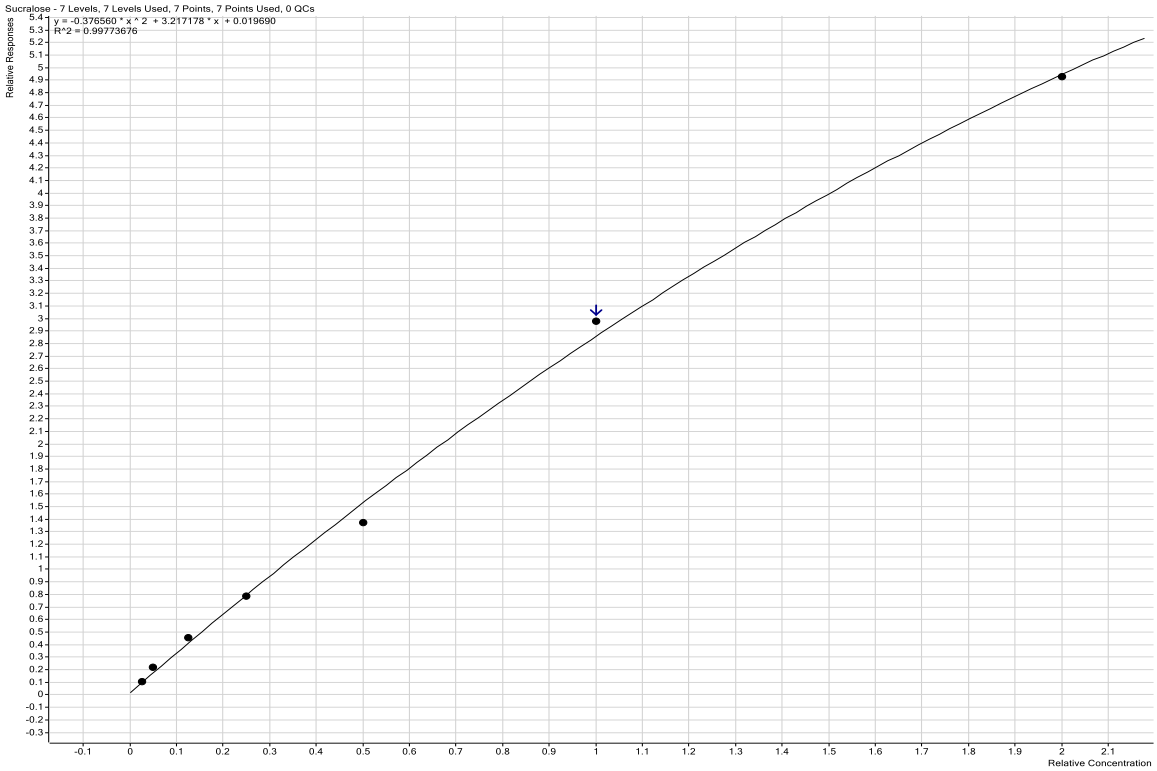
Sulfamethazine



Standard Level	Expected concentration ug/L	Actual Concentration ug/L	Percent Accuracy
1	0.5	0.7	138
2	1.0	1.1	113
3	2.5	2.4	97.4
4	5.0	4.9	97.7
5	10.0	10.1	101
6	20.0	19.6	98.0
7	40.0	40.2	100

Sample Description	Extract Concentration ug/L	Sample Concentration ng/L	Spike % Recovery
W3	0.2	<LOD	--
W2	0.2	<LOD	--
W1	0.2	<LOD	--
W1 duplicate	0.2	<LOD	--
Blank	0.2	<LOD	--
Spike	3.3	16.7	84%
Spike	3.1	15.7	78%
Spike	2.7	13.7	68%

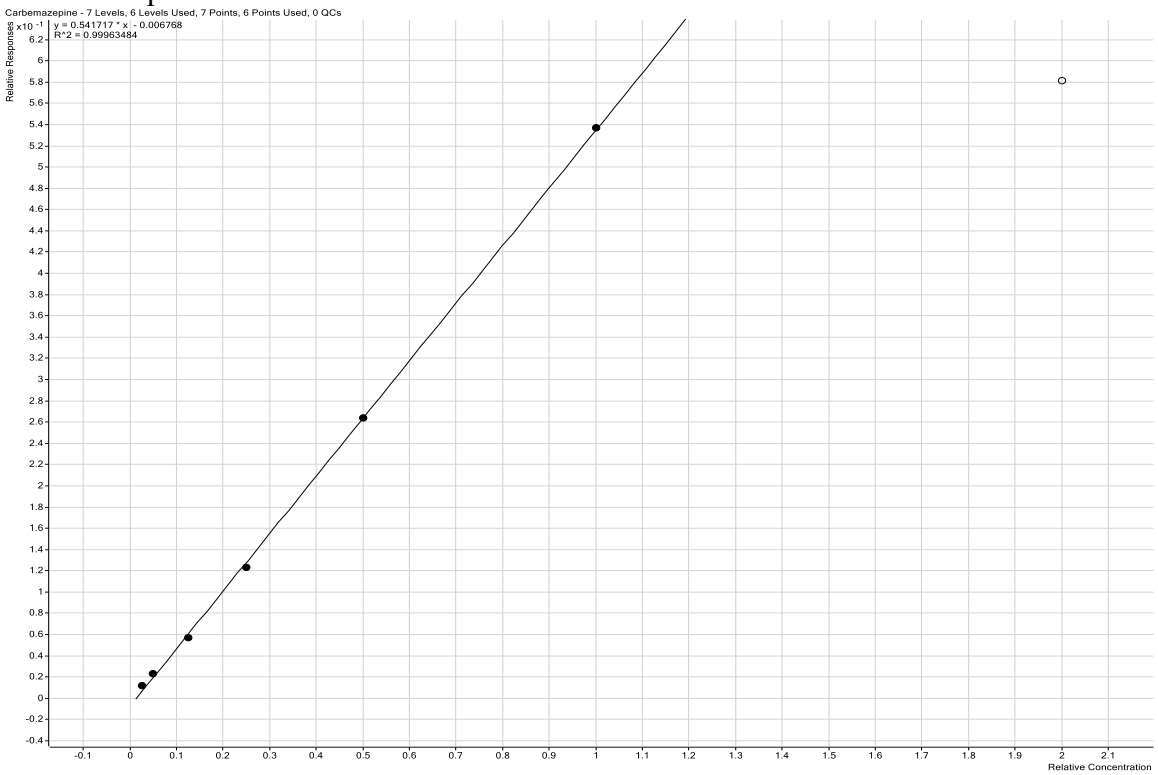
Sucralose



Standard Level	Expected concentration ug/L	Actual Concentration ug/L	Percent Accuracy
1	2.5	2.8	110
2	5.0	4.7	94.6
3	10.0	12.2	97.7
4	25.0	28.7	115
5	50.0	44.2	88.4
6	100.0	103	103
7	200.0	200	99.8

Sample Description	Extract Concentration ug/L	Sample Concentration ng/L	Spike % Recovery
W3	2.4	<LOD	--
W2	2.1	<LOD	--
W1	79.5	397	--
W1 duplicate	121	606	--
Blank	0.0	<LOD	--
Spike	24.9	124.3	62%
Spike	20.8	104.2	52%
Spike	23.8	119.2	60%

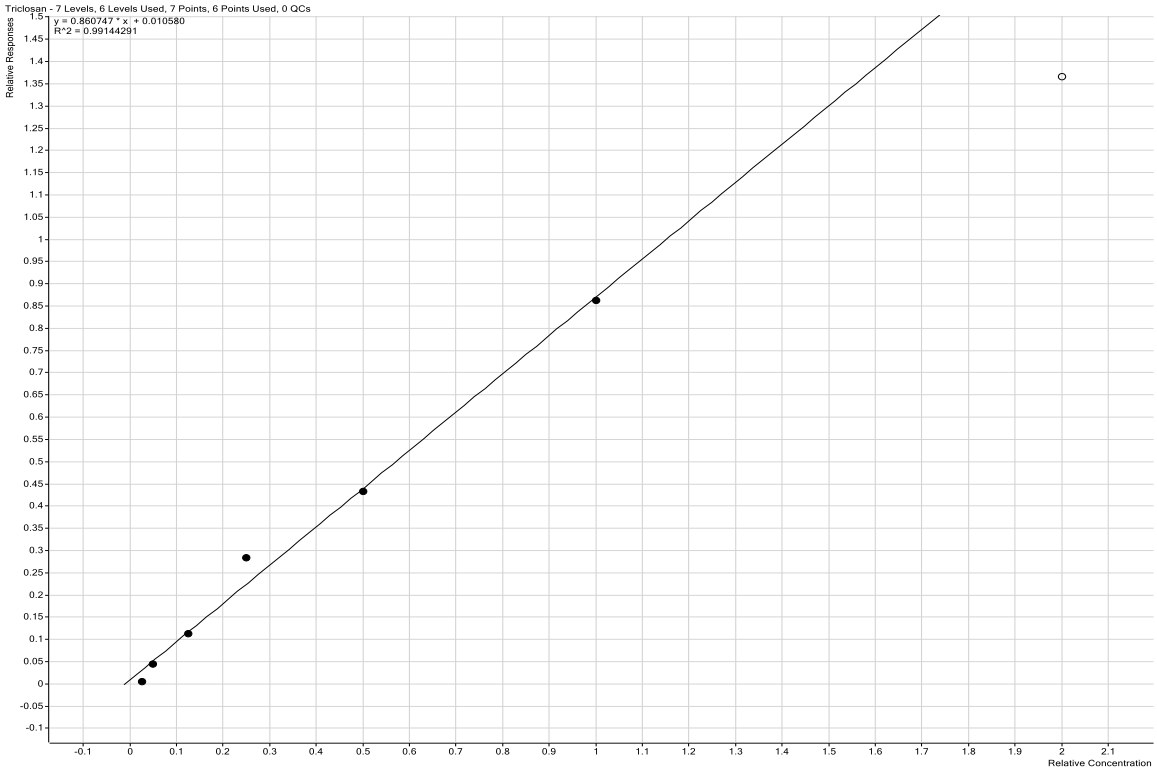
Carbamazepine



Standard Level	Expected concentration ug/L	Actual Concentration ug/L	Percent Accuracy
1	0.25	0.3	123
2	0.5	0.5	106
3	1.0	1.2	94.4
4	2.5	2.5	98.7
5	5.0	5.0	100
6	10.0	10.0	100
7	20.0	11.1	55.4

Sample Description	Extract Concentration ug/L	Sample Concentration ng/L	Spike % Recovery
W3	0.1	<LOD	--
W2	0.1	<LOD	--
W1	0.1	<LOD	--
W1 duplicate	0.1	<LOD	--
Blank	0.1	<LOD	--
Spike	2.0	9.9	50%
Spike	1.7	8.6	43%
Spike	1.7	8.7	44%

Triclosan



Standard Level	Expected concentration ug/L	Actual Concentration ug/L	Percent Accuracy
1	2.5	4.0	160
2	5.0	5.4	109
3	10.0	11.1	89.1
4	25.0	24.6	98.3
5	50.0	49.2	98.5
6	100.0	101	101
7	200.0	143	71.7

Sample Description	Extract Concentration ug/L	Sample Concentration ng/L	Spike % Recovery
W3	1.5	<LOD	--
W2	1.4	<LOD	--
W1	1.1	<LOD	--
W1 duplicate	2.0	<LOD	--
Blank	2.1	<LOD	--
Spike	27.1	135.3	68%
Spike	11.0	55.2	28%
Spike	7.9	39.6	20%

Chloracetanilide Metabolite (CAAM) results using HPLC

The 2 Fraction Collect solid phase extraction method was used with Sep-Pak 6cc (500 mg) C18 cartridges to extract samples for chloracetanilide metabolite analysis. No internal standard was used in this method. Analyte detections were confirmed by analysis on a second column. The spike sample contained 400 uL of 5.0 ng/uL each alachlor OA, acetochlor OA, alachlor ESA, metolachlor OA, and metolachlor ESA in 1000 mL of RO water. [E] = estimated value

Sample Description	Alachlor OA ug/L	Acetochlor OA ug/L	Alachlor ESA ug/L	Metolachlor OA ug/L	Acetochlor ESA ug/L	Metolachlor ESA ug/L
LOD	0.03	0.04	0.03	0.05	0.03[E]	0.03
W1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
W2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
W3	<LOD	<LOD	0.58	0.46	<LOD	1.16
Blank	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Spike	1.10	1.12	1.30	1.34	--	1.37
Spike % Recovery	55%	56%	65%	67%	--	69%

Chloracetanilide Metabolite (CAAM) results using ESI-LC/MS/MS

The 2 Fraction Collect solid phase extraction method was used with Sep-Pak 6cc (500 mg) C18 cartridges to extract samples for chloracetanilide metabolite analysis. Butachlor ESA was used as an internal standard for all analytes. The spike sample contained 200 uL of 10.0 ng/uL each alachlor OA, acetochlor OA, alachlor ESA, metolachlor OA, and metolachlor ESA in 1000 mL of RO water.

Sample Description	Alachlor OA ug/L	Acetochlor OA ug/L	Alachlor ESA ug/L	Metolachlor OA ug/L	Acetochlor ESA ug/L	Metolachlor ESA ug/L
LOD	0.87	0.65	1.16	0.31	0.67	2.04
W4	<LOD	<LOD	0.30	0.59	<LOD	2.90
W5	<LOD	<LOD	0.39	0.92	<LOD	3.20
Blank	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Spike	1.23	1.48	1.90	1.49	--	1.91
Spike % Recovery	61%	74%	95%	74%	--	95%

APPENDIX G – Acronyms

ACE	acesulfame
AMN	acetaminophen
B	boron
BNZ	benzoylecgonine
CAAM	chloroacetanilide herbicide metabolites
CAF	caffeine
COT	cotinine
CRB	carbamazepine
DPH	Division of Public Health
DWS	drinking water standard
ESA	ethane sulfonic acid (form of herbicide metabolite)
EPA	Environmental Protection Agency
ESI	electrospray ionization
FIA	flow injection analysis
ICP-OES	inductively coupled plasma – optical emission spectrometry
GW	groundwater
HA	SPE method using HLB cartridges and acidified samples
HD	SPE method using HLB cartridges and unacidified samples; samples are dried down completely before reconstituting in mobile phase
HDPE	high-density polyethylene
HLB	hydrophilic-lipophilic balanced
HPLC	high performance liquid chromatography
HU	SPE method using HLB cartridges and unacidified samples; samples are dried down to < 100 uL before reconstituting in mobile phase
Koc	soil organic carbon water-partitioning coefficient
LC/MS/MS	liquid chromatography coupled to tandem mass spectrometry
LOD	limit of detection
MDL	method detection limit
MRM	multiple reaction-monitoring

MW	molecular weight
N	nitrogen
NO_3^-	nitrate
NU	SPE method using C8+Aminopropyl cartridges and acidified samples
OA	oxanilic acid (form of herbicide metabolite)
P	phosphorus
PDA	photodiode array detector
PPCP	pharmaceuticals and personal care products
PXN	Paraxanthine
RO	Millipore reverse osmosis purified water
SDB	styrene divinylbenzene
SLF	sulfamethazine
SPE	solid phase extraction
SUC	sucralose
SW	surface water
TRI	triclosan
USGS	United States Geological Survey
UWSP	University of Wisconsin Stevens Point
WAX	weak anion exchange
WEAL	Water and Environmental Analysis Lab
WI DNR	Wisconsin Department of Natural Resources
WWTP	Waste Water Treatment Plant