AN ABSTRACT OF THE DISSERTATION OF

Will J. Backe for the degree of Doctor of Philosophy in Chemistry presented on July 18, 2012

Title: Development of Novel Analytical Methods to Detect Emerging Contaminants in Aqueous Environmental Matrices using Large-Volume Injection

Abstract approved: _____
Jennifer A. Field

It is the responsibility of humans, as environmental stewards, to monitor our impact on the environment so that efforts can be made to remediate the effects of our actions and change behaviors. To better understand our environmental footprint, sensitive and simple analytical methods are needed to quantify the contaminants that we discharge into our natural surroundings. Emerging environmental contaminants are of particular concern because there is limited or no information available on their occurrence, fate, and toxicity. As a result, the implications of using these chemicals are not well understood. Therefore, accurate environmental data are needed to help scientists and government policy-makers make informed decisions on research directions and chemical regulation. However, challenges exist for the analysis of emerging contaminants, including a lack of suitable analytical standards and internal standards, their broad range of chemical properties, and that they are frequently present at trace levels and in complex environmental matrices.

The work presented within this dissertation focuses on the development, validation, and comparison of analytical methodologies based on large-volume injection high-performance liquid chromatography (HPLC) tandem mass spectrometry (MS/MS) for the analysis of emerging environmental contaminants in aqueous environmental matrices. Large-volume injection (e.g. 900 µL to 4,500 µL) is an analytical technique that eliminates sample preparation associated with pre-concentration by injecting larger-than-traditional volumes of sample directly onto a HPLC column.

In Chapter 2, a direct aqueous large-volume injection method was developed and validated for the quantification of natural and synthetic androgenic steroids in wastewater influent, wastewater effluent, and river water. This method was then applied to hourly composite samples of wastewater influent that were taken over the course of a single day. This work expands on the research of the endocrine-disrupting chemicals that occur in wastewater and provides an estimate of the community use/abuse of synthetic androgenic steroids.

Environmental analytical methods should be as environmentally friendly as possible and efforts should be made to reduce the waste generated during analysis while maintaining analytical performance. In Chapter 3, a method based on large-volume injection was compared to two methods based on solid-phase extraction. The purpose of this comparison was to demonstrate that the same method performance could be achieved by large-volume injection as that by solid-phase extraction while reducing waste, labor, and costs. Estrogens and perfluorinated chemicals were used as model

analytes and wastewater influent was used as a model matrix. The results of this study provide convincing reasons for analysts to adopt large-volume injection as an alternative to solid-phase extraction.

In Chapter 4, a novel analytical method was developed and validated to quantify newly-identified and legacy fluorinated chemicals in groundwater. The final method combined micro liquid-liquid extraction, non-aqueous large-volume injection, and orthogonal chromatographic separations. Ground water samples collected from six different U.S. military bases was used to demonstrate the method. This is the first report on the occurrence of these newly-identified fluorinated chemicals in any environmental media and serves as a rational for conducting future research on their environmental fate and toxicity.

The breadth of the research presented in this dissertation advances the field of environmental analytical chemistry in several areas. First, classes of environmental contaminants for which there is limited (synthetic androgenic steroids) or no (newly-identified fluorochemicals) environmental data were studied. Second, novel methods based on direct-aqueous and non-aqueous large-volume injection were developed and validated to identify and quantify those contaminants. Third, it was demonstrated that solid-phase extraction is not a “necessary evil” needed to develop methods for emerging environmental contaminants in aqueous matrices. Finally, this work is a platform on which other environmental chemists can use to develop large-volume injection methods in the future.

Development of Novel Analytical Methods to Detect Emerging Contaminants in Aqueous Environmental Matrices using Large-Volume Injection

by
Will J. Backe

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APPROVED:

_____

Major Professor, representing Chemistry

_____

Chair of the Department of Chemistry

_____

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Will J. Backe, Author

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CONTRIBUTION OF AUTHORS

Christoph Ort and Alex J. Brewer designed and implemented the sampling protocol to acquire the wastewater samples that the analytical method outlined in Chapter 2 was demonstrated on.

Kaneen E. Christensen was responsible for acquiring and sending the groundwater samples, as described in Chapter 4, from Site A to Oregon State University. Additionally, she coauthored the sample collection section of Chapter 4 and provided insights integral to the completion of the manuscript.

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Development of Novel Analytical Methods to Detect Emerging Contaminants in Aqueous Environmental Matrices using Large-Volume Injection

Chapter 1 - Introduction

1.1 Background

The overarching theme of this dissertation is the development, validation, and application of analytical methods based on large-volume injection for the quantitative analysis of emerging contaminants in aqueous environmental samples. Emerging environmental contaminants are anthropogenic chemicals that have recently been identified (within the last two decades) in the environment or are expected to occur in the environment based on production volume, chemical properties, and human-use practices. However, "emerging" does not necessarily mean that the chemicals are new contaminants to the environment. Pharmaceuticals, hormones, personal care products, life-style drugs (e.g. caffeine), wastewater disinfection byproducts, per- and poly-fluorinated chemicals, benzotriazoles, siloxanes, and napthenic acids are all examples of emerging environmental contaminants (1), but by no means do they constitute an exhaustive list. The identification of "new" emerging contaminants is due in part to the evolution of increasingly sensitive analytical methods that are capable of quantifying chemicals at sub to low nanograms-per-liter levels in environmental matrices. (2) The emerging contaminants described in this dissertation include natural and synthetic androgenic and estrogenic steroids, as well as newly-identified and legacy per- and poly-fluorinated chemicals.

Quantifying anthropogenic contamination in aquatic environments presents many analytical challenges. One such challenge is that contaminants are often present at

trace levels in complex aquatic matrices. As such, analytical methods are needed that are sensitive and selective in the face of potential matrix interferences. The methods described in this thesis (Chapters 2-4) were developed around large-volume injection to circumvent the need for extraneous off-line extractions. Large-volume injection is an HPLC technique that reduces sample pre-treatment by concentrating analytes on-column through the injection of larger-than-normal sample volumes (e.g. 900 to 4,500 µL). (3) A complete description of large-volume injection, how it is performed, and examples of its use is presented in Chapters 2-4. Tandem mass spectrometry (MS/MS) is commonly interfaced with high-performance liquid chromatography (HPLC) for the analysis of emerging environmental contaminants. (2) Indeed, all analyses presented in this dissertation employ HPLC-MS/MS. Tandem mass spectrometric detectors operated in multiple-reaction-monitoring mode allow analytical methods to be both sensitive and selective. (4) However, analytical interferences caused by compounds in the sample matrix can still result in decreased sensitivity and selectivity when using MS/MS. (5). In a perceived effort to reduce matrix interferences and preconcentrate analytes, solid-phase extraction remains the most popular sample preparation technique. (1) Nevertheless, a reoccurring point made throughout this dissertation is that solid-phase extraction is laborious, costly, prone to contamination and analyte loss, and redundant with subsequent separations performed via HPLC. Additionally, solid-phase extraction results in the generation of unnecessary hazardous liquid and solid waste. (3)

Estrogenic hormones, pharmaceuticals, and perfluorinated chemicals are known to occur almost ubiquitously in surface waters. (6-9) The high incidence of these compounds in the aqueous environment is of concern, especially considering the persistence of perfluorinated chemicals and the potential of some to biomagnify. (10) Estrogens and pharmaceuticals do not persist as long as perfluorinated chemicals but they are released almost continuously to surface waters via wastewater treatment effluent. (9, 11, 12) Additionally, the estrogens that occur in wastewater effluents are at concentrations sufficient to elicit endocrine-disrupting effects on aquatic wildlife, such as the feminization of male fish. (13) While some environmental implications of perfluorinated chemicals, pharmaceuticals, and estrogens are well known, the relevance of other emerging contaminants is not well understood due to a lack of data on their occurrence, fate, and toxicity. (8, 14, 15) Therefore, more research needs to be conducted on emerging contaminants for which little data exists. This dissertation reports on emerging contaminants for which little or nothing is known about their environmental occurrence. For example, there is a paucity of data on natural and synthetic androgenic hormones in surface and wastewaters (the focus of Chapter 2). Additionally, Chapter 4 describes the analysis and detection of newly-identified fluorinated contaminants, for which no previous environmental data existed.

Surface and wastewaters are the most studied aquatic matrices in emerging environmental contaminant research. (15) A narrowed focus is logical considering that the release of environmental contaminants into the aquatic environment is primarily from wastewater effluents into receiving waters. (14) However, other sources also

introduce emerging contaminants into aquatic environments including direct applications, (16) accidental releases, (17) agricultural runoff, (18) landfills (19) and septic tanks. (20) Many of these sources result in groundwater contamination; however, less is known about emerging contaminants in groundwater compared to that of surface and wastewater. (15) In a recent study, Barnes et al. reported that 81% (n = 41 sites) of the groundwater acquired from across the United States had measurable concentrations of emerging environmental contaminants. (21) This study brings to light the need to focus research on groundwater, in addition to surface and wastewater. In response, the analytical methods described in this dissertation were applied not only to waste and surface waters (Chapters 2 and 3) but to groundwater as well (Chapter 4).

1.2 Overview of Chapters 2 through 4

Chapter 2 of this dissertation is a research article that was published in 2011 in the journal *Analytical Chemistry*. This chapter reports on the development and validation of a large-volume injection based method to quantify natural and synthetic androgenic steroids in wastewater (influent and effluent) and river water. The reported method provided enhanced accuracy over existing methods, while reducing analysis time and cost. The detection limits of the method were generally in the low nanogram-per-liter range and capable of detecting environmentally-relevant concentrations. An application of the method was performed on hourly composite samples of wastewater influent that were collected over 24 hours. This sampling strategy allowed androgen loads (mg/hr) in wastewater to be profiled over the course of a day. Along with the

endogenously-produced androgens (testosterone and androstenedione) and two synthetic androgens (boldenone and nandrolone) were detected in the wastewater samples. Interestingly, the temporal profile of boldenone correlated significantly with those of the endogenously-produced androgens. This correlation indicated that boldenone came from an endogenous source, possibly due to transformation of a related endogenous compound. However, *in-situ* transformation of testosterone to boldenone was tentatively ruled out.

The third chapter of this dissertation is a manuscript recently (2012) accepted for publication in *Environmental Science and Technology*. The focus of Chapter 3 was to challenge the preconceived belief that sample concentration by solid-phase extraction is necessary for the development of sensitive environmental analytical methods. To demonstrate this, a method based on large-volume injection was compared against two different methods based on solid-phase extraction for the analysis of well-studied environmental contaminants (estrogens, perfluoroalkyl acids, and perfluoroalkyl sulfonates). Matrix effects were used as an indicator of analytical signal quality and compared across each method by two approaches. The study demonstrated that the same performance can be achieved by methods using large-volume injection as those from the literature that use solid-phase extraction. In addition, large-volume injection was shown to be cost- and time- effective over solid-phase extraction and "greener" because less chemical and material waste was generated.

Chapter 4 of this dissertation is a research article currently being prepared for submission to *Environmental Science and Technology*. Chapter 4 outlines the development and validation of a non-aqueous large-volume injection method that employs orthogonal coupled-column HPLC-MS/MS to quantify newly-identified and legacy classes of fluorinated chemicals in groundwater. These newly-identified fluorinated chemicals differ from legacy fluorinated chemicals in that they contain not only anionic but also zwitterionic and cationic functionalities. Groundwater was first micro liquid-liquid extracted into an organic solvent to eliminate issues with fluorinated chemical loss from aqueous solutions. The liquid-liquid extracts were then analyzed via non-aqueous large-volume injection without further sample preparation Method recovery and accuracy was around 100% and the detection limits ranged from sub to low nanograms-per-liter. The analytical method was demonstrated on groundwater samples obtained from six different military bases across the United States. The application of the method revealed that newly-identified fluorinated chemicals contaminate groundwater along with legacy classes of fluorinated chemicals at sites where fire-training exercises occurred. This is the first report of these newly-identified fluorinated chemicals as environmental contaminants.

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Chapter 2 - Analysis of Androgenic Steroids in Environmental Waters by Large-Volume Injection Liquid Chromatography Tandem Mass Spectrometry

Will J. Backe, Christoph Ort, Alex J. Brewer, and Jennifer A. Field

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2.1 Abstract

A new method was developed for the analysis of natural and synthetic androgenic steroids and their selected metabolites in aquatic environmental matrixes using direct large-volume injection (LVI) high-performance liquid chromatography (HPLC) tandem mass spectrometry (MS/MS). Method accuracy ranged from 87.6 to 108% for analytes with well-matched internal standards. Precision, quantified by relative standard deviation (RSD), was less than 12%. Detection limits for the method ranged from 1.2 to 360 ng/L. The method was demonstrated on a series of 1 h composite wastewater influent samples collected over one day with the purpose of assessing temporal profiles of androgen loads in wastewater. Testosterone, androstenedione, boldenone, and nandrolone were detected in the sample series at concentrations up to 290 ng/L and loads up to 535 mg/h. Boldenone, a synthetic androgen, had a temporal profile that was strongly correlated to testosterone, a natural human androgen, suggesting its source may be endogenous. An analysis of the sample particulate fraction revealed detectable amounts of sorbed testosterone and androstenedione. Androstenedione sorbed to the particulate fraction accounted for an estimated 5 to 7% of the total androstenedione mass.

2.2 Introduction

Wastewater treatment plants (WWTPs) can act as point sources of anthropogenic pollutants to receiving waters. (1) Sensitive and simplified methods are needed to quantify pollutants in complex wastewater and environmental matrixes, especially

endocrine-disrupting chemicals (EDCs) that are bioactive at low (nanogram/liter) concentrations. (2-5) Analyses of EDCs focus primarily on estrogens and their conjugates in wastewater, (6-13) due to observed estrogenic effects on aquatic wildlife. (7, 14, 15) Conversely, analyses of androgens are focused on wood pulp mill effluent (14, 16) and agricultural runoff, (2, 3, 17) which exhibit androgenic effects on aquatic wildlife. Recently, WWTP influent and effluent are reported to have androgenic activities. (18-21) However, the limited analyses of androgenic steroids in wastewater focuses on compounds endogenous to humans and a few select synthetic compounds, such as methyl-testosterone and stanozolol. (22-25) It is possible that some of the androgenic activity detected in wastewater is due to synthetic androgens which have been overlooked by current androgenic-activity and chemical analyses. Synthetic androgens are used medically and abused illicitly, and because of this, consumption data on them are incomplete. Therefore, it is largely unknown which synthetic androgens might occur in municipal wastewater systems, so analyses incorporating a broader range of synthetic androgens are needed. Androgenic steroids and their phase-I metabolites are, in most cases, excreted from humans as glucuronic acid or sulfate conjugates. (26, 27) Others report that estrogenic steroids are largely deconjugated back to their parent form in-route to, and during, wastewater treatment. (6, 9, 11, 28) However, there have been no studies to support that this phenomenon also applies to androgens in wastewater and, as such, remains a data gap in the literature.

Solid-phase extraction (SPE) is the conventional method for steroid extraction, cleanup, and concentration from environmental and wastewater matrixes. (22-25, 29, 30) However, sample pretreatment by SPE requires the use of large quantities of solvents and materials and is laborious and expensive. In contrast, direct large volume injection (LVI) of analytes in aquatic environmental and wastewater matrixes reduces the amount of labor, solvents, and materials required because the only sample pretreatment step is centrifugation (31-33) or filtration. (34, 35)

Most wastewater sampling approaches use 24 h composite samples, while very few studies are conducted using sampling approaches based on higher temporal sample resolution. (36, 37) To date, sampling protocols for the study of androgens in wastewater rely on grab samples (22) and 24 h volume-proportional composites collected with an unknown sampling frequency. (24) Wastewater influent is highly heterogeneous; grab sampling can miss analyte events entirely and sampling error for composites comes from sampling frequency and number of wastewater pulses containing the analyte (36, 37) Furthermore, 24 h composites do not reveal daily patterns in analyte loads, (37) which potentially can help discriminate between analytes of endogenous and synthetic origin. Additionally, 24 h composites may dilute analytes that occur only episodically throughout a given day to levels below detection.

The objective of this study was to develop a LVI based method that allows for the analysis of androgenic steroids in waste and surface water that is simplified compared to conventional methods and is sensitive, selective, reproducible, and suitable for a

wide range of androgens. The analytes included in this study are two endogenous androgens and nine synthetic androgens used in human (38, 39) and veterinary medicine (40, 41) and abused illicitly. (42-44) Five major phase-I human metabolites (26) of selected androgens were also included. Additional phase-I metabolites are commercially available; however, they were cost prohibitive. While important, assessing androgen conjugates and the potential for their deconjugation in wastewater was beyond the scope of this study. The occurrences of eight of these analytes have not been studied in wastewater. The method was demonstrated on a series of 1 h composite wastewater influent samples collected over a 24 h period to assess diurnal variation in androgen loads.

2.3 Experimental Section

2.3.1 Chemicals. Standards of nandrolone (Nand), boldenone (Bold), methandienone (Meta), stanozolol (Stan), 16β-hydroxystanozolol (16-Stan), androstenedione (Andro), methenolone (Mete), 17β-trenbolone (Tren), 17α-methyltestosterone (CH_3-Test), and D_3-stanozolol (D_3-Stan) were obtained from Cerilliant Corporation (Round Rock, Texas) as solutions at concentrations of 1 mg/mL in Either acetonitrile or 1,2-dimethoxyethane (DME) except 16-Stan and D_3-Stan, which were 0.1 mg/mL. Testosterone (Test) and D_3-Testosterone (D_3-Test) were purchased from Sigma-Aldrich (Saint Louis, MO) as solutions in DME at 1 and 0.1 mg/mL respectively. 17α-Trenbolone Epi-Tren, 5β-androst-1-en-17β-ol-3-one (5-Andro), 17α-oxandrolone Epi-Ox, 6β-hydroxymethandienone (6-Meta), tetrahydrogesterone (THG), and D_3-

boldeonone (D_3-Bold) were purchased from National Measurement Institute (NMI) (Pymble, New South Wales). THG was available only as a qualitative standard (purity 63.3%). Attempts to find a higher purity, commercially available standard were unsuccessful. Standards made from THG were adjusted to compensate for purity. 17β-Oxandrolone (Ox) was purchased from Steraloids Incorporated (Newport, RI). Primary parent standards were made in the solvent recommended by the manufacturer at 10 or 100 μg/mL; compounds with no recommendation were made in DME.

HPLC grade methanol (MeOH) and acetone were purchased from Sigma-Aldrich (Saint Louis, MO), formic acid was bought from Fisher Scientific (Fair Lawn, NJ), and ammonium formate was obtained from Mallinckrodt Chemical (Saint Louis, MO). Ultrapure water was made using a Barnstead Easepure water filtration system (Dubuque, IA).

2.3.2 Sample Collection and Treatment. Wastewater samples were obtained from a wastewater treatment facility located in the Pacific Northwest that serves a population of approximately 55 000. For the demonstration study, 24 1 h wastewater influent composites were collected via an ISCO 3700 autosampler (Teledyne Isco Inc., Lincoln, NE) on March 17, 2010 starting at 8 a.m. The influent flow during the sampling period was relatively constant throughout the day (average of 1.6 ML/h, hourly minimum 1.0 and maximum 1.9 ML/h). The autosampler was set to collect a wastewater subsample every 6 min over each 1 h period. The samples were collected in 350 mL clear glass vials and kept on ice at 4 °C during collection. The samples

were shaken, and approximately 40 mL from each 1 h composite was transferred to a 50 mL HDPE centrifuge tube and stored at −20 °C until analysis. For method development purposes, grab samples of wastewater influent and effluent were collected in 1 L baked (450 °C) and solvent rinsed (MeOH and acetone) amber glass vials and stored at 4 °C. A river water sample was collected in a 0.5 L high-density polyethylene (HDPE) bottle, stored at 4 °C, and used for method development. Zebra fish housing (FH) water from a recirculating system was collected in a 1 L baked and solvent rinsed amber glass vial and stored at 4 °C. The FH water was treated with sodium bicarbonate and Instant Ocean salt, to maintain pH and conductivity, and contained 15 000 to 20 000 fish.

Wastewater samples were centrifuged in an IEC clinical centrifuge (Thermo IEC, Nutley, NJ) at 1625 relative centrifugal force (RCF) for 15 min. Supernatant aliquots of 2.5 mL were transferred to a 6 mL glass autosampler vial and spiked with 188 pg of each stable-isotope internal standard available during the study (D_3-Stan, D_3-Bold, D_3-Test). River and FH water samples were allowed to settle and required no centrifugation. A volume of 5 mL of each river water sample was placed in a 6 mL glass autosampler vial and spiked with 375 pg of each internal standard.

2.3.3 Liquid Chromatography. An Agilent 1100 series HPLC system (Santa Clara, CA) was modified with a 900 μL Injection Upgrade Kit (Agilent part no. G1363-90100) and a Multidraw Upgrade Kit (Agilent part no. G1313-90100) that came with a 1 400 μL seat capillary. Additionally, a 5 000 μL seat capillary (Agilent part no. 0101-

0301) was purchased. The HPLC was controlled via Agilent ChemStation (Rev. A 10.02 [1757]).

Injection volumes of 1 800 µL were employed for wastewater influent and effluent, as described in Chiaia et al. (31) Briefly, one 900 µL sample volume was loaded in the 1 400 µL seat capillary, and a second 900 µL sample volume was drawn into the needle loop for a total of 1 800 µL. For river and FH water, 4 500 µL injection volumes were performed by ejecting five 900 µL sample volumes into the 5 000 µL seat capillary.

Analyte separations were performed on a 4.6 mm × 12.5 mm × 5 µm particle diameter C18 ZORBAX Eclipse Plus guard column combined with a 4.6 mm × 150 mm × 3.5 µm particle diameter ZORBAX Eclipse Plus C18 analytical column (Agilent, Santa Clara, CA). The mobile phase consisted of 0.02% formic acid in methanol (A) and 0.5 mM ammonium formate in ultrapure water (B). Upon injection, the LC injection valve was set to direct the mobile phase through the injection assembly, and a postcolumn valve (model E90, Valco Insturments Co. Inc., Houston, TX) was set to direct the column eluent to waste. For 1 800 µL injections, the gradient started at 15% A at 1 mL/min and was held for 5.6 min to load the sample on the column and then to wash the column. Next, the flow was reduced to 0.5 mL/min over a tenth of a minute, and the injection valve was set so that the mobile phase bypassed the injection assembly, which reduces the mobile phase dwell time. The injection valve was switched after a sufficient amount of mobile phase had passed through the seat capillary (wash time) to

quantitatively transfer the entire analyte sample to the column which eliminated system carryover. Over the next 8.4 min, the gradient was ramped to 70% A and held for 8.3 min. Finally, the gradient was then ramped to 97.5% A over 5.6 min and held for 10 min. All the analytes eluted before 29 min, and the extra 9.4 min of 97.5% A acted to elute the most hydrophobic matrix components off the column. At 16 min, the postcolumn valve directed the column eluent to the mass spectrometer, and at 32 min the postcolumn valve diverted it back to waste. The column re-equilibrated to initial conditions during the subsequent run's injection sequence. The gradient profile allowed for the separation of the two early eluting isomers of trenbolone. The gradient was similar for the 4 500 μL injections, except that the initial 15% A was held for 10 min at 1 mL/min and the postcolumn valve redirects the column eluent to the mass spectrometer at 22.5 min and back to waste at 38.5 min.

2.3.4 Tandem Mass Spectrometry. A SCIEX API 3000 (Applied Biosystems; Foster City, CA) tandem mass spectrometer was equipped with an electrospray ionization (ESI) interface (Turbo Ionspray) and controlled via Analyst (version 1.5.1.). All sample analyses were performed by multiple reaction monitoring (MRM) in positive ionization mode. The source conditions for temperature, nebulizing gas, and Turbo Ionspray gas were 500 °C, 35 bar, and 8000 mL/min, respectively. Analyte standards were made in MeOH at approximately 1 mg/L and infused into the mass spectrometer at 10 to 20 μL/min via a syringe pump (Harvard Apparatus; Holliston, MA) to determine the most intense precursor $[M + H]^+$ and product ions and to optimize the declustering and focusing potential (DP and FP), collision energy (CE), and collision

cell exit potential (CXP) for each compound (Table 2.1). The precursor and product ions identified for the analytes are consistent with previously published literature. (45, 46)

2.3.5 Quantitation and Identification. Calibration standards were made in 5 mM ammonium formate in 10% MeOH/ultrapure water that was adjusted to pH 8.1 using 0.5 N NaOH. Calibration standards ranged from concentrations of 2.3 to 6 000 ng/L. For 1 800 and 4 500 μL injection volumes, an internal standard solution was spiked into each sample yielding an analyte mass of 188 or 375 pg in sample volumes of 2 500 and 5 000 μL, respectively. Analyte responses were normalized to internal standards and quantified from calibration standards (n = 5 or 6) by linear least-squares regression. All regression curves had a coefficient of determination (R2) > 0.99. Positive analyte identification required that its retention was ±0.25 min from the average retention time of authentic standards. Two product ions were selected for each compound, one for quantitation and one for qualitative analyte confirmation (Table 2.1). Quantitative to qualitative ion ratios were required to be within 20% of those in overspiked duplicate samples because some analytes that were spiked into blank wastewater and river water produced product ion ratios that were different from analytical standards.

2.3.6 Injection Volume Optimization. The HPLC autosampler was configured to perform injections up to 5 000 μL. Wastewater and FH water samples were spiked with selected analytes to final concentrations of 150 ng/L. Injection volumes were

varied from 900 to 5 000 µL in order to assess the optimal injection volume for each matrix. Optimal injection volumes were defined as the minimum volume injected at which the highest signal-to-noise (sensitivity) is achieved.

2.3.7 Standard Addition, Accuracy, and Precision. Analyte concentrations in wastewater influent were determined from standard addition and compared to values obtained by internal standard calibration for the purpose of assessing the validity of using solvent-based calibration for analyte quantification. A working analyte stock was prepared at 375 000 ng/L and spiked into 25 mL of wastewater influent that gave no detectable analyte signals, yielding analyte concentrations ranging from 60 to 1 600 ng/L. Standard addition was performed using a nine-point calibration curve, which included four samples at the initial spike concentration and five standard additions corresponding to an increase of 0.5, 0.75, 1, 1.25, and 1.5-fold over the initial spike concentration. Standard additions were spiked with a separate working analyte stock made in ultrapure water containing 5 mM ammonium formate and adjusted to pH 8.1 with 6 M NaOH. The four samples at the initial spike concentration were also quantified using three different internal standard calibration curves, one for each of the three internal standards that were available. For each analyte, the internal standard whose calibration values provided the best agreement to quantification derived by standard addition was used for subsequent analysis (Table 2.1).

Accuracy was determined for each analyte in wastewater influent, effluent, and river water by analyzing each matrix in quadruplicate using internal standard based

calibration. Accuracy was defined as the percent of the calculated analyte concentration over the nominal spiked concentration. Spiked samples in each matrix were prepared as described above at analyte concentrations ranging from 10 to 2 000 ng/L. Within-run method precision was quantified by calculating the RSD of quadruplicate samples.

2.3.8 Limit of Detection and Quantitation. There is currently no unified method for reporting detection limits for HPLC analyses. (47) The limit of detection (LOD) and limit of quantitation (LOQ) in this study were calculated by multiplying the standard deviation of the area of the background noise (n = 10) in the matrix of interest by 3.3 and 10, respectively. Then, that number was divided by the slope of an analyte's calibration curve prepared near detection in the same matrix. (48) Calibration curves used to calculate LOD and LOQ for each analyte contained no less than 13 points and were made in matrixes characterized by no detectable background signal.

2.3.9 Storage Stability. A storage stability study was performed to determine the stability of androgens stored in wastewater influent at −20 °C over 60 days. A 100 mL wastewater influent grab sample was analyzed for native steroids and tested positive for Andro, Test and Bold. The sample was then spiked with analytes that were not present above the detection producing concentrations ranging from 150 to 1000 ng/L. Seven vials were filled with 12 mL of sample and analyzed in quadruplicate at 0, 1, 2, 8, 19, 31, and 60 days.

2.3.10 Boldenone Production in Wastewater. To test the hypothesis that Test is converted to Bold in wastewater influent during transit to the WWTP, 500 mL of influent was spiked with D_3-Test to give a final concentration of 300 ng/L (1.03 nM) and monitored for the production of D_3-Bold over 24 h. An experimental duration of 24 h was selected because it exceeded the maximum estimated transit time of wastewater (8 h) for the municipal system studied. The sample was kept between 18 and 22 °C in a sealed 500 mL glass amber bottle with minimal head space and placed on a rotary shaker for the duration of the experiment. The bottle was only opened to collect samples (n = 3 per time point) for analysis at 0, 2, 6, 12, and 24 h. Since D_3-Test and D_3-Bold were analytes, D_3-Stan was used as an internal standard for all analytes. Experimental conditions were meant to assess the potential for analyte formation in wastewater, not to fully simulate in-situ conditions.

2.3.11 Suspended Solids Extraction. Extraction of the solid-phase associated with wastewater influent was performed to assess possible analyte loss due to sample centrifugation. Five single solid samples from selected 1 h wastewater composites were removed, blotted on an absorbent tissue to remove residual water, placed in a 1.5 mL microcentrifuge vial, and frozen at −20 °C until analysis. Methanol was chosen as an extraction solvent because it has been used for extraction of estrogenic steroids from sediment and sludge samples.(29) Extractions were carried out in triplicate and performed by adding 200 μL of MeOH to each sample, vortexing for 30 s, and sonicating for 6 min. Samples and their extracts were then centrifuged at 8154 RCF for 4 min in a microcentrifuge (5415 C, Eppendorf, Hauppauge, NY). The supernatant

was transferred to a 6 mL autosampler vial, the final volume brought to 2 500 μL with 5 mM ammonium formate in pure water adjusted to a pH of 8.1, and the sample analyzed as described above.

2.4 Results and Discussion

2.4.1 Large-Volume Injection Liquid Chromatography. The LVI chromatography method employed produced good analyte peak shape and separation for analytes at or near their detection limit in wastewater influent (Figure 2.1), effluent (Figure A.1.1 in Appendix 1), and river water (Figure A.1.2 in Appendix 1) Solid-phase extraction was eliminated by the use of large sample volumes (1 800 and 4 500 μL) that are directly injected onto the analytical column. Large-volume injection reduces the amount of solvent necessary to process the sample, which can be over 50 mL per sample for just the SPE step in environmental androgen analysis. (22, 23)

The same column was used throughout the entirety of this study, and column performance did not noticeably decrease compared to more traditional chromatography utilizing small (20 to 100 μL) injections. However, it was necessary to replace the guard column after an average of 50 injections to prevent degradation in the chromatography.

Carryover of two analytes Stan and 16-Stan was observed initially only for injections of standards made in 10% MeOH/water. Carryover was resolved by increasing the wash time of the seat capillary and making all analytical standards in buffered 10% MeOH/water (see the Experimental Section 2.3).

2.4.2 Injection Volume. Systematically increasing the injection volume of wastewater (Figure A.1.3 in Appendix 1) demonstrated that the signal-to-noise (S/N) did not increase appreciably above 1,800 μL. For this reason, 1,800 μL sample injection volumes were selected for the analysis of wastewater. In FH water, S/N increased with increasing injection volumes up to 4,500 μL (Figure A.1.4 in Appendix 1). River water replaced FH water as a matrix of interest. Injection volumes of 4,500 μL were chosen for river water even though similar experiments were not performed. However, volumes of 5,000 μL of surface water have injected via HPLC for the analysis of pesticides. (35)

2.4.3 Standard Addition, Accuracy, and Precision. Internal standard calibration provided statistically (p-value > 0.05, two sided t test) equivalent concentration values to concentrations derived from standard addition for 13 analytes (Table A.1.1 in Appendix 1), which validated their use for subsequent analyte quantification (Table 2.1). 5β-Androst-1-en-17β-ol-3-one, 6-Meta, and Epi-Ox differed significantly (p value < 0.05, two tailed t test) from the concentrations calculated by internal standard calibrations to concentrations calculated by standard addition (Table A.1.1 in Appendix 1), so the internal standard that provided the closest agreement (Table 2.1) was used for subsequent analyses. These differences are most likely attributed to matrix components affecting the ionization of 5-Andro, 6-Meta, and Epi-Ox differently than the internal standards available. (49)

Analytes with well-matched internal standards provided whole-method accuracy ranging from 87.6 to 108% for influent, 96.3 to 107% for effluent, and 93.9 to 108% for river water (Table 2.2). Analytes not well represented by their internal standard (5-Andro, 6-Meta, and Epi-Ox) gave modest method accuracy for influent (62.3 to 84.8%) but improved in the less complex matrixes, including effluent (76.4 to 93.4%) and river water (80.7 to 103%) (Table 2.2). Improved accuracy in wastewater effluent and river water is most likely due to a reduction of matrix effects from fewer matrix components when compared to wastewater influent. (49) Whole-method accuracy obtained by LVI is improved compared to whole-method accuracy by online- and offline-SPE for the analysis of steroids in similar matrixes. (12, 22-25) The within-run precision of this method ranged from 1.2 to 8.9% for river water, 2.2 to 9.8% for effluent, and 1.2 to 8.8% for influent (Table 2.2). The within-run precision is comparable to those published for estrogens (12) and androgens (22, 24, 25) in similar matrixes.

2.4.4 Limit of Detection and Quantitation. The limit of detection for analytes ranged from 1.2 to 28 ng/L for river water, 4.8 to 150 ng/L for effluent, and 6.2 to 360 ng/L for influent (Table 2.2). Comparisons of LOD are difficult due to differences in analytes, matrixes, detectors, calculation, and unreported experimental details. However, the method presented here has comparable LODs to other studies when the masses of the analyte delivered to the detector are compared. For example, reported LODs for Test in wastewater influent are 1.0 pg, (22) 4.0 pg, (25) and 11 pg (current

study), and reported LODs for Andro in wastewater influent are 7.5 pg, (25) 11 pg (current study), and 13 pg. (22)

2.4.5 Storage Stability. Over the 60 day time period of the storage stability study, analyte concentrations plotted as a function of time yielded slopes that were not statistically different from zero ($p > 0.05$, at 95% CI) (50) for all analytes, with the exception of Test and Epi-Ox. This indicated that there was no degradation over 60 days for a majority of the analytes. Testosterone (p value = 0.01) and Epi-Ox (p value = 0.04) had a slight positive slope, which indicates either no degradation or formation of Test and Epi-Ox. The latter seems plausible for Epi-Ox since it is a metabolite of Ox, and the concentration of Ox decreased slightly, although not significantly (p value = 0.09). The storage stability study allowed for an analysis of the inter- and intra- day RSDs for the method applied to the wastewater influent. (Appendix 1).

2.4.6 Method Demonstration: Temporal Trends of Androgens in Wastewater Influent. Testosterone, Andro, and Bold were above detection in each 1 h composite at concentrations up to 45, 290, and 110 ng/L, respectively, over the 24 h sampling period. Nandrolone was detectable in seven samples at concentrations up to 70 ng/L (Figure 2.2). Analyte concentration values were multiplied by the hourly flow to calculate analyte loads (milligrams/hour) (Figure A.1.5 in Appendix 1).

Androstenedione is a direct precursor in the human endogenous production of Test; therefore, it is not surprising that Andro and Test were detected in every sample. A 1 day Test load of 1 023 ± 10 mg was computed by summing the individual 1 h loads

and propagating the uncertainty about their error (loads multiplied by within-run RSD) (Figure A.1.5 in Appendix 1). However, 1 023 mg is a conservative estimate given that Test may be present in the wastewater as conjugated species. The computed total load is in general agreement with an estimated load of 1 744 mg of Test calculated from the assumptions that, one, the average excretion of Test for males is 56.65 μg/day and 6.78 μg/day for females, (51) two, the WWTP served a static population of 55 000 with a 1:1 male to female ratio, and three, no analyte degradation. Data for ando excretion by humans is limited; (51) therefore, a similar comparison was not performed. However, Andro is six times more concentrated on average in the wastewater samples compared to Test, which is in agreement with trends reported previously for the two analytes in wastewater. (21, 22)

Boldenone was detected in all the 1 h composite influent samples over the 24 h study period with concentrations (Figure 2.2) and loads (Figure A.1.5 in Appendix 1) greater than Test. Boldenone was previously reported in 24 h flow-proportional influent samples at concentrations up to 2 419 ng/L. (24) Boldenone had temporal concentration and load profiles similar to Test and Andro, which was somewhat unexpected. Boldenone is a synthetic anabolic steroid of abuse, that can, in rare cases, be produced endogenously in humans. (26, 52) About 3 out of 10 000 doping control samples test positive for Bold. (26) It is hypothesized that microbes in the gut with 1,2-steroid dehydrogenase activity convert Test to Bold and are responsible for the endogenous excretion of Bold in humans. (26, 52, 53) It seems unlikely that the ubiquitous presence of Bold reported in this study is only from rare endogenous

production. Boldenone is also used in equine veterinary medicine (40, 41) but not commonly employed by local veterinarians, (54) so veterinary use was ruled out as a potential source. Boldenone is one of the most commonly abused anabolic steroids. (42-44) Although, it seems unlikely that illicit abuse alone is responsible for its widespread presence, since it is estimated that only 1% of the United States population abuse androgenic steroids. (44) A compound illicitly abused by only a small population of users is assumed to be intermittently excreted and contained in a few discrete number of wastewater pulses. (36) However, Bold is detected at all time points and has loads higher than endogenous Test (Figure A.1.6 in Appendix 1). This is a unique temporal trend that would have been missed without high temporal resolution sampling.

Hourly influent loads of Test and Bold are statistically correlated at 99% CI ($r = 0.94$, p value < 0.0001) (Figure A.1.7 in Appendix 1), which suggests that Bold loads are related to Test loads in wastewater. Therefore, we hypothesized that 1,2,-steroid dehydrogenase activity present in wastewater influent was converting Test to Bold in-situ. An experiment was carried out to Test this hypothesis by spiking D_3-Test in influent and monitoring for D_3-Bold production over time. A steady decrease was seen in D_3-Test concentrations over time with no corresponding increase in D_3-Bold (Figure A.1.8 in Appendix 1), which indicate that Bold is not a transformation product of Test.

In contrast, there was a significant (one sided t test, p value < 0.05) increase in the native concentration of Bold over 6 h (Figure A.1.8 in Appendix 1). An explanation for the rise in the concentration of Bold over time could be due to a gradual deconjugation of glucuronide and sulfate conjugated Bold in wastewater. Estrogens undergo deconjugation in wastewater, presumably due to Escherichia coli which produce glucuronidase and sulfatase enzymes. (6, 9, 11, 28) Further study is needed to elucidate if Bold is an in situ transformation product of a related compound, an endogenous human excretion product, from illicit use, from a potential unknown source, or from a combination of sources. Interestingly, Andro followed a similar trend with a significant (one sided t test, p value < 0.05) increase in concentration over 6 h (Figure A.1.8 in Appendix 1). Androstenedione is a biological oxidation product of Test in soils and biological waste (55-57) and could explain why concentrations of Andro increased over time during the experiment. This is supported by the fact that concentrations of Test, while below quantitation, decreased to below detection over 24 h.

Nandrolone was quantified in one sample and detected in six others (Figure 2.2). The occurrence of Nand, in this study, is likely from illicit and/or medical use. Nandrolone is widely abused as a doping agent to improve athletic performance and body image (42-44) and can be used medically as a treatment for anemia associated with renal insufficiency. (38) Although, Nand is a required metabolic intermediate in estrogen synthesis, (58, 59) it is not known to be endogenously excreted by humans. Further study needs be performed to elucidate its source. Detection of the trace levels of Nand

was made possible by the high frequency (samples taken every 6 min) and high resolution (1 h composites) sampling protocol. Pooling the influent samples into a single 24 h composite would have resulted in an estimated Nand concentration of 10 ng/L, which is below its LOD in wastewater influent (Table 2). Furthermore, a grab sample, or less frequent sampling, may have missed the Nand pulses completely.(36, 37) To the best of our knowledge, only one other study documents the detection of nand in wastewater (a single sample) at 1.7 ng/L, near their reported LOD for Nand (1.6 ng/L). (25)

2.4.7 Analysis of Solids. Centrifugation of wastewater samples results in a small solid pellet that is left in the bottom of the centrifuge tube (approximately 1 to 10 mg per 2.5 mL of wastewater). To the best of our knowledge, currently there is no work on the sorption of androgenic steroids to the particulate phase in wastewater and very little involving estrogens. (29, 30) However, previous research indicates that androgens sorb to solid organic matter in soil and sediment. (56, 60, 61) It is possible that the sorption of androgens to the solid phase is a potential source of analyte loss during wastewater analyses when centrifugation or filtration is used to remove the solid phase. To test this hypothesis, five solid samples from the 1 h composite influent samples were extracted as described above. Tesosterone was detectable in one sample but below the LOQ. Androstenedione was detected in every sample and quantified in two. The quantified Andro mass sorbed to the solids was 5 and 7% of the total andro mass. The calculated K_d (solid−water partition coefficient) values for quantified andro

concentrations were 31.5 and 46.3 L/kg, which fell within a range of reported K_d values of andro sorbing to soil. (56)

2.5 Conclusions

The LVI analytical method described above produces analyses of androgens in wastewater and environmental matrixes without the laborious and expensive sample cleanup and preconcentration steps associated with SPE. The method yields results that are precise and reproducible and that require only minor hardware modifications to commercially available LCs. LODs and LOQs are in the low nanaogram/liter range and are suitable for detection of androgens at environmentally relevant concentrations. Furthermore, application of this method to related compounds (estrogens) or matrixes (urine) would require only slight method modifications.

The method described here was used to analyze 24 wastewater influent samples taken as 1 h composites. This high-temporal resolution approach to sampling allowed for an analysis of analyte concentrations and loads over time. Four analytes of interest were detected: testosterone, androstenedione, boldenone, and nandrolone. Testosterone and androstenedione are endogenous compounds that were detected in all samples.

Without the use of high-temporal resolution sampling, the temporal trends in wastewater influent loads of boldenone could have been missed. Boldenone's ubiquity in the sample set is somewhat of an anomalous finding, considering it is a synthetic androgen of abuse and has loads that correlate strongly to testosterone loads. The study presented here ruled out in situ transformation of testosterone to boldenone as a

potential source of boldenone's ubiquity. Further investigation into the source of boldenone is needed.

The low and infrequent wastewater loads of nandrolone observed in this study may have been diluted below detection if a lower temporal resolution sampling strategy had been performed. The detection of nandrolone in wastewater was likely from its use either medically or illicitly.

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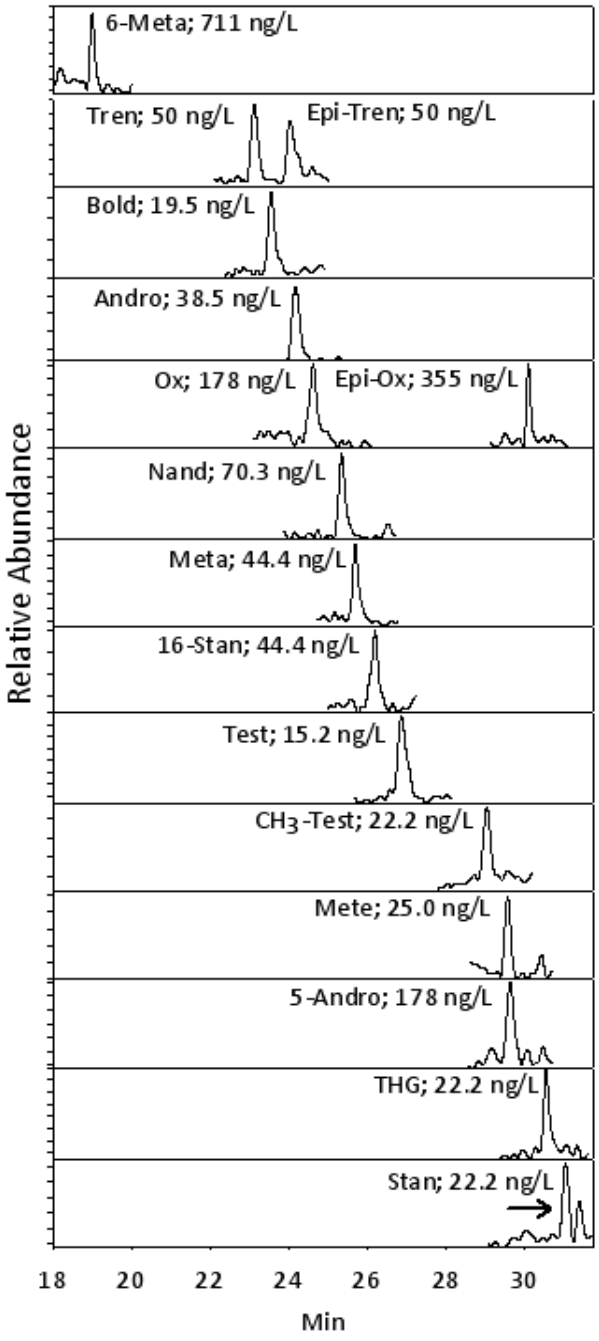

Figure 2.1) Chromatograms of analytes at or near their LOD in wastewater influent. Test, Andro,. Bold, and Nand are native signals.

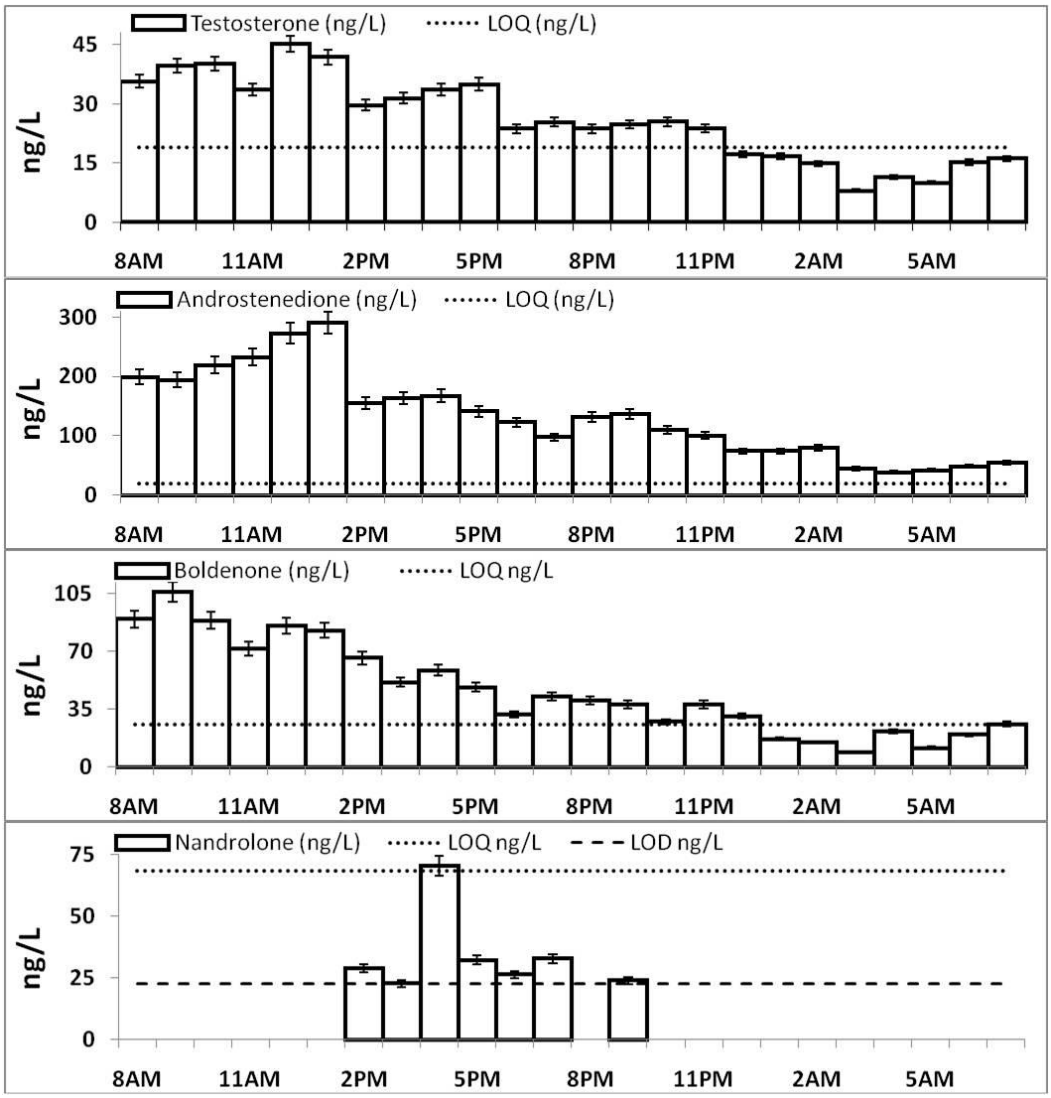

Figure 2.2) Diurnal profiles of analyte concentrations (± RSD) present in the one hour composite influent samples. The error bars are represented by concentration values (nanogram/liter) multiplied by the within run RSD.

Table 2.1) Analyte, precursor and product ions, compound-dependent mass spec parameters†, compound class* and internal standard used for quantification.

Analyte	Parent Ion (m/z)	Frag Ions (m/z)	C.E. (V)	C.X.P. (V)	D.P. (V)	F.P. (V)	Class	Internal Standard
Test	289	97 109	33 35	6 18	61	240	P	D_3 -Test
D_3 -Test	292	97 109	33 35	6 18	61	240	IS	NA
Andro	287	97 109	34 34	16 5	104	282	P	D_3 -Test
Bold	287	121 135	31 19	8 12	70	265	P	D_3-Bold
D_3-Bold	290	121 138	33 23	22 7	70	265	IS	NA
5-Andro	289	187 69	27 43	16 12	101	210	M_{Bold}	D_3-Bold
Meta	301	149 121	21 35	8 6	51	180	P	D_3 -Test
6-Meta	317	281 299	18 13	18 18	56	250	M_{Meta}	d_3-Bold
Stan	329	81 95	71 59	14 16	66	220	P	D_3-Stan
d_3-Stan	332	81 95	76 60	14 16	159	301	IS	NA
16-Stan	345	81 95	73 61	14 16	71	240	M_{Stan}	D_3-Stan
Tren	271	253 199	30 33	16 16	58	268	P	D_3-Bold
Epi-Tren	271	253 199	30 33	16 16	58	268	M_{Tren}	D_3-Bold
Mete	303	83 187	35 29	14 16	51	230	P	D_3-Bold
CH_3-Test	303	97 109	37 35	16 18	56	240	P	D_3 -Test
Nan	275	109 257	37 17	18 42	66	180	P	D_3-Bold
Tetra	313	295 241	21 31	20 14	126	344	P	D_3-Bold
Ox	307	289 271	17 19	24 26	86	340	P	D_3 -Test
Epi-Ox	307	289 271	17 19	24 26	86	340	M_{OX}	D_3-Bold

*P, Parent. IS, Internal Standard. M_x, Human Metabolite of compound X. † C.E. (collision energy), C.X.E. (cell exit potential), D.P. (declustering potential), F.P. (focusing potential).

Table 2.2) Whole-method accuracy, determined for spiked concentrations ([Spike]), and relative standard deviation (RSD) outline the method performance in each matrix. Limit of detection (LOD) and quantitation (LOQ) values for analytes in each matrix.

	Waste Water Influent				
Analyte	**Accuracy* (%)**	**[Analyte] (ng/L)**	**RSD† (%)**	**LOD (ng/L)**	**LOQ (ng/L)**
Test	108 ± 5.5	90	4.5	6.2	19
Ando	87.6 ± 6.2	60	6.3	6.2	19
Bold	98.4 ± 6.3	60	5.7	8.5	26
5-Andro	78.6 ± 7.8	1600	8.8	120	360
Meta	108 ± 8.9	200	7.3	18	54
6-Meta	62.3 ± 3.9	1600	5.6	360	1100
Stan	107 ± 7.6	200	6.4	11	33
16-Stan	101 ± 5.7	200	5.1	19	58
Tren	96.6 ± 5.2	200	5.6	28	85
Epi Tren	93.9 ± 2.2	200	2.1	31	95
Mete	94.3 ± 8.5	200	8.1	22	67
CH_3-Test	102 ±2.9	200	2.5	13	40
Nand	104 ± 8.3	200	7.2	23	68
THG	102 ± 9.9	200	8.7	8.1	24
Ox	103 ± 1.4	750	1.2	100	310
Epi-Ox	84.8 ± 7.2	1600	7.6	210	620

*Accuracy is calculated as the average (n = 4, ± 95% CI) percent calculated internal standard calibration concentration over the spiked concentration ([Spike]). † Relative standard deviation was calculated from concentration values used to determine accuracy (n = 4).

Table 2.2) (Continued)

Waste Water Effluent					
Analyte	**Accuracy* (%)**	**[Analyte] (ng/L)**	**RSD† (%)**	**LOD (ng/L)**	**LOQ (ng/L)**
Test	101 ± 2.5	75	2.2	4.8	15
Ando	101 ± 5.1	75	4.6	5.0	15
Bold	101 ± 3.8	75	3.4	6.2	19
5-Andro	86 ± 3.5	2000	3.7	100	300
Meta	104 ± 2.5	250	3.7	13	41
6-Meta	76.4 ± 8.4	2000	9.8	150	470
Stan	107 ± 3.3	250	2.8	17	52
16-Stan	103 ± 4.2	250	3.6	17	76
Tren	96.3 ± 6.9	250	6.3	19	57
Epi Tren	100 ± 5.0	250	4.5	20	62
Mete	96.3 ± 6.0	250	5.6	20	60
CH_3-Test	102 ± 4.3	250	3.7	7.9	24
Nand	96.8 ± 3.7	250	3.4	19	57
THG	97.6 ± 7.0	250	6.4	8.0	24
Ox	102 ± 4.8	1000	4.2	82	250
Epi-Ox	93.4 ± 9.6	2000	9.2	120	360

*Accuracy is calculated as the average (n = 4, ± 95% CI) percent calculated internal standard calibration concentration over the spiked concentration ([Spike]). † Relative standard deviation was calculated from concentration values used to determine accuracy (n = 4).

Table 2.2) (Continued)

Willamette River Water					
Analyte	**Accuracy* (%)**	**[Analyte] (ng/L)**	**RSD† (%)**	**LOD (ng/L)**	**LOQ (ng/L)**
Test	99.2 ± 6.3	10	5.7	1.2	3.7
Ando	106 ± 3.4	15	2.9	1.5	4.5
Bold	108 ± 5.0	10	4.1	2.3	7.1
5-Andro	95.7 ± 3.8	89	3.5	12	35
Meta	105 ± 6.3	22	3.3	3.8	12
6-Meta	80.7 ± 7.5	89	8.2	28	83
Stan	103 ± 4.6	22	4.0	3.2	9.8
16-Stan	104 ± 4.2	22	3.6	3.3	10
Tren	93.9 ± 5.1	22	4.8	3.7	11
Epi Tren	98.2 ± 7.0	22	6.4	4.4	13
Mete	105 ± 8.4	22	7.1	4.3	13
CH_3-Test	99.5 ± 4.8	22	4.3	1.8	5.3
Nand	101 ± 2.7	22	2.4	3.2	9.8
THG	105 ± 2.3	22	1.9	1.8	5.6
Ox	107 ± 8.1	89	6.8	19	58
Epi-Ox	103 ± 6.8	89	5.9	18	54

*Accuracy is calculated as the average (n = 4, ± 95% CI) percent calculated internal standard calibration concentration over the spiked concentration ([Spike]). † Relative standard deviation was calculated from concentration values used to determine accuracy (n = 4).

Chapter 3 - Is SPE Necessary for Environmental Analysis? A Quantitative Comparison of Matrix Effects from Large-Volume Injection and Solid-Phase Extraction Based Methods.

Will J. Backe and Jennifer A. Field

Environmental Science and Technology

1155 16th St. NW

Washington, DC 20036

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3.1 Abstract

Environmental analysis by large-volume injection (LVI) was compared to solid-phase extraction (SPE) based methods using matrix effects as a quantitative indicator of analytical signal quality. LVI was performed by the direct injection of 900 μL of wastewater onto an high-performance liquid chromatography (HPLC) column while SPE-based methods utilized octadecyl silane (C18) and hydrophobic-lypophilic balance (HLB) solid phases to pre-concentrate wastewater prior to analysis. Model analytes from three classes of environmental contaminants were selected for study including four estrogens (estrone, estradiol, estriol, and ethinylestradiol), eight perfluoroalkyl carboxylates (C4-C11), and five perfluoroalkyl sulfonates (C4, C6-C8, & C10). The matrix effects on analytes were assessed by two approaches (quantitatively by calculating percent matrix effects and qualitatively with post-column infusions) and compared across LVI- and SPE-based methods at constant (high and low) analyte-to-matrix mass ratios. The results from this study demonstrated that the LVI-based method produced analytical signals of similar quality to the two SPE-based methods. Furthermore, LVI presented a clear advantage over SPE because it was performed at lower cost, required fewer materials, involved less labor, and eliminated the analyte loss associated with SPE.

3.2 Introduction

Large-volume injection (LVI) is an analytical technique that is performed by the direct-injection of a large sample volume (e.g. > 10% of the analytical column void

volume) onto a high-performance liquid chromatography (HPLC) column. LVI requires only minimal sample pre-treatment such as centrifugation (1-5) or filtration, (6-12) which reduces costs when compared to those associated with extraction materials, extraction solvents, and labor. Furthermore, because samples are injected directly onto the analytical column, the risk of analyte loss is diminished. (1-4, 6-10) Large-volume injection is employed in a number of trace analytical methods including those for isothiazolinones in shampoo and in drinking, surface, and wastewater; (11) illicit drugs in wastewater; (1, 6) acrylamide in surface and groundwater; (5, 12) fluorochemicals in waste and groundwater; (2, 3) androgenic steroids in waste and surface water; (4) pesticides in surface water; (7, 10) and surfactants in seawater. (13) However, because LVI involves minimal sample clean-up it is perceived as a "dirtier" analysis technique that is thought to cause instrument fouling, decreased column lifetimes, and matrix effects. (13) In an effort to broaden the acceptance of LVI as a cost-effective analytical tool, quantitative data are needed to determine how LVI compares with established analytical techniques.

Solid-phase extraction (SPE) is one such established analytical technique that dates back to the 1970s, (14) and is still the current convention for pre-concentrating analytes in complex environmental matrices (13, 15-18) Despite its history and perceived advantages, SPE suffers from drawbacks including it is laborious; it generates liquid and solid waste; it requires additional hardware; and it has the potential for analyte loss due to incomplete elution, breakthrough, and volatilization during solvent exchange. (13, 14) Alternatively, online SPE-based methods gained

popularity in environmental analysis because they reduced the labor associated with offline SPE. However, online-SPE requires the addition of extra equipment modules and parts which must be interfaced with the HPLC such as high-pressure pumps, switching valves, and columns. (19)

Matrix effects present challenges for HPLC tandem mass spectrometry (MS/MS) interfaced with electrospray ionization (ESI), which is considered a gold standard for environmental analysis. Matrix effects arise from the compounds of the sample matrix that coelute with analytes during HPLC and suppress or enhance their ionization. These ionization interferences can cause decreased sensitivity, selectivity, reproducibility, and produce erroneous results. (18, 20) The magnitude of matrix effects are influenced by analyte and matrix properties such as proton affinity, surface activity, polarity, as well as the ratio of analyte to matrix mass (21-24) and instrument parameters including the desolvation temperature and ionization polarity of the mass spectrometer and the flow rate of the HPLC. (25-27) However, the mechanisms by which matrix effects occur in the ESI source are still not fully understood. (18, 20) Although ESI is reported to be more prone to matrix effects than atmospheric-pressure chemical ionization, (28-31) ESI is the most commonly used interface due to its soft ionization and its amenability to polar and ionized analytes. (20, 32)

Matrix effects are reported for both LVI- and SPE-based methods. (1, 18) However, SPE is reported to contribute additional matrix effects, (6, 33) which may originate from plasticizers or other contaminates leaching from SPE cartridges into the sample.

(34) For example, urine directly analyzed by HPLC MS/MS produced less signal suppression on morphine compared to urine pre-concentrated by SPE. (30) Others hypothesize matrix effects from SPE occur because SPE co-concentrates analytes with the matrix components that co-elute during HPLC separations. (18, 30) Coconcentration followed by coelution is likely because many SPE-based methods used for environmental samples employ separation chemistries (e.g. sorbent phases and elution solvents) that are similar, or identical, to those used in subsequent HPLC separations. (35-41) Any attempt to compare matrix effects from LVI and SPE must keep the ratio of analyte-to-matrix mass constant between raw samples (LVI) and extracts (SPE) so a direct comparison can be made. To the authors' knowledge such a comparison has not been made and is needed to compare the perceived advantages provided by both LVI and SPE.

The objective of the current study was to quantify and compare the matrix effects from LVI and SPE using methods for LVI and SPE similar to what is reported for the analysis of well-studied environmental contaminants. The hypothesis tested is that LVI is a cost- and time-effective alternative to SPE and produces data of similar quality for aqueous environmental analysis. Matrix effects were evaluated on seventeen analytes from three compound classes including four estrogens, eight perfluoroalkyl carboxylates, and five perfluoroalkyl sulfonates. These three compound classes were selected because they represent both non-ionic and ionic compounds that are routinely measured by HPLC-ESI-MS/MS in environmental samples. Furthermore, perfluoroalkyl carboxylates and sulfonates are both a homologues series,

which allows for the matrix effects on analytes of increasing hydrophobicity to be quantified across a wide range of elution times. Wastewater influent was used as a model matrix because it is complex and causes matrix effects during HPLC-MS/MS analyses. (31, 36) Analytes were spiked into wastewater for LVI analysis and into SPE extracts at equivalent analyte-to-matrix mass ratios so that direct comparisons between the two approaches could be made. To eliminate confounding factors including incomplete recovery during SPE and the loss of analyte mass to sorption on suspended particles, analytes were spiked in SPE extracts post-extraction and after centrifugation for analysis using LVI. As this is not a method validation study, analytical parameters describing detection limits and recovery were not included; parameters for methods similar to the ones presented can be found elsewhere. (2, 4, 13, 35, 39, 42-48)

3.3 Experimental Section

2.3.1 Chemicals. Analyte and reagent source and purity are presented in Appendix 2. Compound classes and acronyms for the analytes tested are as follows: Estrogens [estrone (E1), estradiol (E2), estriol (E3), and ethinylestradiol (EE2)]. Perfluoroalkyl carboxylates [perfluro-n-butanoic acid (PFBA), perfluoro-n-pentanoic acid (PFPeA), perfluoro-n-hexanoic acid (PFHxA), perfluoro-n-heptanoic acid (PFHpA), perfluoro-n-octanoic acid (PFOA), perfluoro-n-nonanoic acid (PFNA), perfluoro-n-decanoic acid (PFDA), perfluoro-n-undecanoic acid (PFUdA)]. Perfluoroalkyl sulfonates [perfluoro-1-butanesulfonate (PFBS), perfluoro-1-hexanesulfonate (PFHxS),

perfluoro-1-heptanesulfonate (PFHpS), perfluoro-1-octanesulfonate (PFOS), and perfluoro-1-decanesulfonate (PFDS)].

2.3.2 Sample Collection and Treatment. A 1.5 L grab sample of wastewater influent (raw sewage) was collected from a wastewater treatment facility in the Pacific Northwest in 500 mL high-density polyethylene (HDPE) bottles that were solvent-washed (Methanol and Acetone). The wastewater was then filtered in batches through 1 μm borosilicate glass microfiber filters (Whatman plc, Maidstone, Kent, UK), combined, and used for all SPE and LVI experiments.

2.3.3 Large-Volume Injection. Filtered wastewater for LVI analysis was centrifuged at 8154 RCF in a 5415 C microcentrifuge (Eppendorf, Hauppauge, NY) for 15 min to eliminate any particles that might otherwise clog HPLC capillaries. Four samples of unspiked wastewater were analyzed by LVI to quantify the area counts from analyte background. Six additional aliquots (1,500 μL) of the same wastewater were then spiked to yield a final concentration of 1 μg/L (low) or 100 μg/L (high) of each estrogen and 0.01 μg/L (low) or 1 μg/L (high) of each perfluoroalkyl carboxylate and sulfonate. The concentration of the low LVI estrogen spike level is generally two fold greater than the maximum estrogen concentrations in wastewater influent observed by Schlüsener et al. (0.47 μL to 0.51 μL). (31, 49) Higher concentrations were used to minimize the variance introduced by integrating smaller peaks. Standards at the low and high concentration levels were prepared in triplicate in 3% methanol in 1 mM ammonium acetate reagent water. Injecting 900 μL of spiked wastewater by LVI

yields a mass of analyte on the column of 9 ng (low) and 900 ng (high) of each estrogen and 0.009 ng (low) or 0.9 ng (high) of each perfluoroalkyl carboxylate and sulfonate. Standards and samples were analyzed by LVI within 1 hr of analyte addition and were prepared in polypropylene vials, as glass vials have been shown to cause a loss of perfluorinated surfactants due to sorption. (50)

2.3.4 Solid-Phase Extraction. The overall approach was to prepare SPE extracts that had an equivalent analyte-to-matrix mass ratio as spiked wastewater analyzed by LVI. To achieve an equivalent analyte-to-matrix mass, analytes spiked into SPE extracts had to be at a 50-fold higher concentration than analytes spiked into wastewater because SPE extracts were wastewater concentrated 50-fold (See Below).

Solid-phase extraction was performed using octadecyl silane (C18) (Agilent, Santa Clara, CA) and hydrophobic-lipophilic balance (HLB) (Waters, Milford, MA) SPE cartridges on a Supelco 12-position manifold. Originally, the SPE manifold's flow-control valves contained PTFE but for this study they were replaced with flow-control valves containing polyoxymethylene. Both types of SPE cartridges had similar physical properties; each contained 0.5 g of solid phase with an average particle diameter of 47-65 μm. Average pore diameters for both phases were between 60 and 89 Å.

The method for performing SPE was the same for both SPE phases and is similar (eg. mass of solid phase, eluent strength, solvent volumes used, etc.) to those reported for the analysis of fluorochemicals and estrogens in wastewater and surface water. (35-37,

41, 43-45, 48, 51-53) Cartridges were cleaned and conditioned with 4 X 2.5 mL of methanol, followed by 4 X 2.5 mL of reagent water. A 50 mL aliquot of filtered (un-spiked) wastewater was then loaded onto each SPE cartridge at a flow rate of 1-2 drops per second. The cartridges were then washed with 3 X 2.5 mL of 20 % methanol in reagent water, after which they were allowed to dry under vacuum (10 in of Hg) for 45 min. Finally, the cartridges were eluted with 7.5 mL of methanol and the eluate was collected. The eluate was then reduced in volume to approximately 200-300 μL under a gentle nitrogen stream in a water bath at 40^{o}C using a Turbo Vap LV (Caliper Life Sciences, Hopkinton, MA) and reconstituted to 1 mL with reagent water. A precipitate was observed in the samples upon reconstitution so each extract was centrifuged for 5 min at 8154 RCF to remove any suspended precipitate from solution. Eight of these extracts (un-spiked) from both C18 SPE (n = 4) and HLB SPE (n = 4) were used to quantify background analyte area counts.

SPE extracts were then spiked in triplicate to give a final concentration of either 50 μg/L (low) or 5000 μg/L (high) of each estrogen and either 0.5 μg/L (low) or 50 μg/L (high) of each perfluoroalkyl carboxylate and sulfonate. SPE standards (high and low) were prepared in triplicate by spiking analytes into 3% methanol in 1 mM ammonium acetate reagent water to a final concentration equivalent to those analytes in SPE extracts. Injecting 18 μL of a spiked SPE extract yielded an equivalent analyte mass on column as 900 μL of wastewater injected by LVI (see above). All SPE extracts and their standards were stored in polypropylene vials and analyzed within 1 hr of analyte spike addition. Solvent blanks did not reveal any matrix interferences leaching from

polypropylene, and analyte loss was not observed for either SPE- or LVI-based analyses.

2.3.5 Liquid Chromatography Tandem Mass Spectrometry. An Agilent series 1100 HPLC (Santa Clara, CA) set up to perform LVI as previously described (4) was used in this study. The system was modified with a 4.6 x 150 mm 3.5 μm particle diameter ZORBAX Eclipse Plus-C18 analytical column that was placed after the solvent mixing valve to separate systemic perfluoroalkyl carboxylate contamination from injected analytical signals (Figure 3.1). (54) The PTFE solvent line from the MeOH solvent bottle to the degasser was replaced with PEEK tubing. The Vespel injection valve rotor seal (Agilent part no. 0101-0623) was replaced with one made of PEEK (Agilent part no. 0101-1255) which is reported to be less sorptive of hydrophobic analytes. (55) An 18 μL sample loop was created by joining a 2.3 μL needle seat capillary (Agilent part no. G1313-87101) with a 15.7 μL stainless steel capillary (Agilent part no. 79835-87638).

Analytical separations were performed with a ZORBAX Eclipse Plus-C18 guard column (4.6 x 5 mm 5 μm particle diameter) in tandem with a 75 x 3.5 mm 3.5 μm particle diameter ZORBAX Eclipse Plus-C18 analytical column. For SPE extracts, injection volumes of 18 μL were performed by injecting 54 μL (3x overfill) of sample through the 18 μL sample loop (excess sample goes to waste) to ensure accurate and reproducible sample delivery. Large-volume injections were performed by drawing 900 μL into the 900 μL needle loop and initiating the sample run. The HPLC was

designed to accurately draw and inject large volumes (900 μL) of sample so a loop overfill was not required.

The mobile phase consisted of 1mM ammonium acetate in reagent water (A) and 100 % methanol (B). The gradient profile was convex with respect to mobile phase B (Figure A.2.1 in Appendix 2). The convex gradient produced well resolved and evenly spaced peaks in the chromatogram (Figure 3.1). The flow rate was 0.35 mL/min for 31.5 min and then increased to 0.6 mL/min over 0.5 min and held for 8 min to reequilibrated the column (Figure A.2.1 in Appendix 2). For 18 uL injections, a valve directed the mobile phase flow past the injection assembly (needle loop + needle + sample loop + injection valve) at 1.5 min post injection to reduce gradient dwell time. After 1.5 min post injection, the injection assembly was washed with 900 μL of 66 % MeOH in reagent water brought to pH 12 with NaOH and then washed with 1,800 μL of 3% methanol in 1 mM ammonium acetate reagent water to eliminate analyte carryover (E1 and > C8 perfluoroalkyl carbolxylates and sulfonates). Chromatographic conditions were similar for 900 μL injections, except that for 900 μL injections the initial gradient composition was held for 2 min to load the sample and the injection valve redirected the mobile phase flow around the injection assembly at 5.1 min after injection. Analytical column eluate was diverted to waste for the first 8 min after injection to protect the mass spectrometer from involitile salts for both 18 μL and 900 μL injections.

Analytes were detected with a TQ Detector (Waters Corporation, Milford, MA) triple quadrupole mass spectrometer with an ESI source controlled via MassLynx (Version 4.1). Sample acquisitions were performed by multiple-reaction monitoring using a negative ionization potential. Mass spectrometer parameters were as follows: a capillary potential of -2,800 V, an extractor potential -3 V, a source and desolvation temperature of 150 and 450 ^{o}C respectively, and a desolvation gas and cone gas flow of 1,100 and 75 L/hr respectively. The precursor and product ions used in this study are consistent with previously published literature (Table A.2.1 in Appendix 2). (56, 57) Quantitative data for PFOS was acquired from the transition, 499 > 99, which is less intense than the 499 > 80 transition but is less affected by signal interferences from samples. (36, 43)

2.3.6 Quantification of Matrix Effects. Matrix effects (% ME) were quantified by subtracting background analyte area counts (Table 1) from those detected in spiked wastewater (LVI) and spiked SPE extracts, dividing by the area counts of analytes in standards, and multiplying by 100 (Equation 1). (32, 58) The error for each measurement was calculated at the 95% confidence interval (CI) and propagated to determine the error about the computed % ME. Internal/surrogate standards were not used in this study to because they compensate for matrix effects.

(Equation 1)

2.3.7 Assessment of Matrix Effects by Post-Column Infusions. The compounds E2, PFDA, and PFHpS were not detected in un-spiked wastewater samples (Table 3.1) and

they also represent analytes from each compound class. For those reasons they were selected to qualitatively examine matrix effects by the post-column infusion method as described by Bonfiglio et al. (59) Briefly, concentrations of 500 µg/L of E2 and 5 µg/L of PFDA and PFHpS made in MeOH were infused into the column eluate flow (between the analytical column and mass spectrometer) at a rate of 10 µL/min, while the HPLC performed analytical separations on un-spiked C18 and HLB extracts (18 µL injection) and un-spiked wastewater samples (900 µL injection by LVI).

2.4 Results and Discussion

2.4.1 Background Analyte Area Counts. Ten of the 17 analytes investigated in this study were detected in unspiked wastewater influent (LVI) and in unspiked HLB SPE extracts (Table 1). It is not surprising to find these analytes as they are frequently present in wastewater and surface water. (2, 35, 42, 43) In contrast, only 8 of the 17 analytes were detected in C18 SPE extracts (Table 3.1). The absence of PFBS and PFPeA and the low area counts of PFHxA in C18 extracts are attributed to their breakthrough during sample loading and/or elution during the wash step. Many studies report that short-chain perfluoroalkyl carboxylates and sulfonates are either unretained or weakly retained on C18 and HLB SPE phases, yielding low analyte recovery. (42, 43, 45, 60) In one instance, it was reported that the C18-based method was unsuitable for perfluoroalkyl chemicals with carbon backbones of less than six. (45) The breakthrough and loss of short-chain perfluorinated analytes reveals one of the potential weaknesses of sample preconcentration by SPE. (42, 43, 45, 60) In contrast,

previous work confirms that short-chain perfluorinated analytes can be quantitatively analyzed by LVI. (2, 3)

2.4.2 Quantitative Comparison of Matrix Effects. Percent matrix effects are a quantitative measure of signal suppression or enhancement. A value of 100% indicates no sample matrix effects, while values below and above 100% indicate signal (ionization) suppression and enhancement, respectively. (18) The matrix effects on eight of the 17 analytes at the high analyte-to-matrix mass ratio were statistically equivalent at the 95% CI (confidence interval) in wastewater (LVI) and in both C18 and HLB SPE extracts (Figure 3.2 (A), Table A.2.2 in Appendix 2). These eight analytes span three compound classes and demonstrate that, for both nonionic and homologous ionic LVI results in analytical signal quality equivalent to that from C18 and HLB SPE.

An additional 3 of the 17 analytes (E1, E2, and E3) exhibit statistically equivalent signal suppression at the 95% CI in wastewater analyzed by LVI and in HLB extracts (Figure 3.2 (A), Table A.2.2 in Appendix 2). For E1, E2, and E3, LVI produced matrix effects statistically different from C18 extracts; however, the calculated difference was only 4−8%. Signal enhancement of two other analytes (PFOA and PFNA) and suppression of one (PFOS) were statistically equivalent at the 95% CI in wastewater analyzed by LVI and in C18 extracts (Figure 1A, Table S2). For PFOA, PFNA, and PFOS, analysis by LVI and extraction by HLB SPE produced statistically different matrix effects. However, the difference in matrix effects was minor (5−9%).

Only three analytes (PFBA, PFDA, and PFHpS) at the high analyte-to-matrix mass ratio had matrix effects from LVI that were statistically different from the matrix effects of C18 and HLB extracts (Figure 3.2 (A), Table A.2.2 in Appendix 2). However, the PFHpS signal was improved (less suppressed) when analyzed by LVI, (96% ± 2.4%) compared to C18 SPE (86% ± 3.8%) and HLB SPE (88% ± 1.5%). For PFDA, although the matrix effects were statistically different between LVI and both types of SPE, the differences were small (107% vs 96% and 99%).

Although PFBA was suppressed at a much greater magnitude in wastewater analyzed using LVI than in both types of SPE extracts (Figure 3.2 (A), Table A.2.2 in Appendix 2), it is not retained during C18 and HLB SPE. (42) Therefore, it is important to note that the matrix components that coelute with PFBA during HPLC are also not retained during SPE and, therefore, are not present in the extracts. Whereas C18 and HLB SPE resulted in lower matrix effects for PFBA, this finding is an artifact resulting from the postextraction addition of PFBA into extracts. This experimental artifact is also observed at the low analyte-to-matrix mass ratio for PFPeA, PFHxA, and PFBS, all of which were either unretained or marginally retained by C18-based SPE (Table 3.1). However, this difference is only significant (95% CI) for PFPeA.

At the low analyte-to-matrix mass ratio, the matrix effects on 14 of the 17 analytes were statistically the same between raw wastewater analyzed by LVI and both types of SPE extracts (Figure 3.2 (B), Table A.2.2 in Appendix 2). The 14 analytes include two estrogens (E3 and EE2), all the perfluoroalkyl acids except PFPeA (discussed earlier),

and all the perfluoroalkyl sulfonates. The larger numbers of analytes that do not display statistical difference in matrix effects for LVI and C18 and HLB at the low analyte-to-matrix ratio are due, in part, to the increased uncertainty about measurements at low analyte concentrations. However, these data agree with the data at the high analyte-to-matrix ratio and reinforce the conclusion that C18 and HLB SPE do not reduce matrix effects over direct analysis by LVI. Signals of both E1 and E2 were improved (less suppressed) in wastewater analyzed by LVI (E1 = 47% ± 3.0% and E2 = 37% ± 2.6%) compared those in C18 (E1 = 39% ± 2.3% and E2 = 28% ± 2.0%) and HLB SPE extracts (E1 = 38% ± 1.5% and E2 = 23% ± 2.0%) (Figure 1B, Table S2). The increase in matrix effects on E1 and E2 in SPE extracts observed here supports the conclusion that SPE can cause additional matrix effects. (30, 33)

Matrix effects were not reduced by SPE because the C18 and HLB SPE phases coconcentrated analytes and matrix components by the same hydrophobic-partitioning mechanisms that result in separations on a reverse-phase (e.g., C18) analytical column. Therefore, performing C18 or HLB SPE is “chemically redundant” with subsequent reverse-phase analytical separations because the same separation chemistry is performed twice. Although HLB has other retention mechanisms, such as pi−pi interactions, (61) these act only to increase the retention of additional chemicals that might normally break through during C18-based SPE. The use of SPE phases that are orthogonal (e.g., normal phase, immunoaffinity, etc.) to reverse-phase HPLC separations, such as for the analysis of estrogens in organic extracts, are reported to reduce matrix effects. (62) However, the use of orthogonal SPE phases is not

commonly reported in environmental analytical methods. Research is currently being completed that adapts the advantages of orthogonal separations with LVI.

To the best of our knowledge, environmental analysis of estrogens has not been performed by LVI before. In contrast, methods based on C18 and HLB SPE are the standard for estrogen analysis. (46) Previous work by our lab demonstrated that structurally similar androgens can be quantitatively determined by LVI (4) and the present work demonstrates that quantitative evaluation of estrogens in complex matrices is also possible by LVI. The degree to which estrogens were subject to matrix effects in this study was dependent on analyte-to-matrix mass ratio. As the amount of estrogen mass increased there was a decrease in magnitude of the matrix effects on the estrogen signals (Figure 3.2, Table A.2.2 in Appendix 2). This same trend also was observed by others for the analysis of clenbuterol in urine. (24) While the purpose of this study was not to specifically test the effect of ionization polarity on matrix effects, an analysis of published studies that report on androgenic steroids (compounds that ionize in positive polarity) in wastewater shows that matrix effects are similar between LVI- and SPE-based methods (Table A.2.3 in Appendix 2).

Perfluoroalkyl carboxylates and sulfonates were less affected by the sample matrix than estrogens, regardless if the sample was directly injected or concentrated by SPE before analysis. A proposed reason behind the reduced matrix effects of perfluoroalkyl carboxylates and sulfonates reported here and elsewhere (54) may be due to their surface active properties. During ESI surfactant ions, such as perfluoroalkyl

carboxylates and sulfonates, are thought to be enriched at the droplet surface, leading to their preferential transfer to smaller droplets and finally to the gas phase. (63, 64) In contrast, estrogens are nonsurface active compounds, therefore their ionization during electrospray is more likely to be subject to modification by coeluting matrix components. However, it is possible that there are also other factors that influence the sensitivity of perfluorinated chemicals and estrogens to matrix effects duringionization.

2.4.3 Postcolumn Infusions. The matrix effects for three analytes (E2, PFDA, and PFHpS) were assessed by postcolumn infusion. Postcolumn infusion offers a qualitative assessment of the magnitude and presence of the matrix components that cause signal interferences across a chromatogram. The chromatographic traces from the postcolumn infusion of E2 reveal a suppression of the 271 > 145 signal from wastewater samples injected by LVI and from injections of SPE extracts occurring after 13 min (Figure 3.3). Interestingly, there are only minor differences in the E2 traces between LVI and both types of SPE. This finding indicates that not only at the point of E2 elution, but across the entire chromatogram, matrix effects are not reduced by C18- and HLB-based SPE, which reinforces the quantitative results presented earlier.

As with the postcolumn infusion of E2, there is little difference between LVI and SPE on the signal of PFDA during postcolumn infusions (Figure 3.3). An enhancement of the PFDA signal was observed from 15 to 32 min (Figure 3.3), which is consistent

with the signal enhancement observed from the LVI-based analysis of wastewater at the high analyte-to-mass ratio (Figure 3.2 (A), Table A.2.2 in Appendix 2). Additionally, the signal of PFDA appeared to be enhanced in both types of SPE extracts and in wastewater (LVI) at the low analyte-to-mass ratio; however, it was not statistically significant (Figure 3.2 (B), Table A.2.2 in Appendix 2). Signal enhancement of PFDA also was reported for SPE extracts of soils and sludge. (54) In contrast, Sinclair et al. reported signal suppression for PFDA in SPE extracts of wastewater. (36) The difference in the results presented here from from those of Sinclair et al. may be due to the heterogeneity of wastewater matrices. (65) Finally, the results from PFHpS postcolumn infusions further indicate that perfluoroalkyl carboxylates and sulfonates are subject to minor matrix effects regardless of whether LVI or SPE is used (Figure 3.3).

2.5 Practical Implications and Future Directions.

Once data quality obtained by LVI are found comparable to that of SPE, a critical examination of LVI reveals additional practical benefits. For example, the time needed to prepare a sample for LVI in this study was less than 30 min, while the preparation of SPE extracts required several hours. Unless SPE is automated by additional, often expensive, equipment, the labor costs associated with SPE are significant whereas the equivalent analyte concentration was performed by LVI during the first 2 min of sample loading in this study. The additional materials (e.g., SPE cartridges, solvents, nitrogen, etc) that are needed for sample preparation makes SPE-based methods

inherently expensive. Additionally, column lifetimes are not reduced by LVI as indicated by a contract laboratory (ISO 17025 accredited) that performs LVI with column lifetimes of 3 months and 2800−3700 injections per column, (13) which is similar to those reported in a survey of HPLC users. (66) Guard columns that are used for 50−100 injections/samples in LVI provide the same function as SPE (1 cartridge per sample) in protecting analytical columns from matrix compounds that irreversibly adsorb and deteriorate performance. (4)

Direct injection of sample by LVI is a flexible technique because the volume of sample injected can be easily "tuned" to achieve the same analyte concentration factor used by SPE to match the sensitivity of a detector. Simply put, less volume is injected for more sensitive detectors. Ultra-high-pressure LC (UPLC) offers increased sensitivity over conventional HPLC because analytes are eluted in smaller volumes that result in narrower taller peaks. Interfacing LVI with UPLC would result in increased sensitivity, higher throughput, and the use of less solvent when compared with LVI using HPLC; however, employing LVI with UPLCs has yet to be fully explored for environmental analyses. (13)

Another practical implication of LVI is the elimination of sample hold times. SPE serves to stabilize analytes in samples which allows samples to be held for a long period of time before analysis. The elimination of SPE will require that alternative sample storage procedures be established such as freezing or acidifying to increase hold times. (1, 4) For SPE, it is common to adjust sample pH outside of the normal

operating range of analytical columns (pH 2−9) to facilitate analyte retention. The same pH adjustment would need to be made for LVI-based methods. Analytical columns designed for pH conditions outside pH 2−9, such as double end-capped columns, should be explored for LVI. Alternatively, pH adjustments are commonly combined with liquid−liquid and liquid−solid extractions for the analysis of analytes in biological samples, soils, sediments, and other particulate phases. (4, 30, 59, 60) These organic extracts are incompatible with reverse-phase LVI due to the low breakthrough volumes of analytes in solvents of high elutopic strength. However, using two-dimensional (e.g., orthogonal) LVI with normal-phase guard columns in line with reverse-phase analytical columns is under development for analytes in organic liquid−liquid extracts of acidified ground-water and landfill leachates.

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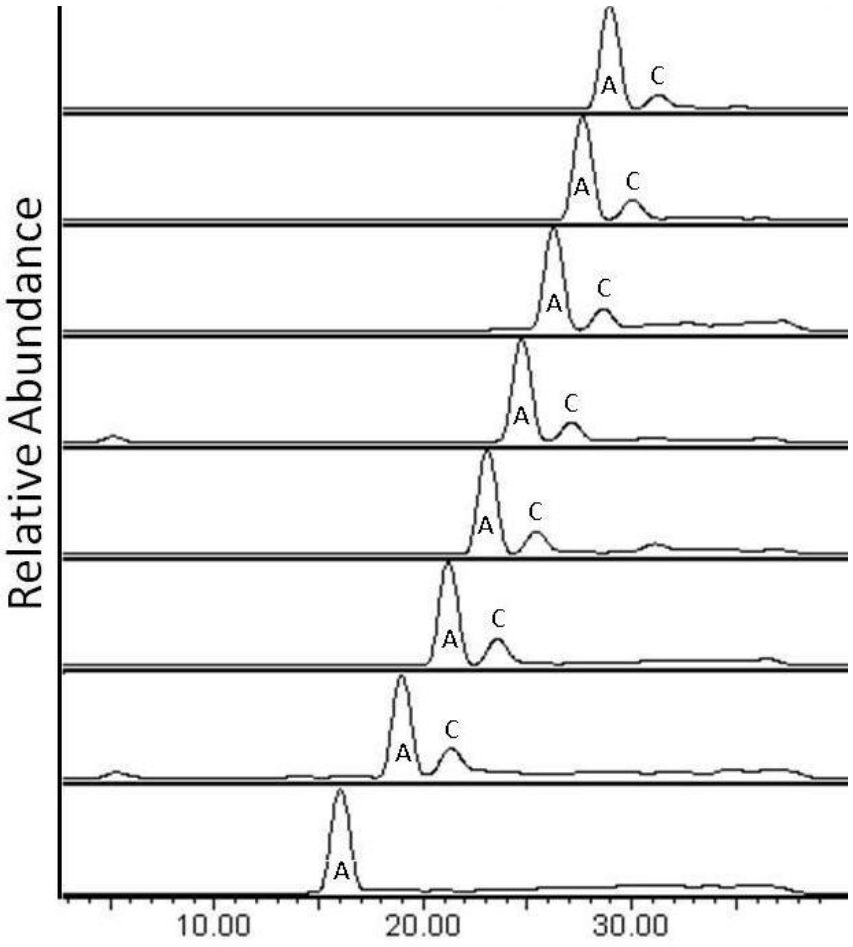

Figure 3.1) Chromatograms of analyte (A) and contamination (C) peaks of perfluoroalkyl carboxylates, from C4 (bottom) to C11 (top).

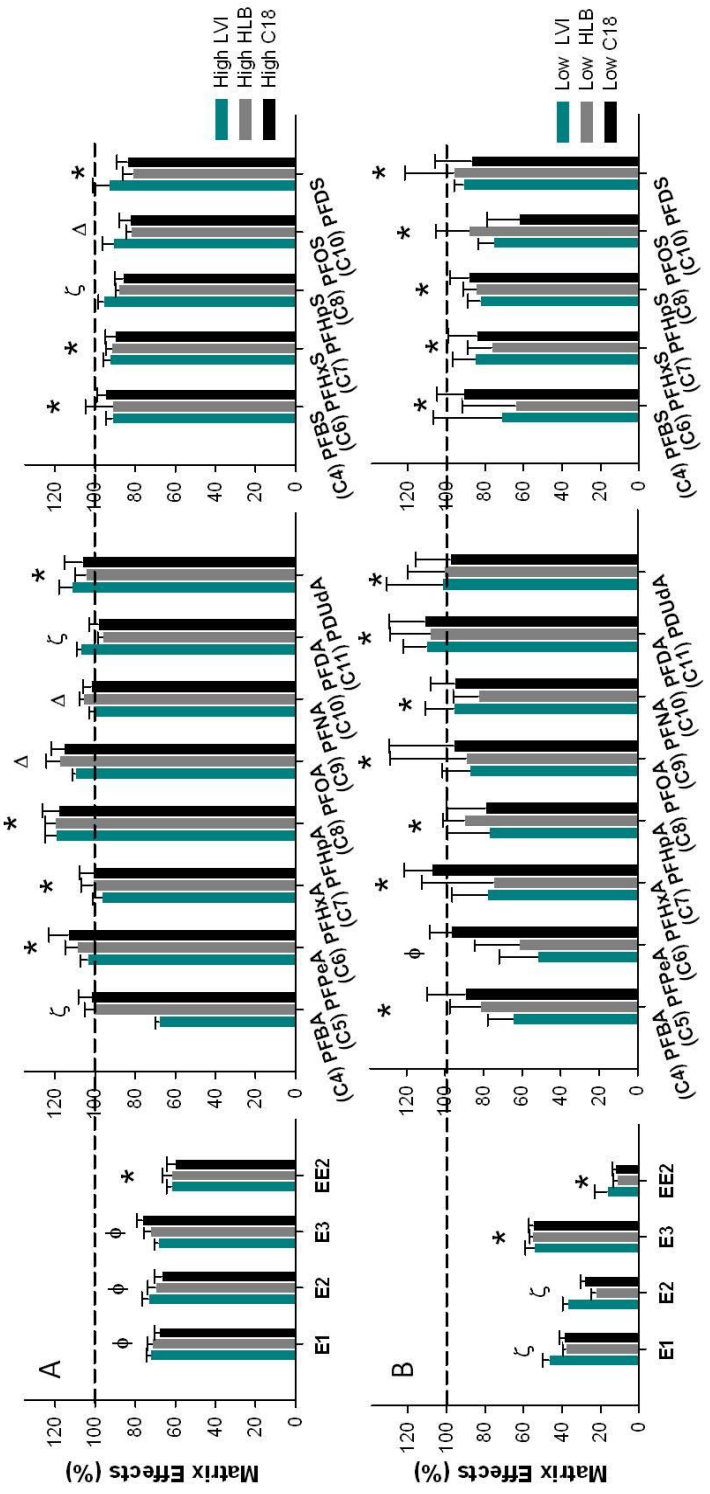

Figure 3.2) Percent matrix effects for LVI-, HLB SPE-, and C18 SPE-based analyses of wastewater at high (A) and low (B) ratios of analyte-to-matrix mass. 100% = no matrix effects, <100% = signal suppression, >100% = signal enhancement. Error bars represent ± 95% CI. * Indicates no statistical difference between LVI and C18 and HLB. Δ Indicates no statistical difference between LVI and C18. ϕ Indicates no statistical difference between LVI and HLB. ζ Indicates that LVI is statistically different than both C18 and HLB.

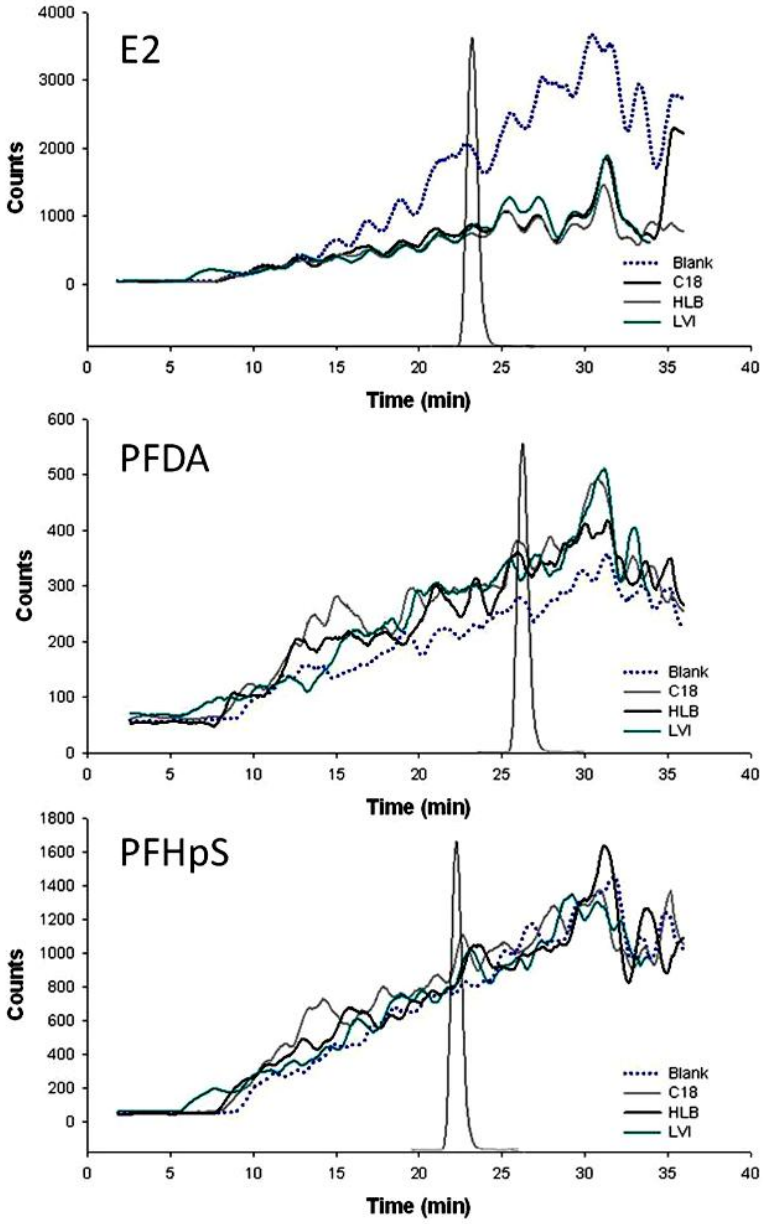

Figure 3.3) Matrix effects of E2, PFDA, and PFHpS evaluated across the chromatogram by post-column infusions. The peaks represent where the compound would normally elute. The LVI chromatographic traces were adjusted back 2 min to compensate for the 2 min sample loading time of 900 µL.

Table 3.1) Background area counts of analytes in un-spiked wastewater analyzed by LVI and un-spiked HLB and C18 SPE Extracts [a]. (n = 4)

	LVI	HLB	C18
E1	109 ±13	101 ± 25	131 ± 26
E3	43 ± 7	70 ± 22	73 ± 16
PFPeA	1171 ± 43	1278 ± 23	ND
PFHxA	1147 ± 63	1651 ± 59	338 ± 58
PFHpA	460 ± 88	514 ± 28	604 ± 5
PFOA	1014 ± 86	1520 ± 65	1443 ± 134
PFNA	212 ± 39	244 ± 20	241 ± 17
PFBS	6543 ± 171	9436 ± 439	ND
PFHxS	937 ± 35	1177 ± 55	1048 ± 46
PFOS	290 ± 16	420 ± 87	638 ± 84

[a] ± 95% Confidence Interval.

Chapter 4 - Newly-Identified Cationic, Anionic, and Zwiterionic Fluorinated Chemicals in Groundwater at U.S. Military Bases by non-Aqueous Large-Volume Injection HPLC-MS/MS

Will J. Backe, Kaneen E. Christensen , and Jennifer A. Field

Environmental Science and Technology

1155 16th St. NW

Washington, DC 20036

Submitted

4.1 Abstract

A new analytical method was developed for the purpose of quantifying 24 newly-identified and 19 legacy (e.g PFOS and PFOA) fluorinated chemicals in groundwater. Fluorinated chemicals were extracted from groundwater by micro liquid-liquid extraction. Extracts were then analyzed by non-aqueous large-volume injection (900 μL) with orthogonal inline high-performance liquid chromatography tandem mass spectrometry. Method detection limits ranged from 0.71 ng/L to 67 ng/L and whole-method accuracy ranged from 96 % to 106 % for analytes with authentic analytical standards. For analytes without authentic analytical standards, whole-method accuracy ranged from 78% to 144%. Method precision for all analytes was less than 15%. Twenty-two groundwater samples collected from six U.S. military bases were analyzed to demonstrate the method. Eight of the 24 newly-identified fluorinated chemicals were detected in groundwater from five of the military bases at concentrations up to 6,900 ng/L. Additionally, legacy fluorinated chemicals were measured in groundwater from all six sites at concentrations up to 360,000 ng/L. Aqueous film-forming foams were used for fire-training exercises at all but one of the military bases studied. Toward this end, the aqueous film-forming foams mixtures commonly used at military bases were also analyzed for the targeted fluorinated chemicals.

4.2 Introduction

Recently, several new classes of zwitterionic, cationic, and anionic per- and poly-fluorinated alkyl substances (PFASs) were identified in the aqueous film-forming foams (AFFFs) used by the US military (Figure 4.1). (1) Aqueous film-forming foams are used to extinguishing hydrocarbon-fuel fires (1, 2) and contain PFASs to lower surface tension. (3) The U.S. military accounts for 75% of all the AFFF used in the United States (3) and historical fire-training exercises at military bases resulted in the discharge of AFFFs into the environment on a weekly to monthly basis. (3, 4) At these and other sites, such as municipal airports, where AFFFs were released, elevated (up to > 1,000,000 ng/L) concentrations of perfluoroalkyl carboxylates , perfluoroalkyl sulfonates, and fluorotelmer sulfonates are shown to occur in ground- and surface-water. (4-9) Groundwater at sites impacted by AFFF use has the largest contamination of legacy PFASs of any aqueous environment. (9) Therefore, it is likely that the "newly-identified" PFASs contaminate groundwater at sites where AFFF was used and that previous studies underestimated the breadth of PFAS contamination. (4-6, 10) However, there are no analytical methods that quantify the newly-identified PFASs in groundwater.

Most of the analytical methods developed to quantify legacy PFASs in aqueous samples are based on pre-concentration by solid-phase extraction (SPE). (9, 11) However, SPE-based methods generate unnecessary solid and liquid waste, are laborious, and are prone to analyte loss. (12, 13) Additionally, PFASs from SPE materials can leach into and contaminate extracts. (14, 15) Furthermore, C18- and HLB- SPE phases are unable to retain C_4F_9-C_6F_{13} perfluoroalkyl carboxylates and

perfluoroalkyl sulfonates due to breakthrough. (14, 16-18) Although weak anion-exchange (WAX) phases retain C_4F_9-C_6F_{13} perfluoroalkyl carboxylates and perfluoroalkyl sulfonates, (17, 19) they would not retain many of the newly-identified PFASs that are cationic and zwitterionic (Figure 4.1). WAX SPE could be stacked with weak cation-exchange SPE for a comprehensive extraction of anionic, cationic and zwitterionic PFASs; however, this would generate twice the solid waste and increase the labor and solvents needed to process a sample.

Other analytical methods used for legacy PFAS analysis are based on direct-aqueous large-volume ($\geq$ 500 μL) injection (LVI) (5, 20) and liquid-liquid extraction. (21) Direct-aqueous LVI is advantageous because only small sample volumes (< 5 mL) and minimal pretreatment is required, such as filtration or centrifugation. (20, 22) Although direct-aqueous LVI is shown to be equally effective as SPE-based methods, (23) many PFASs are surface active in aqueous solutions. This surfaces activity causes PFASs to stratify, (24) adsorb to surfaces, (9, 11, 25) and aggregate (26) which will result in analyte loss from the sample over time. Previous direct-aqueous LVI methods used isotope labeled internal standards for perfluoroalkyl carboxylates and perfluoroalkyl sulfonates which compensated for loss. (5, 20, 27) However, isotope labeled internal standards do not exist for fluorotelomer sulfonates so it is possible that previous direct-aqueous LVI methods underestimated their concentrations. (5, 20, 27) Additionally, isotope labeled internal standards are not available for the newly-identified PFASs.

Issues associated with PFAS stratification, aggregation, and sorption in aqueous solutions are eliminated by liquid-liquid extraction (LLE) because analytes are extracted into an organic solvent. Another benefit of LLE is that no sample filtration is required so analytes sorbed to suspended particulate matter can potentially be extracted. (21) However, the drawbacks to LLE are that uses large volumes sample (400 mL to 1,000 mL) and organic extract (100 to 120 mL). (21, 28) Additionally, LLE requires extraneous preparation steps like sample blow down and solvent exchange. (21, 28, 29) Similar to SPE-based methods, the labor that goes into LLE is wasted by only injecting a small fraction of the final extract (1% to 10%). (21, 28, 29) Large-volume injection uses a larger fraction (> 50%) of the sample for analysis; (22) however, because organic solvents have high elution strengths on reverse-phases (e.g. C18) the LVI of an organic extract would result in analyte loss due to breakthrough on a C18 HPLC column. Therefore, new approaches are needed that successfully integrate LLE and LVI to maximize the benefits of each approach while eliminating their respective drawbacks.

The purpose of this study was to develop and validate a comprehensive analytical method for the analysis of the newly-identified PFASs, perfluoroalkyl carboxylates, perfluoroalkyl sulfonates, and fluorotelmer sulfonates (Figure 4.1) in groundwater and AFFF formulations. Groundwater (3 mL) was LLE with 1.2 mL of organic extract (micro-LLE). Direct analysis of the extract without sample blow down or solvent exchange was performed by non-aqueous LVI (900 μL) tandem mass spectrometry. To successfully employ non-aqueous LVI, three HPLC columns of orthogonal

retention mechanisms (weak cation exchange, weak anion exchange, and reverse phase) were used in series (orthogonal inline HPLC). All 43 analytes were acquired in a single run using positive/negative electrospray ionization (ESI) tandem mass spectrometry. The final method was demonstrated on groundwater obtained from six different military bases within the United States and 12 different AFFF formulations.

4.3 Experimental Section

4.3.1 Chemicals. Chemical and reagent source and purity as well as descriptive scientific names for the target analytes and internal standards used are provided in the Appendix 3 (Table A.3.1 in Appendix 3). Additionally, for the purpose of brevity individual analytes and internal standards will be referred to by their acronym (Figure 4.1, Table 4.1)**.** For reference, ***poly***fluorinated chemicals are chemicals with alkyl chains that are partially-fluorinated up to the functional group. (2) An example of C6 ***poly***fluorinated chemicals are 6:2 FtS, 6:2 FtSaB, and 6:2 FtTAoS (Figure 4.1). Alternatively, ***per***fluorinated chemicals are chemicals that are fully fluorinated up to the functional group. (2) For example, PFOA, PFOS, PFOSaAm, and PFOSaAmA (Figure 4.1) are all C8 ***per***fluorinated chemicals. Additionally, the compound classes perfluoroalkyl carboxylates, perfluoroalkyl sulfonates, and fluorotelmer sulfonates will be referred to a **legacy PFASs** because they were previously studied, while the compound classes fluorotelomer thioamido sulfonates, fluorotelomer thiohydroxy ammonium, fluorotelomer sulfonamido betaines, fluorotelomer sulfamido amines,

fluorotelomer betaines, perfluoroalkyl sulfonamido amines, and perfluoroalkyl sulfonamide amino carboxylates will be referred to as **newly-identified PFASs.**

4.3.2 Sample Collection. Groundwater sampling was conducted by third parties as outlined below. Groundwater samples from sites A, B, and C were collected in collected in 250 mL solvent rinsed (methanol and reagent water) high-density polyethylene (HPDE) bottles, placed in a coolers filled with ice, and shipped overnight to Oregon State University.

Site A. Prior to groundwater collection, the well was purged using a peristaltic or bladder pump until water quality parameters (e.g. pH, specific conductivity, temperature, turbidity, oxidation/reduction potential and dissolved oxygen) stabilized. The depth to the groundwater ranged from 0.50 m to 8.8 m. The tubing that came in contact with the sampled groundwater was fluoropolymer free, and new tubing was used for each sample location.

Site B. Groundwater samples were collected from this site following the U.S. EPA's Groundwater Sampling procedures. (30) The depth to the groundwater ranged from 6.7 to 10 m. Groundwater was collected at each monitoring well using new silicone and polyethylene tubing. The monitoring wells sampled were purged first by pumping with a peristaltic pump until stabilization of water quality parameters occurred.

Site C. The groundwater from this site was collected following the U.S. EPA's Low Stress (low flow) Purging and Sampling Procedure (31) (different than Site B). The

depth to groundwater ranged from 2.9 m to 3.0 m. Groundwater was collected using PTFE tubing and bladder pumps that were dedicated to each sampling well. Deionized water that was rinsed through the PTFE tubing did not reveal the presence of any PFAS contamination (data not shown). The sampling wells were purged until water quality parameters stabilized after which groundwater was sampled.

Tyndall Air Force Base, Naval Air Station Fallon, and Wurtsmith Air Force Base. Samples from Tyndall Air Force Base, Naval Air Station Fallon, and Wurtsmith Air Force Base were collected in 1999 and archived since that time at -4^{o}C in 125 mL HDPE bottles. The specific details of the sample collection are described elsewhere. (4, 6)

Aqueous Film-Forming Foam. AFFF formulations were collected from military bases around the United States as previously described. (1) AFFF formulations were sampled from their original product containers into 60 mL HDPE bottles from military bases within the United States and shipped to Oregon State University. The AFFF samples received dated from between 1984 to 2011 and are from the different manufacturers whose AFFF formulations are or were available to the military.

4.3.3 Groundwater Micro Liquid-Liquid Extraction and AFFF Sample Preparation.

To obtain a representative subsample (see Appendix 3), groundwater in HDPE bottles was repeatedly (4x) sonicated in a heated (60^{o}C) Model 75HT sonication bath (VWR, Radnor, PA) for approximately 20 seconds then gently swirled and inverted. Next, a 3 mL subsample was taken from approximately 3.0 cm to 3.5 cm below the meniscus

and delivered to a 5 mL polypropylene microtube (Argos Technologies, Elgin, IL) that contained 0.97 g to 1.0 g of sodium chloride. The subsamples were then spiked with 1.05 ng of each isotopically-labeled internal standard (Table 4.1). Then, the samples were acidified with 10 μL of 6 N HCl and extracted with 10% 2,2,2-trifluoroethanol in ethyl acetate, henceforth referred to as extractant. Each sample was extracted in triplicate by adding extractant (500 μL for the first extraction, 370 μL for the second extraction, and 330 μL for the third extraction) to the sample, shaking for 45 s, allowing the phases to separate, and transferring the supernatant to a clean 1.5 mL polypropylene vial (MicroSolv, Eatontown, NJ, Part # 9502S-PP-Amber). The total volume of extractant extract collected was 1 mL. This collected extract was then brought to 1.5 mL with methanol in the same vial and analyzed without further sample preparation.

AFFF samples were prepared by diluting the AFFF formulations in methanol between 100,000 and 4.5 million fold, transferring 1.5 mL of the dilute AFFF into a clean polypropylene autosampler vial, adding 1.05 ng of each isotopically-labeled internal standard, and analyzing as described below.

4.3.4 Analysis by non-Aqueous LVI Orthogonal Coupled-Column HPLC MS/MS. Chromatographic separations were performed by an Agilent 1100 series HPLC (Santa Clara, CA) capable of performing 900 μL LVI (Agilent part # G1363A) and retrofitted as described previously to eliminate contamination from sources of perfluorinated chemicals. (23) Chromatographic separations were achieved using three columns of

orthogonal stationary phases in tandem. In the following order, an Agilent 4.6 x 12.5 mm x 5 μm Zorbax Silica (Sil) guard column (Agilent part # 820950-901) was connected to an Agilent 4.6 x 12.5 mm x 5 μm Zorbax propylamine (NH_2) guard column (Agilent part # 820950-908) which was connected to a Zorbax Eclipse Plus C18 4.6 x 50 mm x 1.8 μm analytical column.

The composition of the mobile phases was 10 mM ammonium acetate in HPLC grade water (A) and 10 mM ammonium acetate in HPLC grade MeOH (B). Analytes were separated and eluted using a convex gradient program (Figure A.3.1 in Appendix 3). The autosampler valve was programmed to direct the mobile phase flow past the flow-path of the injection assembly (needle loop + needle + injection valve) at 2.4 min after sample injection to reduce gradient dwell time. By 2.4 min the sample had been completely transferred from the needle loop and onto the columns. Column eluate was diverted away from the mass spectrometer and into a waste container for the first 7 min after injection in order to wash early-eluting matrix components and inorganic salts to waste. This step is analogous to the wash step used for SPE. (13) After 7 min, the column eluate was directed into the mass spectrometer for sample acquisition.

Analytes were detected by an ESI interfaced TQ Detector (Waters Corporation, Milford, MA) triple quadrupole mass spectrometer operated in multiple-reaction monitoring (MRM) mode and controlled by MassLynx (Version 4.1). Two transitions were acquired for each analyte, except PFBA and PFPeA, (Table A.3.2 in Appendix 3) and were detected in either positive or negative polarity within a single acquisition

(positive/negative polarity switching). Mass spectrometer parameters were as follows: a capillary potential of ± 2,800 V, an extractor potential of ± 2 V, a source and desolvation temperature of 150 ^{o}C and 450 ^{o}C, respectively, and a desolvation gas and cone gas flow of 1,100 L/hr and 75 L/hr, respectively.

Compound-dependant acquisition parameters (e.g. cone voltage and collision energy) (Table A.3.2 in Appendix 3) were optimized by infusing analytical standards or mixtures (see below) diluted in methanol to yield analyte concentrations of approximately 0.1 to 1 mg/L. For analytes without an available characterized source (see below), compound-dependant mass spectrometer parameters were optimized by infusing dilute (10 to 20 thousand fold in methanol) AFFF formulations that contained those analytes (Table A.3.2 in Appendix 3). (1)

4.3.5 Analyte Identification and Quantitation. Due to the differences in the availability and the quality of the standards with which to determine analyte concentrations in samples, analytes and the confidence of their respective data were classified into three groups. The first analyte/data group is **quantitative** (Qn) and was assigned to analytes that had commercially-available authentic analytical standards (perfluoroalkyl carboxylates, perfluoroalkyl sulfonates and fluorotelomer sulfonates) (Figure 4.1). The next analyte/data group is **semi-quantitative** (Sq) and included analytes that were donated (see Appendix 3) in characterized mixtures that are used to produce AFFF formulations (fluorotelomer betaines, 6:2 FtTAoS, 6:2 FtTHN^{+}, 6:2 FtSaB, and 6:2 FtSaAm) (Figure 4.1). Analyte concentrations in characterized mixtures were

determined from manufacturer MSDSs (32-34) and patent data. (35) However, the uncertainty about their concentrations is undoubtedly larger than those of authentic analytical standards. The third analyte/data group is **qualitative** (Ql), to which all the other target analytes belong (all of the perfluoroalkyl sulfonamide amines; all of the perfluoroalkyl sulfonamide amino carboxylates; C_4F_9 and C_8F_{17} fluorotelomer thioamido sulfonates; C_8F_{17}, $C_{10}F_{21}$, and $C_{12}F_{25}$ fluorotelomer sulfonamide betaines; and C_8F_{17} fluorotelomer sulfonamide amines) (Figure 4.1). There are no authentic analytical standards or other sources of Ql analytes available at this time and an approach to estimate their concentrations in groundwater is outlined below.

Calibration standards were prepared by first extracting reagent water (B&J Brand®, Morristown, NJ), as described above for groundwater, to generate a blank extract. Calibration standards for Qn and Sq analytes were then prepared in 1 mL of blank extract diluted with 500 μL of methanol. 1.05 ng of each internal standard was added to each calibration standard. Analytes were quantified using 1/x weighted internal-standard calibration. The range, number of points, and coefficient of determination (R^2), for each calibration curve is presented in the Appendix 3 (Table A.3.3 in Appendix 3). Concentrations of qualitative analytes were estimated by assuming equal molar response to a related Qn or Ql analyte (Table A.3.3 in Appendix 3); see the Appendix 3 for an example. Samples that produced analytical signals above that of the highest calibration standard were reanalyzed by subsampling 15μL to 60 μL of the original groundwater, diluting the subsample to 3 mL with reagent water (a 50 to 200

fold dilution, respectively) and then micro LLE the diluted subsample as described above.

For positive identification analytes were required to have retention times that varied less than ± 0.25 min of those in calibration standards (Qn and Sq). However, because calibration standards were not available for Ql analytes, those analytes had to have retention times that varied less than ± 0.25 min when compared to the retention times of those same analytes in dilute AFFF formulations. Additionally, the ratio of the two product ions (transitions) for each analyte (not applicable for PFBA and PFPeA) (Table A.3.2 in Appendix 3) were required to be within 20% of those from calibration standards or from dilute AFFF formulations.

4.3.6 Method Limits of Detection and Quantitation. To determine the method limits of detection (LOD), calibration points for Qn and Sq analytes were prepared in blank groundwater around their estimated LODs (from preliminary experiments) and then extracted as outlined above. Actual LODs were calculated by multiplying 3.3 by the 1/x-weighted regression-residual standard deviation ($\sigma_{x/y}$) of the analyte's calibration in the extracted groundwater, then subtracting by the y-intercept, and finally dividing by the regression slope. This approach for determining LODs is similar to that recommended by Vial and Jardy (36) and is approved by the International Conference on Harmonization (ICH) for the validation of analytical procedures. (37) The limit of quantitation (LOQ) was then defined as 3.3 times the LOD.

4.3.7 Whole-Method Accuracy, Precision, and Absolute Extraction Efficiency. To demine whole-method accuracy and precision, 3 mL aliquots of groundwater (n = 11) with no detectable analyte signal was spiked with analytes (Ql and Sq) to give final concentrations within ten fold of their LOQs (Table 4.1) and with 1.05 ng of each internal standard. The spiked groundwater was used as a matrix that contained an artificial "*background*" concentration of all the Qn and Sq analytes. The samples were then extracted as described above. Accuracy was defined as the analyte concentration in spiked groundwater as determined by internal-standard calibration divided by the analyte concentration in groundwater determined from standard addition multiplied and by 100. Standard-addition analysis was performed using 11 points from the 11 groundwater samples, including 6 points (six replicate samples) at the Y-intercept ("*background*" concentration) and five standard additions at 0.5, 0.75, 1.0, 1.5, and 2.0 fold above the "*background*" concentration. Internal-standard calibration was carried out on the same six replicate groundwater samples that were used to determine the Y-intercept for standard addition. For analytes without well-matched isotopically-labeled internal standards, an internal standard was selected for all subsequent analyses that yielded concentrations closest to those obtained by standard addition (Table 4.1). The error about the whole-method accuracy was compounded from each measurement and reported as ± 95 % CI. Within-run precision was calculated by taking the percent relative standard deviation (% RSD) of the six replicate samples at the "*background*" concentration. The procedure for determining absolute extraction efficiency is outlined in the Appendix 3.

4.4 Results and Discussion

4.4.1 Orthogonal Coupled-Column HPLC. The HPLC method outlined above resulted in well resolved and reproducible chromatography for zwitterionic, cationic, and anionic analytes in groundwater (Figure 4.2). Analytes were concentrated out of the injected extract (900 μL) onto the Sil or NH_2 phases during sample loading. Ion exchange interactions are the proposed mechanisms for the retention of ionic analytes on the Sil and NH_2 phases because they were negatively and positively charged, respectively, at the pH of the mobile phase (approximately pH 6). Additionally, the non-ionic PFAS, perfluorooctanesulfonamide (FOSA), broke through the columns and eluted with the injection plug. For this reason, FOSA was not included in the target analyte list and previous work did not detect FOSA in ground- or surface- water at sites impacted by AFFF. (10) An advantage of losing nonionic compounds to breakthrough is that any matrix effects caused by nonionic species in the sample matrix are eliminated. (23, 38) Elution of the analytes off the Si and NH_2 guard columns was promoted by the ammonium acetate (10 mM) in the mobile phases, as methanol and water alone could not elute the analytes from those columns. Finally, the analytes were eluted off the Si and NH_2 guard columns at a low enough organic strength so that they were refocused at the head of the C18 analytical column where they were subsequently separated by reverse-phased mechanisms.

The use of orthogonal inline HPLC is infrequently reported in the literature. The few applications of orthogonal inline HPLC previously reported are for bio-analytical

analyses (39-42) and for determining naturally occurring carboxylic acids in plants. (43) To the authors' best knowledge the use of orthogonal inline HPLC with non-aqueous LVI as well as for the analysis of environmental contaminants is not previously described.

4.4.2 Limit of Detection and Quantitation. The LOD for Qn and Sq analytes was between 0.71 ng/L to 67 ng/L with a median LOD of 1.7 ng/L (Table 4.1). Because this is the first report of the newly-identified PFASs in any environmental media, a comparison to existing LODs could not be made. However, the LODs presented here are similar to those reported elsewhere for the SPE-based analyses of legacy PFAS in groundwater (44, 45) and lower than those of previous direct aqueous LVI-based methods. (5, 46) Jin et al. was able to achieve detection limits for PFOA and PFOS at 0.03 ng/L and 0.05 ng/L in groundwater but had to extract 1,000 mL of groundwater using 16.5 mL of solvent by SPE. (47) In comparison, the method presented here requires only 3 mL of a sample and 1.2 mL of solvent for extraction. Additionally, micro-LLE requires less time to perform (minutes vs. hours) because it eliminates the steps associated with sample loading and elution off SPE cartridges. (13) A different LLE-based method that used 900 mL of sample and 120 mL of organic extractant gave LODs of selected legacy PFASs in wastewater that are an average 3 fold lower than the ones presented here, however that method also required extract blow down and solvent exchange. (21)

4.4.3 Whole-Method Accuracy, Precision, and Absolute Extraction Efficiency. Whole-method accuracy ranged between 96 % and 106 % for Qn analytes and between 78 % and 144 % for Sq analytes (Table 4.1). For Qn analytes, whole-method precision as indicated by % RSD ranged between 2.8% and 12% and ranged between 5.6% and 15% for Sq analytes (Table 4.1). While comparisons cannot be made for the newly-identified PFASs, the accuracy and precision of the legacy PFASs reported here is similar (5, 47) to or improved (46) over that of previous direct aqueous LVI- and SPE-based analyses of groundwater. Finally, the absolute extraction efficiency of the micro-LLE procedure was between 87% to 99% for the Qn and Sq analytes tested (Table A.3.4 in Appendix 3). A comparison of absolute extraction efficiencies to other methods is presented in the Appendix 3.

4.4.4 Method Demonstration: Newly-Identified and Legacy Fluorochemical Contamination in Groundwater at Military Sites.

Site A.

Polyfluorinated Chemicals.

Two fluorotelomer thioamido sulfonates, 4:2 FtTAoS and 6:2 FtTAoS, were detected in two of the four samples from Site A (Table 4.2). 4:2 FtTAoS and 6:2 FtTAoS was present in groundwater at concentrations up to 490 ng/L and 6,900 ng/L, respectively (Table 4.2).The detection of 4:2 FtTAoS and 6:2 FtTAoS is consistent with the time that AFFF formulations containing fluorotelomer thioamido sulfonate were available to U.S. military bases (1976-present) (1) and the range of time when Site A was used for fire-training (1942 to 1990). (48) Six different manufacturers, National Foam,

Chemguard, Ansul, Buckeye Fire Equipment, Angus, and Fire Service Plus, formulate telomerization-based AFFFs that are or were available to the U.S. military. (1) AFFFs from Chemguard, Ansul, and Angus contain fluorotelomer thioamido sulfonate, although, only Ansul and Angus AFFFs had detectable concentrations of both 4:2 and 6:2 FtTAoS (Table A.3.5 in Appendix 3). Additionally, Angus AFFF contains a PFAS (6:2 $FtTHN^{+}$) that was not detected in groundwater (Table A.3.5 in Appendix 3) which implicates Ansul AFFFs as the source of the FtTAoS at Site A. Additionally, Ansul formulated the only AFFFs that contained fluorotelomer thioamido sulfonate and were available to the military before 1990, (1) which is the year fire-training ceased at Site A. (48)

The ratio of 6:2 FtTAoS/4:2 FtTAoS in Ansul AFFF is 235 to 1 (Table A.3.5 in Appendix 3). However, in the two groundwater samples in which 6:2 and 4:2 FtTAoS were detected, their ratios are 33 to 1 (Sample 3) and 0.18 to 1 (Sample 4), respectively (Table 4.2). 4:2 FtTAoS is more water soluble than 6:2 FtTAoS and, therefore, will migrate with the groundwater faster. Samples taken down gradient from where fire-training occurred are likely to have lower ratio of 6:2 FtTAoS/4:2 FtTAoS compared to AFFF, however, this site information is unavailable. 8:2 FtTAoS is also present in Ansul AFFF (Table A.3.5 in Appendix 3), however, this compound more hydrophobic than 4:2 and 6:2 FtTAoS and likely sorbed onto soil. The inconsistencies between the ratios of fluorotelomer thioamido sulfonates detected in AFFF versus those in groundwater reveal the need for additional research in their environmental fate.

The fluorotelomer sulfonates, 4:2, 6:2, and 8:2 FtS, were detected in all four samples from Site A at concentrations ranging from 370 ng/L to 11,000 ng/L, 8,900 ng/L to 220,000 ng/L, and 58 ng/L to 370 ng/L, respectively. Fluorotelomer sulfonates are the major source of polyfuorinated chemical contamination in groundwater, even though; they are not detected or occur at reduced concentrations compared to other compounds in telomerization-based AFFFs (Table A.3.5 in Appendix 3). In fact, 6:2 FtS and 8:2 FtS were only detected in National Foam and Fire Service Plus AFFFs, neither of which contain detectable concentrations of fluorotelomer thioamido sulfonates (Table A.3.5 in Appendix 3). The telomerization-based AFFFs analyzed here are expected to be consistent with what was historically produced by each manufacturer (1) and, as such, it is unlikely that fluorotelomer sulfonates were ever a major component in any AFFF used by the military. The source of fluorotelomer sulfonates in groundwater may be from the degradation of the newly-identified PFASs (Figure 4.1). However, because this is the first study to report on the environmental occurrence of these newly-identified PFASs there are no studies on their degradation. Previous studies are focused on the degradation of fluorotelomer alcohols and derivatives of FOSA into stable ***per***fluorinated chemicals like PFOA and PFOS. (49-53) Understanding the degradation pathways of the newly-identified PFASs is an important topic for future research and is needed to determine if they are a source of the fluorotelomer sulfonates in AFFF impacted groundwater.

Perfluorinated Chemicals.

The perfluoroalkyl sulfonamido amines, PFBSaAm, PFPeSaAm, and PFHxSaAm, were present at concentrations of 2.8 ng/L to 54 ng/L in two samples from Site A (Table 4.2). 3M, who ceased production in 2002, was only formulator of AFFFs that were used by the U.S military that contained perfluorinated chemicals as a main active ingredient. (1) 3M AFFFs dating from 1989 to 2001 have detectable concentrations of C4-C8 perfluoroalkyl sulfonamido amines (Table A.3.6 in Appendix 3) so it is possible that these compounds were in AFFFs when fire-training occurred at Site A (1942 to 1990). (48) Perfluoroalkyl sulfonamide amino carboxylates, a related compound to perfluoroalkyl sulfonamido amines, were not detected in groundwater from Site A. However, 3M reformulated the chemical makeup of its AFFFs over the years (1) and AFFFs from 1989 do not contain detectable concentrations of perfluoroalkyl sulfonamide amino carboxylates (Table A.3.6 in Appendix 3).

The majority of PFAS contamination in groundwater from Site A was from perfluoroalkyl sulfonates (40% by mass) and perfluoroalkyl carboxylates (46% by mass) (Table 4.2). The concentrations of PFHxS and PFHxA in groundwater at Site B ranged from 36,000 ng/L to 360,000 ng/L and 19,000 ng/L to 350,000 ng/L, respectively (Table 4.2). Additionally, the levels of PFOS and PFOA ranged from 15,000 ng/L to 78,000 ng/L and 12,000 ng/L to 220,000 ng/L, respectively (Table 4.2). As a reference, the U.S. Environmental Protection Agency set a provisional health advisory of 200 ng/L of PFOS and 400 ng/L of PFOA in drinking water. (54) The magnitude of PFAS contamination in the groundwater at Site A is consistent with

previous studies that report legacy PFASs at μg/L to mg/L levels in groundwater from AFFF impacted sites. (4-6, 10)

Perfluoroalkyl sulfonates are 23 ± 3.3 fold (n=6, 95 % CI) more concentrated in 3M AFFFs than perfluoroalkyl carboxylates (Table A.3.6 in Appendix 3). However, in individual groundwater samples from Site A that ratio reduces to 1.1 ± 0.29 fold (n = 4, 95 % CI) (Table 4.2). It is possible that newly-identified PFAS from AFFF formulations applied at Site A degraded in into perfluoroalkyl carboxylates. For example, certain polyfluorinated chemicals are reported to biodegrade to perfluoroalkyl carboxylates. (51) It is also possible that 3M AFFFs older than 1989 had a larger ratio of perfluoroalkyl carboxylates since they chemical composition of their AFFFs changed over the years (Table A.3.6 in Appendix 3). (1, 2) However, no 3M AFFFs were available to be analyzed that date before 1988. The relatively low concentrations of perfluoroalkyl sulfonamido amines in pre-1993 3M AFFFs (Table A.3.6 in Appendix 3) may be a reason why only small amounts of perfluoroalkyl sulfonamido amines (≤ 54 ng/L) were detected at Site A (1942 to 1990). Additionally, perfluoroalkyl sulfonamido amines are cationic so their concentration in groundwater might be small compared to what is catatonically exchanged onto soils. (55) The development of a method to quantify the newly-identified PFASs sorbed soil is needed to provide information on the environmental fate of these compounds.

Site B.

Polyfluorinated Chemicals.

One newly-identified polyfluorinated chemical, 6:2 FtTAoS, was detected in one of the eight groundwater samples (68 ng/L) from Site B (Table 4.3). Fire-training exercises at Site B began in 1950 and ceased in 1993; however, AFFF use at this site did not begin until the 1970s. (56) Based on the years of operation and reasons discussed previously, the detection of 6:2 FtTAoS is likely from an Ansul AFFF. (1) Fluorotelomer sulfonates were detected in seven of the eight groundwater samples from Site B at concentrations ranging from 5.2 ng/L to 160 ng/L for 4:2 FtS, 210 ng/L to 37,000 ng/L for 6:2 FtS, and 66 ng/L to 2,300 ng/L for 8:2 FtS (Table 4.3). As with Site A, fluorotelomer sulfonates made up the majority of the polyfluorinated chemical contamination (Table 4.3), even though fluorotelomer sulfonates are only a minor product or impurity in some of the telomerization-based AFFF (Table A.3.5 in Appendix 3). A possible reason for this disparity, as discussed on earlier, is from the degradation of the polyfluorinated chemicals in telomerization-based AFFF to fluorotelomer sulfonates. If degradation is responsible for the fluorotelomer sulfonate contamination at Sites A and B, it would be difficult to attribute the contamination to any one AFFF manufacturer. For example, National Foam's AFFF was available to the military since 1976, (1) however, none of the PFASs in National Foam AFFF are detected in groundwater. This could mean either National Foam AFFF was never used at Sites A and B or that the PFASs in National Foam AFFF were completely degraded into fluorotelomer sulfonates. The total concentration of the polyfluorinated chemicals detected in the eight samples from Site B (70,000 ng/L) is approximately 6 fold less than that from the four samples from Site A (390,000 ng/L). This difference suggests

that less telomerization-based AFFF was released onto the ground during fire-training or that the telomerization-based PFASs degraded into a stable "dead end" product like PFOA.

Perfluorinated Chemicals.

Two perfluoroalkyl sulfonamide amino carbaxylates were detected in one of the eight groundwater samples from Site B at trace levels (4.1 ng/L of PFBSaAmA and 8.0 ng/L PFHxSaAmA) (Table 4.3). The detection of these trace concentrations is consistent with the years of fire-training occurred at Site B (1950 to 1993) and when perfluoroalkyl sulfonamide amino carbaxylates were first used in 3M AFFFs.[1] Unlike Site A, perfluoroalkyl sulfonamido amines were not detected at this site even though they cooccur at approximately the same concentrations in 3M AFFFs that date from 1993 (Table A.3.6 in Appendix 3). However, perfluoroalkyl sulfonamide amino carbaxylates are zwitterionic and perfluoroalkyl sulfonamido amines are cationic. Consequently, perfluoroalkyl sulfonamido amines may be strongly cationic exchanged to the soil (55) and not detected in groundwater.

As with Site A, the PFAS contamination in groundwater was dominated by perfluoroalkyl sulfonates (58% by weight) and perfluoroalkyl carboxylates (35% by weight) (Table 4.3). The maximum concentration of PFHxS and PFHxA at Site B was 170,000 ng/L and 99,000 ng/L, respectively, and the maximum concentration of PFOS and PFOA was 65,000 ng/L and 57,000 ng/L, respectively (Table 4.3). Similarly to Site A, PFHxS was the most concentrated analyte in groundwater which is interesting

because PFOS is 6.0 ± 0.4 (n=6, 95 % CI) fold more concentrated than PFHxS in 3M-based AFFFs (Table A.3.6 in Appendix 3). In the eight groundwater samples from Site B PFHxS was an average of 1.3 ± 0.6 (n=8, 95 % CI) times more concentrated than PFOS. However, PFOS's organic carbon adsorption coefficient (k_{oc}) is 4.4 times greater than PFHxS, (57) therefore, a greater portion of PFOS may be associated with the soil fraction. The total concentration of perfluoroalkyl carboxylates and perfluoroalkyl sulfonates in groundwater at Site B was 920,000 ng/L, compared to 2,400,000 ng/L from the four samples at Site A and indicates that either less AFFF was used at Site B or Site B had better measures to capture used AFFF.

Site C.

Five groundwater samples were obtained from Site C. Only two analytes were detected in the samples, PFOA (n = 2) and PFHxS (n = 1) and were both below the limit of quantitation. There was a fire-training area at Site C in the late 1960s; however, the type of fire-training (structure fires) and the years in which they were performed at Site C is inconstant with AFFF use. (58) Military fire-training that employs AFFFs are for hydrocarbon fuel-based fires often from simulated crashes (e.g. aircraft). (4) Additionally, AFFFs that contained PFASs were not listed for military use until 1976. (1) These inconsistencies may explain why PFASs were not detected or were not above quantitation at this site.

Tyndall Air Force Base (TAFB), Naval Air Station Fallon (NASF), and Wuirtsmith Air Force Base (WAFB).

Four archived samples from TAFB, two samples from NASF, and one sample from WAFB were reanalyzed for the newly-identified PFASs (Figure 4.1). It must be noted that the samples from TAFB, NASF, and WAFB were archived at - 4^{o}C since 1999 and periodically reanalyzed. (5, 6) It is possible that during this time additional degradation of newly-identified PFASs occurred.

Seven of the newly-identified PFASs were detected in groundwater from TAFB at concentrations ranging from 2.8 ng/L to 720 ng/L (Table 4.4). These seven newly-identified PFASs included 6:2 FtAoS, three perfluoroalkyl sulfonamido amines (PFBSaAm, PFPeSaAm, and PFHxSaAm) and three perfluoroalkyl sulfonamide amino acids (PFBSaAm, PFPeSaAm, and PFHxSaAm). AFFF formulations were used at TAFB during the time when fire-training activities were conducted at this site (1980 to 1992). (4) The years that fire-training occurred at TAFB is inclusive of the time when the newly-identified PFASs detected at this site were used in AFFF formulations available to the military (Table 4.4). (1) The highest concentrations of the newly-identified PFASs came from the groundwater sample (TAFB PW-10) taken directly adjacent to the "burn pit" where the AFFFs were applied during fire-training (Table 4.4). (4) Shultz et al. previously analyzed for 6:2 FtTAoS in the same samples from TAFB and did not detect it. (5) However, the 6:2 FtTAoS detected here was at 8.8 ng/L and the detection limits of the current method are two orders of magnitude lower than those by Shultz et al for legacy PFASs. (5) The data for fluorotelomer sulfonates, perfluoroalkyl carboxylates, and perfluoroalkyl sulfonates in groundwater from this site were previously reported elsewhere. (5) However, like Sites A and B the

newly-identified chemicals are only a minor fraction of the PFAS contamination. For example, in sample TAFB PW-10 6:2 FtS was reported at 14,600,000 ng/L and PFOS was reported at 2,300,000 ng/L. (5)

At NASF, PFBSaAm, PFPeSaAm, PFHxSaAm, PFBSaAmA, PFPeSaAmA, and PFHxSaAmA were detected in one (NASF 51-U) out of two groundwater samples at concentrations ranging from 5.8 ng/L of PFPeSaAmA to 550 ng/L of PFBSaAm (Table 4.4). Fire-training exercises occurred at NASF from between the mid-1950s to 1988. (5) There is no evidence to show that PFBSaAm, PFBSaAmA, and their longer chained homologs occur in 3M AFFF formulations from before 1989 (Table 4.4). (1) However, no 3M AFFF from before 1988 are available to be analyzed and it is not well documented as to when these compounds were first used in AFFF. (1) Therefore, it is possible that AFFFs containing perfluoroalkyl sulfonamido amines and perfluoroalkyl sulfonamide amino acids were used at NASF. As with TAFB data on legacy PFASs are reported elsewhere. (5) As with every other military site the newly-identified PFASs make up less than 1% by weight of the PFAS contamination. For example, in sample NASF 51-U PFHxS is reported at 880,000 ng/L. Previous analysis of groundwater samples from NASF did not reveal any FtS contamination, (5) which may explain why newly-identified telomerization-based PFASs were also not detected in samples from this site.

Finally, five of the newly-identified PFASs were detected in the one sample from WAFB (WAFB FT-3) at concentrations ranging from <2.7 ng/L to 79 ng/L (Table

4.4). WAFB was a fire-training area and was decommissioned in 1993; (6) this includes the times the newly-identified 3M-based PFASs were put in AFFFs available to the military. (1) As with all other groundwater reported on in this study, legacy PFASs were the major perfluorinated contaminants in sample WAFB FT-3; PFOS was reported at 110,000 ng/L. in this sample. (6)

4.5 Implications

Newly-identified PFASs were detected in groundwater from five out of the six U.S. military bases sampled. While Legacy PFASs made up a majority of the PFAS contamination in those samples, it is possible that many of the newly-identified PFASs are sorbed onto soil. Additionally, it could be that the newly-identfied PFASs were degraded into more stable "dead end" products like 6:2 FtS, PFOS, and PFOA. Degradation would explain why FtS are either not detected or are at low levels in telomerization based AFFFs but make up the majority of telomerization based PFAS contamination in groundwater. Future research should focus on developing a method to quantify newly-identified and legacy PFASs sorbed to soil to determine the fate and transport of these chemicals in groundwater. Additionally, studies should be conducted to understand the degredation pathways of the newly-identified PFASs in effort to determine if newly-identified PFASs are a significant source of legacy PFAS contamination.

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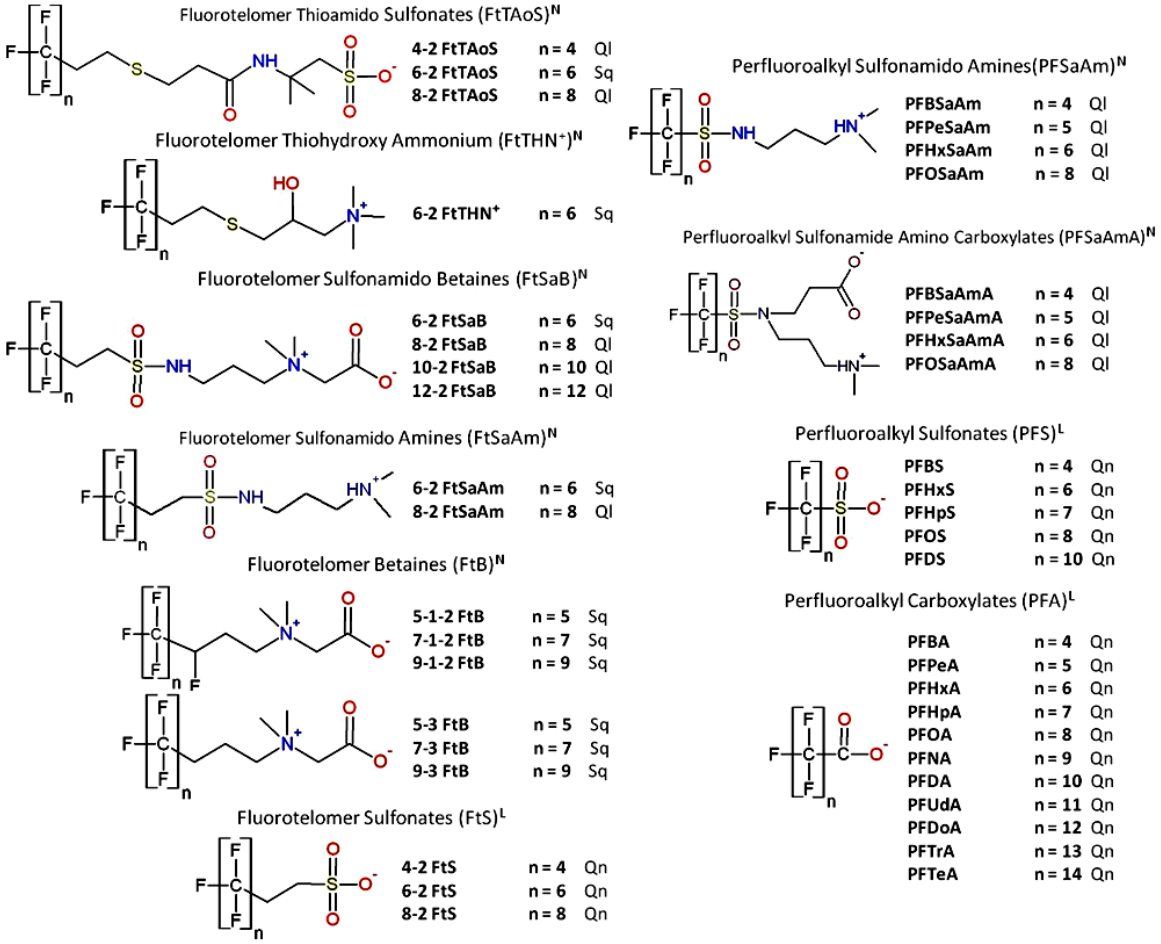

Figure 4.1) Target analyte classes, structures, and acronyms. Compound classes manufactured by telomerization are listed to the left and compound classed manufactured by electrofluorination are listed on the right. [L] Legacy classes of fluorochemical contaminants that have previous environmental data. [N] Newly-identified classes of fluorochemical contaminants for which previous environmental data does not exist. Quantitative (Qn) analytes, semi-quantitative (Sq) analytes, qualitative (Ql) analytes.

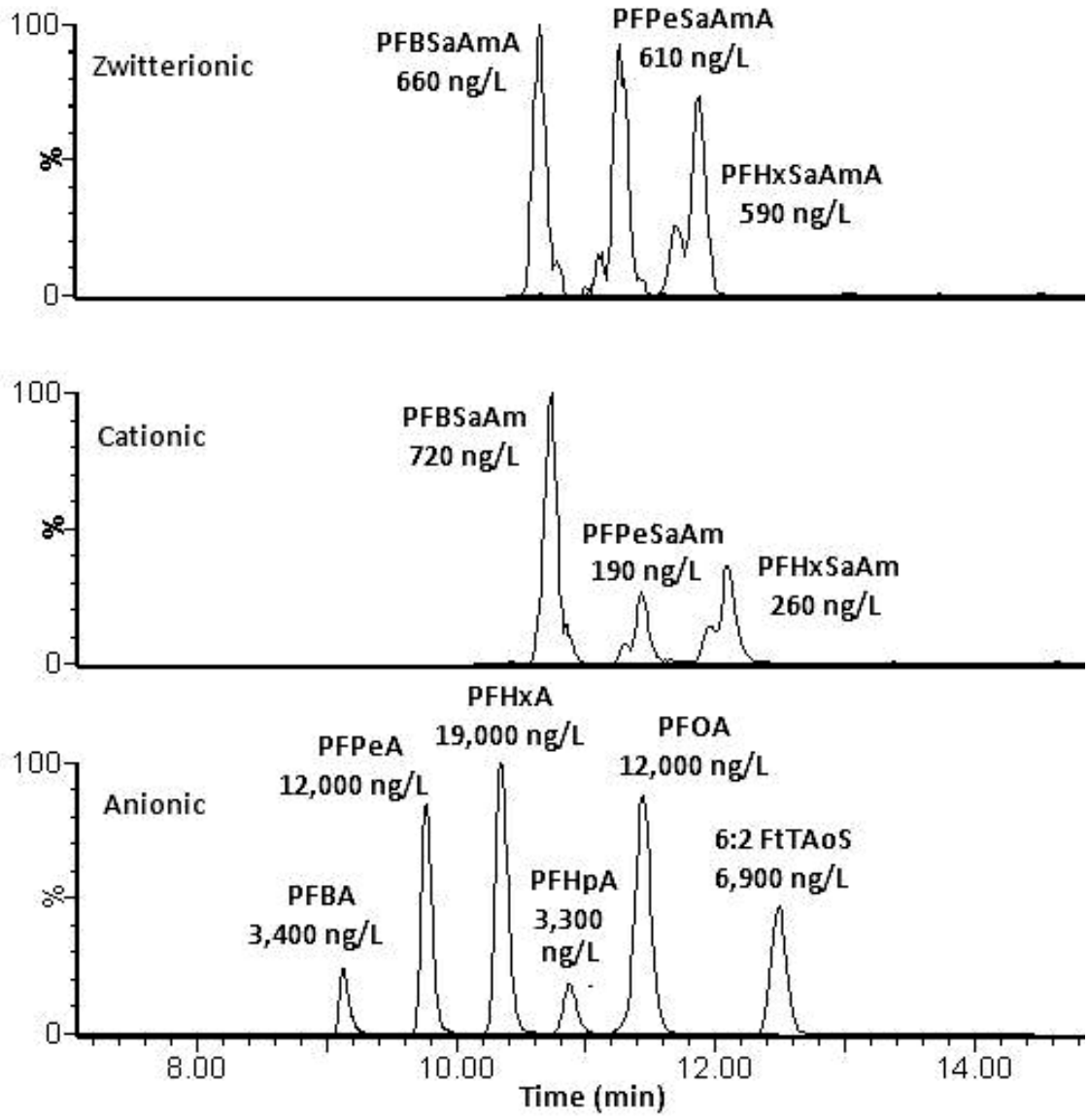

Figure 4.2) Typical chromatograms of selected zwitterionic, cationic, and anionic, and analytes detected in unspiked groundwater samples from military bases.

Table 4.1) The analytical validation parameters limit of detection (LOD), limit of quantitation (LOQ), whole-method accuracy and precision for quantitative and semi-quantitative analytes.

Analyte	LOD (ng/L)	LOQ (ng/L)	Accuracy[a] (%)	Precision[b] (% RSD)	Conc.[c] (ng/L)	Internal[d] Standard
6-2 FtTAoS	2.6	8.5	107 ± 8.7	9.1	40	[$^{13}C_2$]-PFHxA
6-2 FtTHN$^+$	5.0	16	101 ± 6.0	5.6	40	[$^{13}C_4$]-PFOS
6-2 FtSaB	23	76	131 ± 13	11	75	[$^{13}C_4$]-PFOS
6-2 FtSaAm	67	221	117 ± 7.7	8.2	700	[$^{13}C_4$]-PFOS
5-1-2 FtB	3.6	12	144 ± 13	10	35	[$^{13}C_4$]-PFOS
7-1-2 FtB	5.9	19	128 ± 12	11	40	[$^{13}C_4$]-PFOS
9-1-2 FtB	8.7	29	103 ± 5.4	11	170	[$^{13}C_4$]-PFOS
5-3 FtB	4.6	15	101 ± 13	15	75	[$^{13}C_4$]-PFBA
7-3 FtB	7.9	26	96 ± 8.4	10	150	[$^{13}C_4$]-PFBA
9-3 FtB	6.1	20	78 ± 8.3	13	50	[$^{13}C_4$]-PFBA
4-2 FtS	1.6	5.2	105 ± 11	12	40	[$^{18}O_2$]-PFHxS
6-2 FtS	0.84	2.8	99 ± 9.5	11	20	[$^{18}O_2$]-PFHxS
8-2 FtS	1.9	6.3	106 ± 9.1	10	20	[$^{18}O_2$]-PFHxS
PFBS	1.2	4.0	98 ± 9.5	11	40	[$^{18}O_2$]-PFHxS
PFHxS	1.7	5.5	96 ± 4.2	3.7	20	[$^{18}O_2$]-PFHxS
PFHpS	0.88	2.9	100 ± 9.1	11	20	[$^{13}C_4$]-PFOS
PFOS	0.81	2.7	104 ± 5.9	6.3	20	[$^{13}C_4$]-PFOS
PFDS	0.71	2.4	103 ± 2.9	2.8	20	[$^{13}C_4$]-PFOS
PFBA	4.1	14	106 ± 8.8	9.4	25	[$^{13}C_4$]-PFBA
PFPeA	1.1	3.7	102 ± 6.8	4.8	35	[$^{13}C_4$]-PFBA
PFHxA	1.4	4.7	101 ± 3.8	4.2	35	[$^{13}C_2$]-PFHxA
PFHpA	1.8	6.0	106 ± 9.8	11	25	[$^{13}C_2$]-PFHxA
PFOA	1.5	5.0	107 ± 7.7	8.5	25	[$^{13}C_4$]-PFOA
PFNA	1.0	3.3	99 ± 6.4	7.8	25	[$^{13}C_5$]-PFNA
PFDA	0.94	3.1	105 ± 7.3	8.4	25	[$^{13}C_2$]-PFDA
PFUdA	0.93	3.1	104 ± 8.7	9.1	25	[$^{13}C_2$]-PFUdA
PFDoA	1.0	3.4	103 ± 5.9	6.4	25	[$^{13}C_2$]-PFDoA
PFTrA	1.2	4.1	103 ± 6.7	7.1	25	[$^{13}C_2$]-PFDoA
PFTeA	1.7	5.6	106 ± 5.5	5.9	25	[$^{13}C_2$]-PFDoA

[a] Determined as the percentage of the ratio of the concentration determined by internal calibration over the concentration determined by standard addition (n = 6, ± 95 % CI).[b] Precision was calculated as the percent relative standard deviation (% RSD) from the samples used to determine accuracy (n = 6). [c] The nominal concentration (conc.) at which whole-method accuracy and precision were determined. [d] The internal standard used for internal calibration.

Table 4.2) Concentrations of the poly- and per- fluorinated chemicals detected in samples from Site A.

	Sample 1 ng/L	Sample 2 ng/L	Sample 3 ng/L	Sample 4 ng/L
4:2 FtTAoS[a]	<LOD	<LOD	210	490
6:2 FtTAoS	<LOD	<LOD	6,900	86
4:2 FtS	370	6,500	7,500	11,000
6:2 FtS	8,900	36,000	220,000	93,000
8:2 FtS	120	58	370	180
PFBSaAm[b]	2.8[c]	54	<LOD	<LOD
PFPeSaAm[b]	4.4 [c]	8.7	<LOD	<LOD
PFHxSaAm[b]	45	<LOD	<LOD	<LOD
PFBSaAmA[b]	<LOD	<LOD	<LOD	<LOD
PFPeSaAmA[b]	<LOD	<LOD	<LOD	<LOD
PFHxSaAmA[b]	<LOD	<LOD	<LOD	<LOD
PFBS	7,100	24,000	43,000	150,000
PFHxS	36,000	100,000	240,000	360,000
PFHpS	1,100	3,700	11,000	3,700
PFOS	19,000	15,000	78,000	19,000
PFDS	7.0	<LOD	<LOD	<LOD
PFBA	3,400	12,000	24,000	57,000
PFPeA	12,000	21,000	69,000	120,000
PFHxA	19,000	63,000	130,000	350,000
PFHpA	3,300	11,000	15,000	45,000
PFOA	12,000	35,000	51,000	220,000
PFNA	130	40	220	390
PFDA	17	<LOD	<3.1	6.5
PFUdA	<LOD	<LOD	<3.1	<LOD
PFDoA	<LOD	<LOD	<LOD	<LOD

[a] Calculated assuming equal molar response to 6:2 FtTAoS (see main text). [b] Calculated assuming equal molar response to PFOS (see Chapter 4). [c] Concentration above LOQ but below the lowest calibration standard.

Table 4.3) Concentrations of the poly- and per- fluorinated chemicals detected in samples from Site B.

	Sample 1 ng/L	Sample 2 ng/L	Sample 3 ng/L	Sample 4 ng/L	Sample 5 ng/L	Sample 6 ng/L	Sample 7 ng/L	Sample 8 ng/L
4:2 FtTAoS[a]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
6:2 FtTAoS	<LOD	<LOD	<LOD	68	<LOD	<LOD	<LOD	<LOD
4:2 FtS	<LOD	5.2	<LOD	44	<LOD	100	160	99
6:2 FtS	<LOD	1,400	210	860	3,500	15,000	3,900	37,000
8:2 FtS	<LOD	660	660	66	1,200	2,300	620	1,400
PFBSaAm[b]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFPeSaAm[b]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFHxSaAm[b]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFBSaAmA[b]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	4.1[c]	<LOD
PFPeSaAmA[b]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFHxSaAmA[b]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	8.0	<LOD
PFBS	12	1,500	640	7,300	2,900	2,800	17,000	24,000
PFHxS	81	11,000	10,000	9,800	18,000	17,000	74,000	170,000
PFHpS	<LOD	580	410	120	920	490	1,700	4,100
PFOS	88	15,000	23,000	4,000	29,000	20,000	44,000	65,000
PFDS	<LOD	33	<LOD	<LOD	16	<LOD	<LOD	26
PFBA	8.5	1,100	980	3,000	2,000	1,700	5,900	13,000
PFPeA	4.9[c]	2,000	1,800	8,100	3,300	6,000	15,000	35,000
PFHxA	<4.7	5,400	2,400	12,000	11,000	7,700	29,000	99,000
PFHpA	<6.0	480	1,600	860	670	1,200	1,300	7,200
PFOA	8.6	890	2,500	840	1,700	3,700	3,000	57,000
PFNA	<LOD	56	680	15	110	110	130	400
PFDA	<3.1	8.0	19	<3.1	12	10	7.6	17
PFUdA	<LOD	3.7[c]	5.2	<3.1	4.2[c]	<3.1	<3.1	4.9[c]
PFDoA	<LOD	<3.4	<LOD	<LOD	<LOD	<3.4	<LOD	<3.4

[a] Calculated assuming equal molar response to 6:2 FtTAoS (see main text). [b] Calculated assuming equal molar response to PFOS (see Chapter 4). [c] Concentration above LOQ but below the lowest calibration standard.

Table 4.4) Concentrations of the newly-identified perfluorinated chemicals detected in archived samples from Wuirtsmith Air Force Base (WAFB), Naval Air Station Fallon (NASF), and Tyndall Air Force Base (TAFB). See Schultz et. al. for sample descriptions (e.g. FT-3) and data on fluorotelomer sulfonates, perfluoroalkyl sulfonates, and perfluoroalkyl carboxylates. (4)

	TAFB TY22FtA	TAFB T 11-2	TAFB PW-7	TAFB PW-10	NASF MW 16	NASF MW 51-U	WAFB FT-3
	ng/L	ng/L	ng/L	ng/L	ng/L	ng/L	ng/L
4:2 FtTAoS [a]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
6:2 FtTAoS	<LOD	<LOD	<LOD	8.8	<LOD	<LOD	<LOD
PFBSaAm [b]	4.1 [c]	11	<LOD	720	<LOD	550	26
PFPeSaAm [b]	2.8 [c]	7.8	5.1	190	<LOD	61	79
PFHxSaAm [b]	5.7	8.3	6.3	260	<LOD	260	36
PFBSaAmA [b]	<LOD	<LOD	62	660	<LOD	9.7	<LOD
PFPeSaAmA [b]	<LOD	<LOD	7.9	610	<LOD	5.8	<2.7
PFHxSaAmA [b]	<LOD	<LOD	10	590	<LOD	38	<2.7

[a] Calculated assuming equal molar response to 6:2 FtTAoS (see main text). [b] Calculated assuming equal molar response to PFOS (see Chapter 4). [c] Concentration above LOQ but below the lowest calibration standard.

Chapter 5 - Conclusions and Suggested Directions for Future Research

The research presented in this dissertation was based on analytical method development using large-volume injection (LVI) high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) detection. Large-volume injection is an adaptable HPLC technique that can be used to replace extraneous steps that exist within analytical methods based on solid-phase extraction (SPE). Methods were validated and applied for the analysis of emerging environmental contaminants in aqueous matrices. Through the development of these methods, the general perception of what was considered possible by large-volume injection was challenged. For example, injecting 4,500 uL sample volumes without loss of chromatographic performance or successfully applying large-volume injection to organic extracts was shown to be possible. Additionally, similar or improved method performance (e.g. accuracy, percesion, etc.) was achieved by replacing SPE with LVI.

Endocrine-disrupting chemicals are classes of emerging contaminants that have received considerable attention since the mid 1990s. However, most of this attention is focused on estrogenic steroids or estrogen-mimicking chemicals. The focus on estrogens was generated from studies documenting estrogenic effects on aquatic wildlife in waters adjacent to wastewater treatment plant discharge. However, wastewater is also reported to be androgenically active and more research was needed on androgenic steroids in waste- and receiving-water because less is known about

them. In regard to this, a large-volume injection based method was developed to quantify natural and synthetic steroids in waste- and river- water. The method was then used to assess the temporal profiles of androgenic steroids in wastewater influent. Two synthetic androgens, boldenone and nandrolone, on which there is scarce environmental data, were detected in the wastewater samples. Boldenone was present in all the wastewater samples at higher concentrations that testosterone, an endogenous hormone. Additionally, boldenone had a temporal signature in wastewater that indicated it came from an endogenous source. However, whether the source of boldenone was from community steroid abuse, from *in-situ* transformation of a related compound, or endogenous production was not determined. Additional research is needed to determine if boldenone is universally present in wastewater at other locations. Boldenone is more androgenic than testosterone so if it occurs at elevated levels elsewhere and survives wastewater treatment it could adversely affect aquatic wildlife living in impacted ecosystems. Toward that end, additional research is needed to characterize the fate and removal efficiency of both the conjugated and unconjugated fractions of natural and synthetic androgenic steroids during wastewater treatment.

While large-volume injection is applied to a number of analytical methods (see examples reported in chapters 2 through 4), there is still a perception that large-volume injection analysis will result in shorter column lifetimes, instrument fouling, decreased sensitivity, matrix interferences, and poor chromatography. Because of these perceptions, many analysts assume that solid-phase extraction is necessary for

the analysis of environmental samples. To challenge these ingrained perceptions, large-volume injection was compared against solid-phase extraction to determine if they gave similar performance. Three classes of emerging contaminants were chosen as target analytes and wastewater influent as a model matrix because of its complexity. Matrix effects were compared across methods because they are the major non-instrumental and non-laboratory variable that influences sensitivity, accuracy, and precision. It was demonstrated that because redundant separation chemistry is often performed for solid-phase extraction and HPLC, there is no improvement over large-volume injection in reducing matrix effects. Offline preconcentration is eliminated by large-volume injection; therefore, sample preparation was reduced from several hours for solid-phase extraction to a few minutes. Additionally, a reduction in the solvents, materials, labor and costs was achieved by employing large-volume injection instead of solid-phase extraction. This research was aimed at providing a convincing argument for analysts to adopt large-volume injection as a replacement for solid-phase extraction. However, research is still needed to compare other variables between methods that employ large-volume injection and solid-phase extraction including analytical-column lifetimes, frequency of instrument maintenance, and method robustness. Finally, future large-volume injection methods should adapt the power of orthogonal separation chemistries and be applied to the analysis of solids as well as biological fluids.

Per- and poly-fluorinated chemicals (e.g. perfluoroalkyloctane sulfonate [PFOS], perfluoroalkyloctane carboxylate [PFOA], and 6:2 fluorotelomer sulfonate [6:2 FtS])

are becoming well-studied emerging contaminants due to their recalcitrance to degradation, environmental ubiquity, and the debate over their toxicity. Possible degradation precursors to these per- and poly-fluorinated chemicals were recently identified in fire-fighting foams used at military bases. There was no previous environmental data on these newly-identified fluorinated chemicals. However, it is well established that at sites where fire-fighting foams are discharged into the environment, there exists elevated levels of PFOS, PFOA, 6:2 FtS, and their homologs. A non-aqueous large-volume injection method using orthogonal HPLC was developed to assess the occurrence of the newly-identified fluorinated chemicals in groundwater. Groundwater collected from six military sites within the United States was micro liquid-liquid extracted to eliminate issues with analyte stability in aqueous samples. The extracts were then directly analyzed without further sample preparation. The newly-identified fluorinated chemicals were confirmed as occurring in groundwater along with other per- and poly-fluorinated chemicals, albeit at much lower concentrations. This is the first reported account of these chemicals as environmental contaminants. However, this study has resulted in questions concerning the environmental fate and degradation of these newly-identified fluorinated chemicals. More research is needed to determine if these newly-identified fluorinated chemicals occur in other environmental media. For example, an explanation as to why the newly-identified fluorinated chemicals are present at much lower concentrations in groundwater when compared other per- and poly-fluorinated chemicals (e.g. PFOS) is that they are associated with the soils and sediments. Additionally, it is very likely that

through degradation these newly-identified fluorinated chemicals are sources of persistent fluorinated chemicals including PFOS, PFOA, 6:2 FtS, and their homologs. However, because these chemicals were just recently identified, their environmental degradation pathways are not known and this is an area for future research. Finally, the ecotoxicity of those newly-identified fluorinated chemicals that are environmentally relevant should be examined to determine if the concentrations at which they occur in the environment pose a threat to the wildlife or humans.

In final conclusion, the work contained within this dissertation was at times challenging and frustrating but always rewarding and interesting. It was satisfying to see the fruits of my research and conveying the results to others. Additionally, I feel a great sense of accomplishment in that I was able to adapt the knowledge I acquired during my tenure at Oregon State University to develop and solve research questions. I would like to thank everyone who made that possible.

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Appendices

Appendix 1 - Supporting Information: Chapter 2 - Analysis of Androgenic Steroids in Environmental Waters by Large-Volume Injection Liquid Chromatography Tandem Mass Spectrometry

A.1.1 Inter and Intra Day RSD. The inter- and intra-day RSDs in wastewater influent ranged from 1.6 to 6.8 and 3.7 to 6.8 %, respectively, while the combined RSDs ranged from 4.1 to 8.2 % for analytes with stability regression slopes that are statistically equivalent to zero (Table A.2.2). Combining intra and inter-day RSD describes the overall precision of the method within and between days. (1) Combined RSDs are rarely calculated, but the inter-day RSDs presented here are slightly lower than what has been presented for androgens, although the sample matrix of that study was effluent. (2)

A.1.2 Literature Cited.

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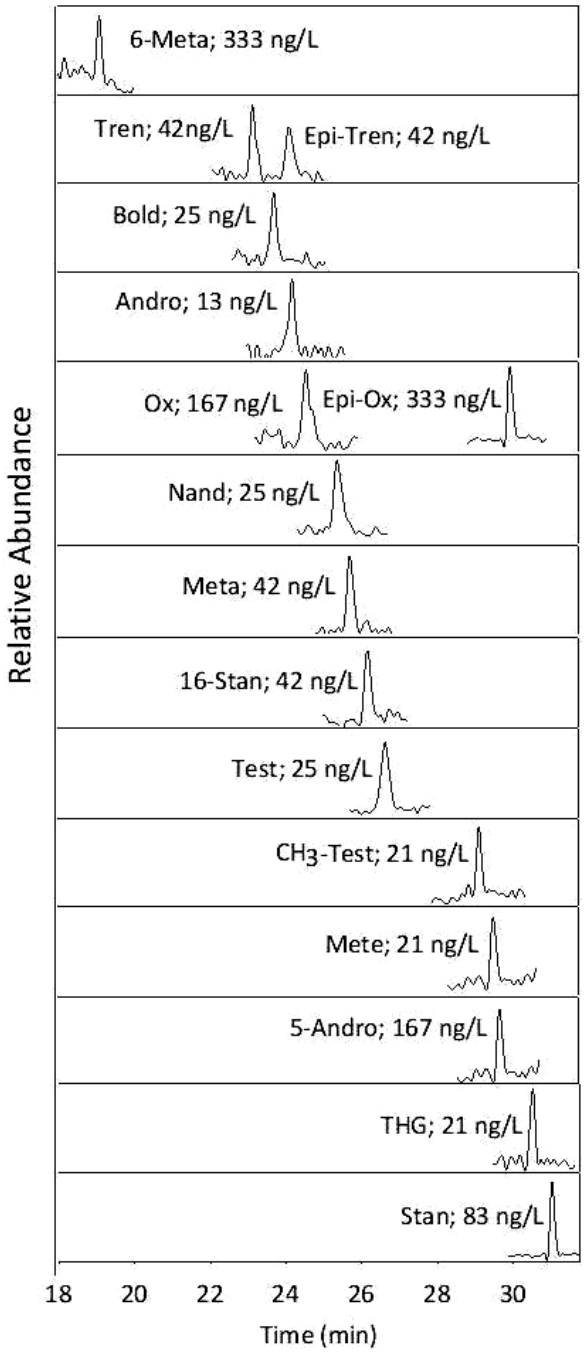

Figure A.1.1) Chromatograms of analytes spiked into wastewater effluent near their LOD or LOQ.

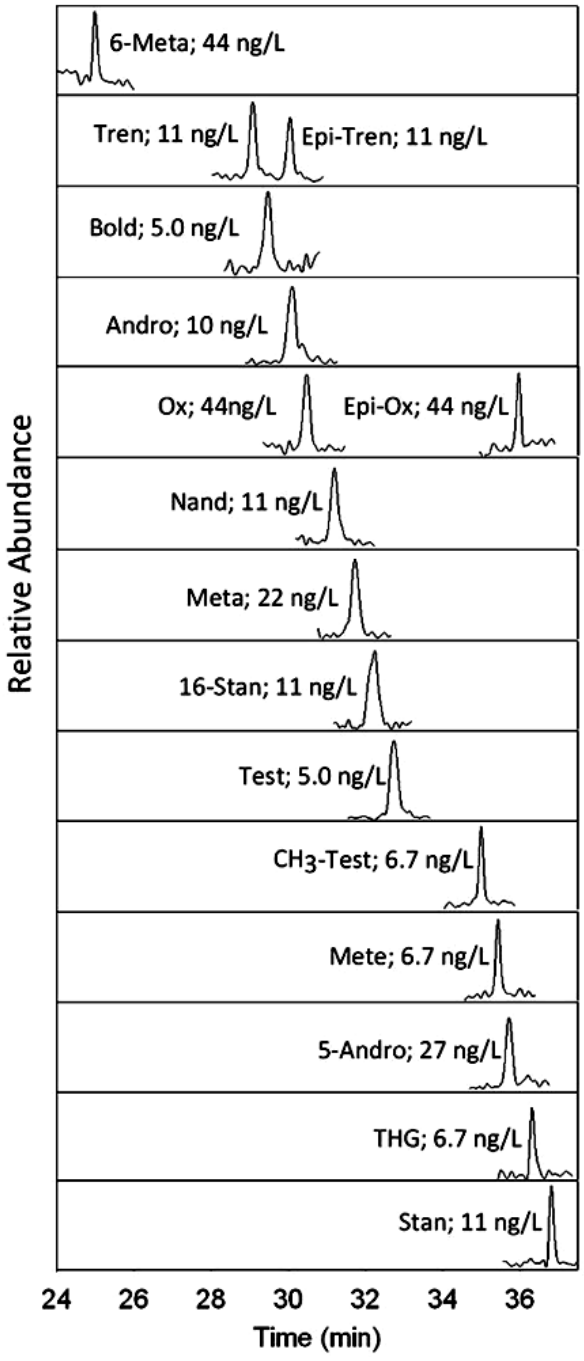

Figure A.1.2) Chromatograms of analytes spiked into river water near their LOD or LOQ.

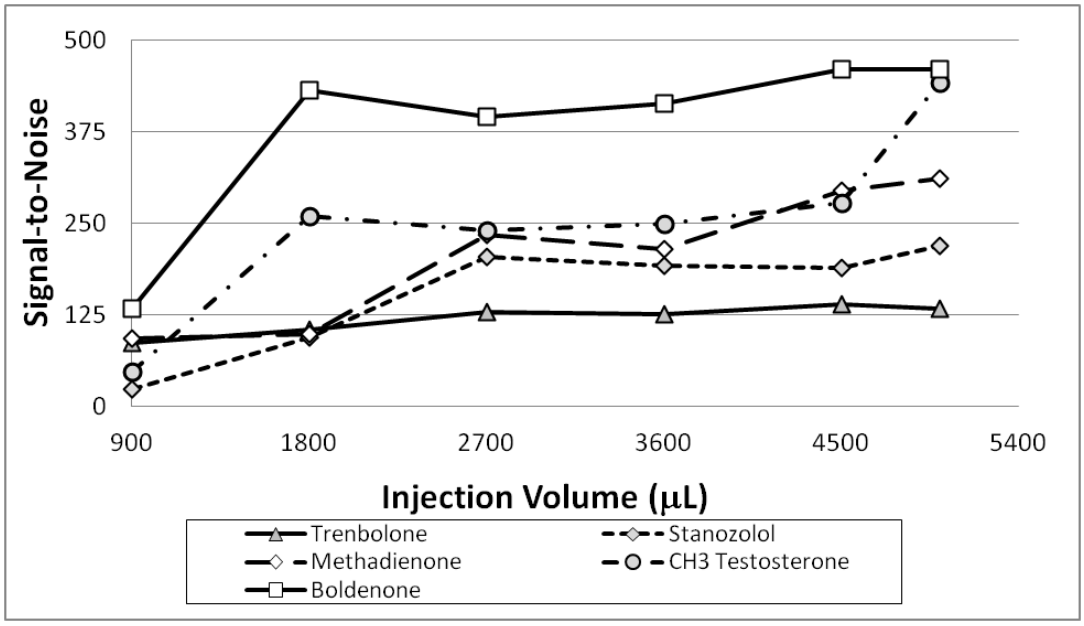

Figure A.1.3) Signal-to-noise as a function of injection volume for selected androgens in wastewater. Analyte concentration was kept constant at 150 ng/L.

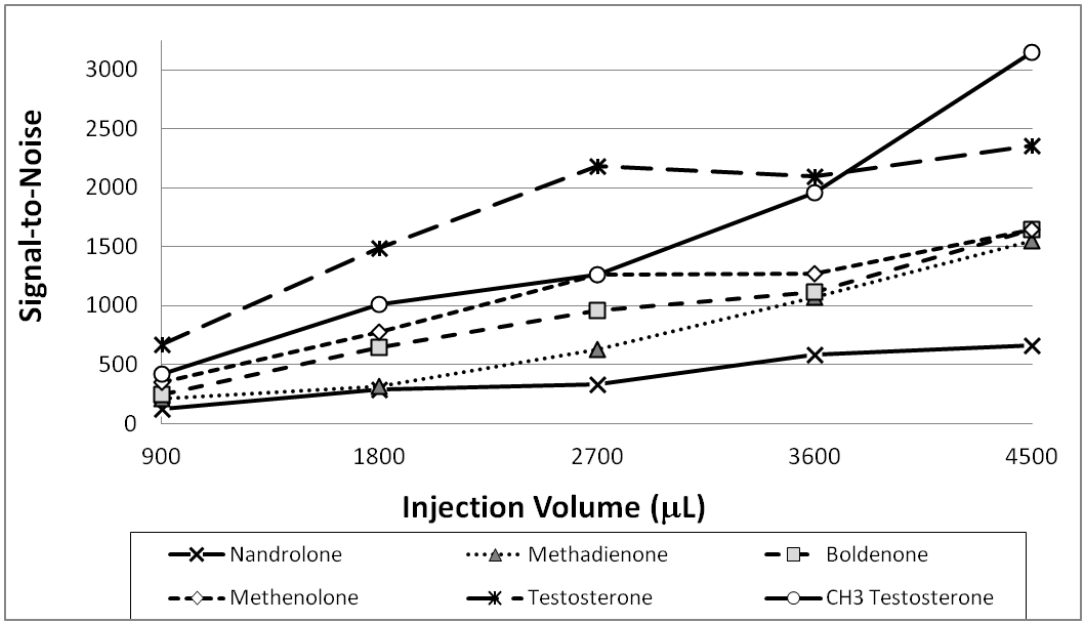

Figure A.1.4) Signal-to-noise as a function of injection volume of selected analytes in fish-housing water. Analyte concentration was kept constant at 150 ng/L.

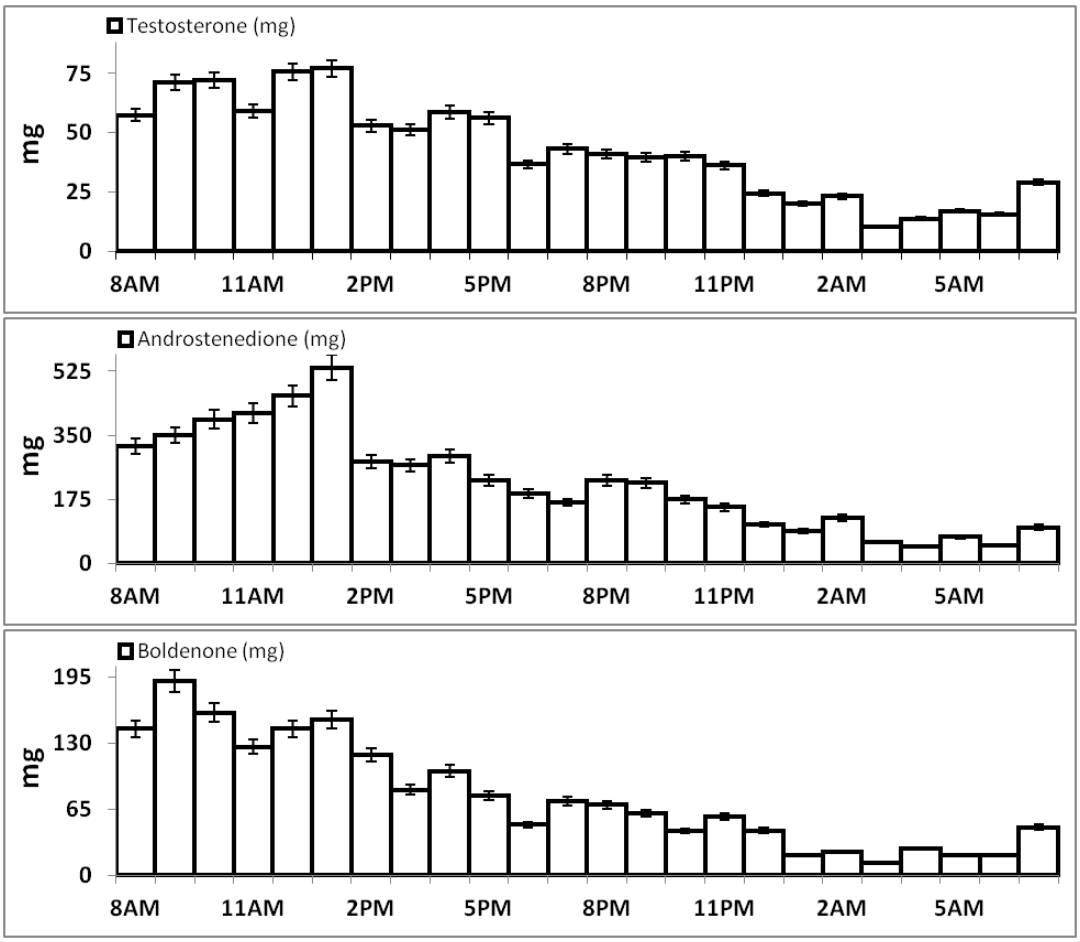

Figure A.1.5) Diurnal profiles of analyte concentrations (± within-day RSD) present in the one hour composite samples of influent. Nandrolone was excluded because most values were below the LOQ. The error bars are represented by mass values (mg) multiplied by the within run RSD.

Table A.1.1) Comparison of concentration values (± 95% CI) obtained by standard addition and internal standard calibration for analytes in wastewater influent.

Analyte	Standard Addition Concentration (ng/L)	Internal Standard Calibration Concentration (ng/L)
Test	102 ± 3.9	97.2 ± 4.9
Ando	54.5 ± 3.0	52.6 ± 3.7
Bold	61.6 ± 7.0	59.0 ± 3.8
5-Andro	1620 ± 40	1260 ± 120*
Meta	215 ± 9.0	216 ± 9.0
6-Meta	1630 ± 130	997 ± 130*
Stan	218 ± 6.7	215 ± 15
16-Stan	209 ± 11	202 ± 12
Tren	203 ± 15	188 ± 7.5
Epi Tren	196 ± 11	188 ± 4.5
Mete	191 ± 6.7	189 ± 17
CH_3-Test	209 ± 6.6	204 ± 5.7
Nand	214 ± 11	208 ± 17
THG	199 ± 9.4	204 ± 20
Ox	790 ± 33	775 ± 11
Epi-Ox	1580 ± 77	1360 ± 120*

*indicates statistical difference between concentrations values at the 95% CI.

Table A.1.2) RSDs calculated from a one-way ANOVA analysis of the stability study data.

	Intra Day RSD %	Inter Day RSD %	Combined RSD %
Test	5.1	6.3	8.1
Ando	4.9	6.2	7.9
Bold	4.8	1.7	5.1
5-Andro	5.9	3.1	6.7
Meta	5.4	4.1	6.8
6-Meta	6.2	5.3	8.2
Stan	4.7	3.4	5.8
16-Stan	5.8	5.8	8.2
Tren	5.5	3.1	6.3
Epi-Tren	5.7	1.6	6.0
Mete	5.4	4.1	6.8
CH3-Test	3.7	1.9	4.1
Nand	4.8	3.7	6.0
THG	6.8	4.5	8.1
Ox	4.6	6.8	8.2
Epi-Ox	6	11	12

Appendix 2 - Supporting Information: Chapter 3 - Is SPE Necessary for Environmental Analysis? A Quantitative Comparison of Matrix Effects from Large-Volume Injection and Solid-Phase Extraction Based Methods.

A.2.1 Chemicals. The estrogens (estrone (E1), estradiol (E2), estriol (E3), and ethinylestradiol (EE2)) were acquired from Steraloids, Inc (Newport, RI). The perfluoroalkyl carboxylates (perfluro-n-butanoic acid (PFBA), perfluoro-n-pentanoic acid (PFPeA), perfluoro-n-hexanoic acid (PFHxA), perfluoro-n-heptanoic acid (PFHpA), perfluoro-n-octanoic acid (PFOA), perfluoro-n-nonanoic acid (PFNA), perfluoro-n-decanoic acid (PFDA), perfluoro-n-undecanoic acid (PFUdA)) were purchased as a mixture in methanol at concentrations of 2 µg/mL each from Wellington Laboratories (Guelph, Ontario). The perfluoroalkyl sulfonates (perfluoro-1-butanesulfonate (PFBS), perfluoro-1-hexanesulfonate (PFHxS), perfluoro-1-heptanesulfonate (PFHpS), perfluoro-1-octanesulfonate (PFOS), and perfluoro-1-decanesulfonate (PFDS)) were purchased as a mixture in methanol at concentrations of 2 µg/mL each from Wellington Laboratories. HPLC grade methanol, HPLC grade acetone, and ammonium acetate (NH4 OAc) were purchased from Sigma-Aldrich (Saint Louis, MO). B&J Brand® water (Morristown, NJ) was pre-treated by filtering the water through a 4.6 x 150 mm 3.5 µm particle diameter ZORBAX Eclipse Plus-C18 analytical column (Agilent, Santa Clara, CA) as a precautionary step to remove any possible perfluoroalkyl carboxylate and/or sulfonate contamination. Treated B&J Brand® water was collected in 20 mL volumes in a baked (450 ^{o}C for 12 hr) glass container. The column used to pre-treat reagent water was regenerated with 10 mL of

methanol. Volumes of 20 mL of water were collected because the weakest retained possible contaminant (PFBA) had a calculated retention volume of 24.4 mL in water (See Below). Filtered reagent water produced no detectable perfluoroalkyl carboxylate or sulfonate contamination.

A.2.2 Calculated Analyte Retention in Water. The retention factor (k') of each analyte in a 4.6 x 150 mm 3.5 µm particle diameter ZORBAX Eclipse Plus-C18 analytical column (Agilent, Santa Clara, CA) was determined under isocratic conditions for each compound. The log k' of each analyte was plotted against the fraction of methanol (ϕ MeOH) used for isocratic separations (Figure A.2.2). From the line log k' over ϕ MeOH the capacity of the analyte in water (k_w') can be solved by the formula of the linear-solvent-strength model, $\log k' = \log k_w' - S \phi$ MeOH, where S is the slope of the line. (1) The retention volume of each analyte in water ($V_{r\ water}$) can then be calculated by $V_{r\ water} = V_o (1 + k_w')$ where V_o is the column void volume.

A.2.3 Literature Cited.

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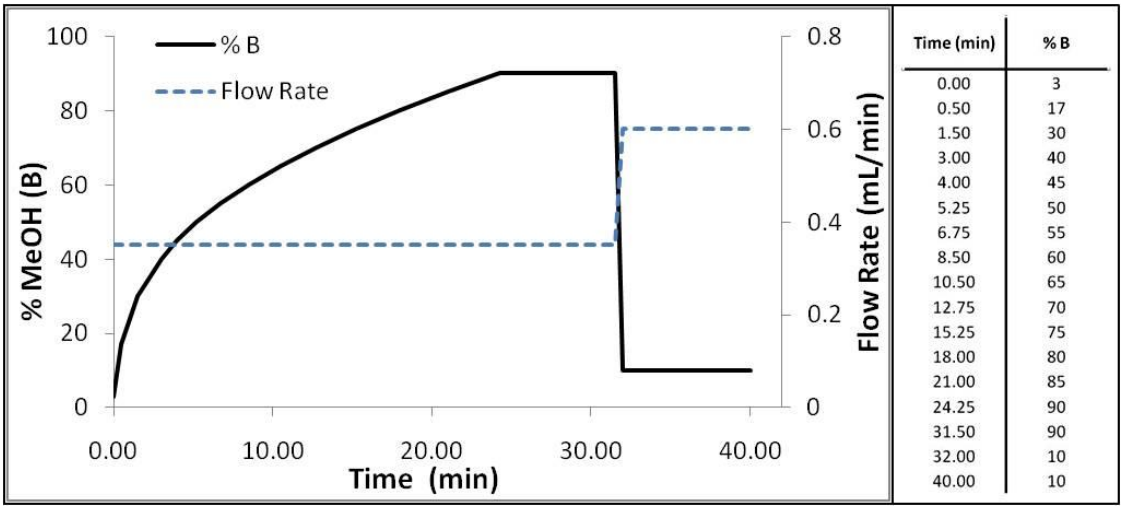

Time (min)	% B
0.00	3
0.50	17
1.50	30
3.00	40
4.00	45
5.25	50
6.75	55
8.50	60
10.50	65
12.75	70
15.25	75
18.00	80
21.00	85
24.25	90
31.50	90
32.00	10
40.00	10

Figure A.2.1) Change in % MeOH (B) and flow rate as a function of time for 18uL Injections. For 900 µL injections, the initial gradient composition was held for 2 min during sample loading.

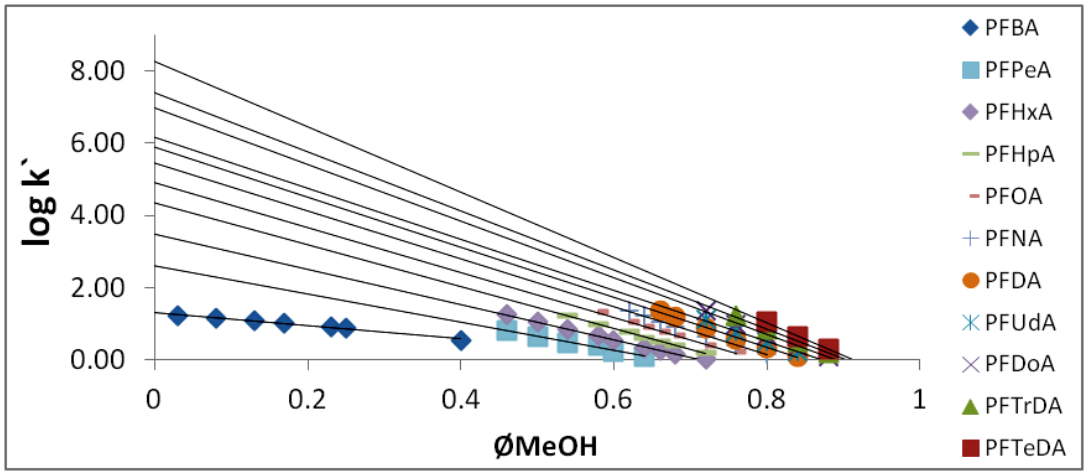

Figure A.2.2) Changes in log k' versus the fraction of methanol (ϕ MeOH) used for the isocratic separations. The y-intercept is k' water.

Table A.2.1) Analyte precursor ions, product ions, and compound dependant mass spectrometric parameters (collision energy (CE) and cone voltage (CV)).

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	CE (V)	CV (V)
PFBA	213	**169**	8	20
		NA	NA	
PFPeA	263	**219**	8	20
		NA	NA	
PFHxA	313	**269**	8	20
		119	22	
PFHpA	363	**319**	8	20
		169	14	
PFOA	413	**369**	8	20
		169	18	
PFNA	463	**419**	8	22
		169	18	
PFDA	513	**469**	10	22
		269	18	
PFUdA	563	**519**	10	22
		169	22	
PFDoA	613	569	10	22
		169	24	
PFTrDA	663	**619**	12	24
		169	26	
PFTeDA	713	**669**	12	24
		169	26	

Product ions in bold indicate the ion used for quantitation. The other ion is used for qualitative analyte conformation.

Table A.2.1) (Continued)

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	CE (V)	CV (V)
PFBS	299	**80**	32	50
		99	26	
PFHxS	399	**80**	36	58
		99	28	
PFHpS	449	**80**	46	64
		99	32	
PFOS	499	80	46	70
		99	34	
PFDS	599	**80**	52	76
		99	36	

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	CE (V)	CV (V)
E1	269	**145**	34	64
		159	34	
E2	271	**145**	36	70
		183	36	
E3	287	**145**	36	70
		171	40	
EE2	295	**145**	40	64
		159	36	

Product ions in bold indicate the ion used for quantitation. The other ion is used for qualitative analyte conformation.

Table A.2.2) Percent matrix effects ± 95 % confidence interval for LVI-, HLB SPE-, and C18 SPE-based analyses of wastewater at low and high ratios of analyte-to-matrix mass. 100% = no matrix effects, <100% = signal suppression, >100% = signal enhancement.

	% Matrix Effects					
	High Spike Level			Low Spike Level		
	LVI	**HLB**	**C18**	**LVI**	**HLB**	**C18**
E1	72.5 ± 2.06	71.6 ± 2.25	68.2 ± 2.17	47.0 ± 3.03	38.0 ± 1.49	39.1 ± 2.28
E2	73.6 ±2.98	69.9 ± 3.78	66.9 ± 3.27	37.2 ± 2.59	22.7 ± 2.04	28.4 ± 1.95
E3	68.7 ± 1.85	72.4 ± 3.04	76.5 ± 2.54	54.6 ± 4.49	55.5 ± 1.23	55.2 ± 2.03
EE2	62.2 ± 2.12	62.1 ± 4.30	60.1 ± 4.03	16.7 ± 6.37	11.6 ± 1.75	12.5 ± 1.29
PFBA	63.8 ± 1.76	101 ± 6.31	102 ± 6.31	64.7 ± 13.2	81.9 ± 15.7	89.6 ± 19.7
PFPeA	104 ± 3.46	109 ± 5.87	114 ± 9.36	52.2 ± 19.8	61.5 ± 23.1	96.8 ± 11.2
PFHxA	96.6 ± 4.67	101 ± 5.66	101 ± 6.72	78.0 ± 18.5	75.1 ± 36.8	107 ± 14.4
PFHpA	120 ± 5.16	120 ± 4.95	118 ± 7.98	77.2 ± 21.5	90.2 ± 10.9	79.0 ± 20.2
PFOA	110 ± 1.22	118 ± 6.44	116 ± 6.40	87.3 ± 14.1	89.2 ± 39.2	95.6 ± 33.3
PFNA	101 ± 1.92	106 ± 1.84	102 ± 3.96	95.7 ± 14.6	82.9 ± 12.5	95.1 ± 12.6
PFDA	107 ± 2.03	96.2 ± 2.19	98.5 ± 4.51	110 ± 11.6	108 ± 20.7	110 ± 18.3
PDUdA	112 ± 6.16	105 ± 5.38	107 ± 8.64	101 ± 28.7	101 ± 19.1	97.6 ± 17.7
PFBS	91.4 ± 3.27	91.2 ± 13.5	95.0 ± 4.05	71.5 ± 35.5	64.5 ± 27.4	91.4 ± 13.6
PFHxS	92.8 ± 3.08	91.7 ± 2.79	89.0 ± 4.90	85.5 ± 11.3	76.9 ± 12.0	84.7 ± 14.3
PFHpS	96.0 ± 2.42	88.3 ± 1.51	86.1 ± 3.82	82.7 ± 6.31	84.8 ± 6.58	88.8 ± 9.29
PFOS	90.9 ± 5.46	82.1 ± 2.49	82.6 ± 5.30	75.6 ± 7.91	88.3 ± 17.3	62.6 ± 16.5
PFDS	93.2 ± 7.91	82.5 ± 4.65	84.1 ± 4.94	91.6 ± 4.61	96.4 ± 25.38	87.1 ± 18.8

Table A.2.3) A comparison of matrix effects on androgenic steroids in wastewater using ESI+. 100% = no matrix effects, <100% = signal suppression, >100% = signal enhancement.

Chemical	Backe et al. [a]	Schröder et al. [b]	Chang et al. [c]
	LVI	HLB SPE	HLB + Si SPE
Testosterone	94% ± 4.0%	> 89%	89%
Boldenone	81% ±1.9%	>86%	NA[d]
Stanozolol	100% ± 7.2%	>88%	95%

[a] Backe, W. J., Ort, C., Brewer, A. J., Field, J. A., *Anal. Chem.* **2011,** *83*. 2622-2630. (n = 4) 75 ng/L. Determined using D_3-Testoterone, D_3-Boldenone, and D_3-Stanozolol. Raw area counts used to calculate matrix effects were previously unreported.

[b] Schroder, H. F.; Gebhardt, W.; Thevis, M. *Anal. Bioanal. Chem*. **2010**, *398*, 1207–1229. (n = 3) 25 ng/L. Adapted from % signal suppression data presented in Table 3.

[c] Chang, H.; Wu, S.; Hu, J.; Asami, M.; Kunikane, S. *J. Chromatogr., A* **2008**, *1195*, 44–51. (n=1) between 1 and 100 µg/L. Calculated from data presented in Figure 2.

Appendix 3 - Supporting Information: Chapter 4 - Newly-Identified Cationic, Anionic, and Zwiterionic Fluorinated Chemicals in Groundwater at U.S. Military Bases by non-Aqueous Large-Volume Injection HPLC-MS/MS

A.3.1 Chemicals. A mixture of perfluoroalkyl carboxylates [perfluro-n-butanoic acid (PFBA), perfluoro-n-pentanoic acid (PFPeA), perfluoro-n-hexanoic acid (PFHxA), perfluoro-n-heptanoic acid (PFHpA), perfluoro-n-octanoic acid (PFOA), perfluoro-n-nonanoic acid (PFNA), perfluoro-n-decanoic acid (PFDA), perfluoro-n-undecanoic acid (PFUdA)] were purchased from Wellington Laboratories (Guelph, Ontario) in a methanol solvent at concentrations of 2 μg/mL each. A mixture of perfluoroalkyl sulfonates [(perfluoro-1-butanesulfonate (PFBS), perfluoro-1-hexanesulfonate (PFHxS), perfluoro-1-heptanesulfonate (PFHpS), perfluoro-1-octanesulfonate (PFOS), and perfluoro-1-decanesulfonate (PFDS)] were purchased from Wellington Laboratories in a methanol solvent at concentrations from 1.77 μg/mL to 1.93 μg/mL. The perfluoroalkyl carboxylates and sulfonates all have purities of > 98%.The fluorotelomer sulfonates 1H,1H,2H,2H-perfluoro-1-hexanesulfonate (4-2 FtS), 1H,1H,2H,2H-perfluoro-1-octanesulfonate (6-2 FtS), and 1H,1H,2H,2H-perfluoro-1-decanesulfonate (8-2 FtS) were generously donated by Chris Higgins at the Colorado School of Mines as individual solutions in methanol at 6 □g/mL. The internal standards [perfluoro-1-hexane[$^{18}O_2$]sulfonate ([$^{18}O_2$]-PFHxS), perfluoro-1-[1,2,3,4-$^{13}C_4$]octanesulfonate ([$^{13}C_4$]-PFOS), perfluoro-n-[$^{13}C_4$]butanoic acid ([$^{13}C_4$]-PFBA) perfluoro-n-[1,2-$^{13}C_2$]hexanoic acid ([$^{13}C_2$]-PFHxA), perfluoro-n-[1,2,3,4-$^{13}C_4$]octanoic acid ([$^{13}C_4$]-PFOA), perfluoro-n-[1,2,3,4,5-$^{13}C_5$]nonanoic acid ([$^{13}C_5$]-

PFNA), perfluoro-n-[1,2-$^{13}C_2$]decanoic acid ([$^{13}C_2$]-PFDA), perfluoro-n-[1,2-$^{13}C_2$]undecanoic acid ([$^{13}C_2$]-PFUdA), and perfluoro-n-[1,2-$^{13}C_2$]dodecanoic acid ([$^{13}C_2$]-PFDoA)] were purchased from Wellington Laboratories as a mixture in methanol at approximately 2 μg/mL and are 94% to 99% isotopically pure.

Commercial source materials containing 6-2 FtSaB, 6-2 FtSaAm, 6-2 FtTAoS, 6-2 $FtTHN^+$, 5-1-2 FtB, 7-1-2 FtB, 9-1-2 FtB, 5-3 FtB, 7-3 FtB, 9-3 FtB (Table S1) were provided by the Fire Fighting Foam Coalition (FFFC). HPLC grade methanol (> 99%) and ethyl acetate (> 99%), GC grade 2,2,2-trifluoroethanol (> 99%), and ammonium acetate (≅ 98%) were purchased from Sigma-Aldrich (Saint Louis, MO). B&J Brand® reagent water (> 99%) was purchased from VWR (Radnor, PA) and sodium chloride was acquired from Mallinckrodt Chemical (> 99%).

A.3.2 Representative Subsampling. Preliminary data (not shown) revealed that area counts of perfluoroalkyl carboxylates (> C6) and perfluoroalkyl sulfonates (> C4) decreased by 50% to 100% over 6 hr while the analytes were in 3% MeOH/97% reagent water in 6mL glass autosampler vials. This loss was attributed to adsorption onto vials and stratification which indicated that samples that sat for a period of time were no longer homogeneous. As such, the ability of the subsampling protocol in obtaining a representative subsample needed to be determined.

To assess representative subsampling, a volume of 200 mL of blank groundwater in a 250 mL HDPE bottle was spiked to final concentrations of 400 ng/L for each quantitative analyte (Figure 1 in main text) and to final concentrations ranging from 84

ng/L to 1,300 ng/L for each semi-quantitative analyte (Figure 1 in main text). Representative subsampling was not assessed for qualitative analytes. The 200 mL groundwater sample was allowed to sit overnight to allow the PFCs to stratify, (1) agitate, (2) adsorb to the container, (3) or any other phenomena that would result in non-representative sub-sampling. The next day right before sub-sampling, the 200 mL sample was repeatedly sonicated in a Model 75HT heated (60^{o}C) sonication bath (VWR, Radnor, PA) then gently agitated and inverted. After sonication and agitation a 3 mL subsample was taken from approximately 3.0 cm to 3.5 cm below the meniscus and delivered to a 5 mL micro tube. The subsample was extracted and analyzed as outlined in Chapter 4.

The representativeness of subsampling was defined as the percentage of the analyte concentration in the subsample over that of spiked concentration in the 200mL sample. (n=5, ± 95% CI). The representativeness of the subsampling ranged from 76% ± 2.8% (PFDoA) to 106% ± 8.1% (4:2 FtS) for quantitative analytes (Table A.3.7) and from 62% ± 2.5% (6:2 FtTHN^{+}) to 126% ± 12% (5:1:2 FtB) for semi-quantitative analytes (Table A.3.7). Overall, the protocol was deemed acceptable and the analyte concentrations in groundwater were not corrected for subsampling.

A.3.3 Estimating Analyte Concentrations Assuming Equal Molar Response. Estimations of qualitative analyte concentrations are performed by assuming equal molar response to a related analyte. For example, for PFBSaAm, the response of PFBSaAm is ratioed to the response of [$^{13}C_4$]-PFOS (Table A.3.3). The concentration

that corresponds to that response ratio is calculated using PFOS's calibration curve (Table A.3.3). Then, the concentration value is multiplied by ratio of the molecular weight of PFOS over the molecular weight of PFBSaAm (499/385) to correct for the difference in the number of molecules per unit weight of each analyte.

A.3.4 Absolute Extraction Efficiency. To determine the absolute efficiency of the extraction procedure, groundwater that gave no detectable analyte signal was ***spiked first*** with analytes to give final concentrations of between 50 and 450 ng/L then extracted (**pre-extraction spikes**). The pre-extraction spikes were compared to groundwater that was ***extracted first*** then spiked with analytes to equivalent concentrations (**post-extraction spikes**). Absolute extraction efficiency was defined as the ratio of pre-extraction spike area counts (n = 5) to post-extraction spike area counts (n = 5) multiplied by 100. The error about each measurement was compounded and reported as ± 95 % CI. Only Ql and Sq analytes were assessed for extraction efficiency. Internal standards were not added before extraction as they correct for incomplete extraction.

The absolute extraction efficiency that ranged from 87% ± 8.3% to 99% ± 8.0% for Qn and Sq analytes **(**Table A.4.4**)**. It is not possible to make comparisons across methods of the extraction efficiencies for the newly-identified PFCs because this is the first method developed for their analysis. Previous methods that report on fluorotelomer sulfonates in groundwater are based on direct-aqueous LVI and extraction efficiency is not reported because the samples were directly injected. (4)

However, the absolute extraction efficiencies reported here for perfluoroalkyl sulfonates (from 92% to 98%) and perfluorocalyl carboxylates (from 91% to 98%) are an improvement over previous LLE-, (5) C18 SPE-, (6) and HLB SPE- (7) based methods and are similar to the WAX SPE-based methods reported by Taniyasu and coworkers (8, 9) on which ISO method 25101 is based. (10) The advantage micro-LLE has over SPE is that it requires less sample and generates less liquid and solid waste. For example, the method by Taniyasu and coworkers, mentioned previously, extracted 100 mL to 200 mL of sample using 20 mL of solvent. (9)

A.3.3 Literature Cited.

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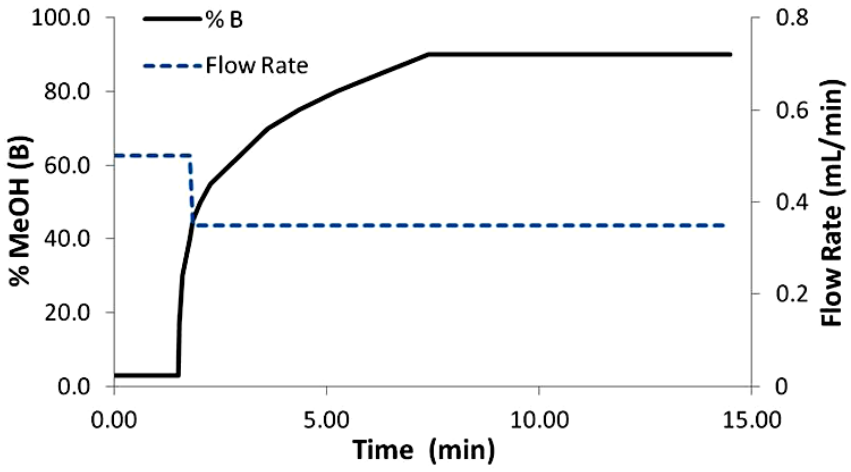

Time	% B
0.00	3.0
1.50	3.0
1.53	17.0
1.60	30.0
1.76	40.0
1.85	45.0
2.02	50.0
2.26	55.0
3.61	70.0
4.34	75.0
5.24	80.0
6.27	85.0
7.38	90.0
14.5	90.0

Figure A.3.1) The gradient conditions used for analyte separation and elution during HPLC.

Table A.3.1) Descriptive names and acronyms of the newly-identified target analytes.

Compound Name of Newly-Identified Analytes	Acronym
2-methyl-2-(3-((1H,1H,2H,2H-perfluoro-1-hexyl)thio)propanamido)propane-1-sulfonate	4-2 FtTAoS
2-methyl-2-(3-((1H,1H,2H,2H-perfluoro-1-octyl)thio)propanamido)propane-1-sulfonate	6-2 FtTAoS
2-methyl-2-(3-((1H,1H,2H,2H-perfluoro-1-decyl)thio)propanamido)propane-1-sulfonate	8-2 FtTAoS
2-hydroxy-N,N,N-trimethyl-3-((1H,1H,2H,2H-perfluoro-1-octyl)thio)propan-1-aminium	6-2 FtTHN$^+$
N-(carboxymethyl)-N,N-dimethyl-3-(1H,1H,2H,2H-perfluoro-1-octanesulfonamido)propan-1-aminium	6-2 FtSaB
N-(carboxymethyl)-N,N-dimethyl-3-(1H,1H,2H,2H-perfluoro-1-decanesulfonamido)propan-1-aminium	8-2 FtSaB
N-(carboxymethyl)-N,N-dimethyl-3-(1H,1H,2H,2H-perfluoro-1-dodecanesulfonamido)propan-1-aminium	10-2 FtSaB
N-(carboxymethyl)-N,N-dimethyl-3-(1H,1H,2H,2H-perfluoro-1- tetradecanesulfonamido)propan-1-aminium	12-2 FtSaB
N-(carboxymethyl)-N,N-dimethyl-3-(1H,1H,2H,2H-perfluoro-1-octanesulfonamido)propan-1-aminium	6-2 FtSaAm
N-(carboxymethyl)-N,N-dimethyl-3-(1H,1H,2H,2H-perfluoro-1-decanesulfonamido)propan-1-aminium	8-2 FtSaAm
N-(carboxymethyl)-1H,1H,2H,2H,3H -N,N-dimethylperfluorooctan-1-aminium	5-1-2 FtB
N-(carboxymethyl)-1H,1H,2H,2H,3H -N,N-dimethylperfluorodecan-1-aminium	7-1-2 FtB
N-(carboxymethyl)-1H,1H,2H,2H,3H -N,N-dimethylperfluorododecan-1-aminium	9-1-2 FtB
N-(carboxymethyl)-1H,1H,2H,2H,3H,3H -N,N-dimethylperfluorooctan-1-aminium	5-3 FtB
N-(carboxymethyl)-1H,1H,2H,2H,3H,3H -N,N-dimethylperfluorodecan-1-aminium	7-3 FtB
N-(carboxymethyl)-1H,1H,2H,2H,3H,3H -N,N-dimethylperfluorododecan-1-aminium	9-3 FtB
N,*N*-dimethyl-3-{[(trideca-perfluorobutyl)sulfonyl]amino}propan-1-aminium	PFBSaAm
N,*N*-dimethyl-3-{[(trideca-perfluoropropyl)sulfonyl]amino}propan-1-aminium	PFPeSaAm
N,*N*-dimethyl-3-{[(trideca-perfluorohexyl)sulfonyl]amino}propan-1-aminium	PFHxSaAm
N,*N*-dimethyl-3-{[(trideca-perfluorooctyl)sulfonyl]amino}propan-1-aminium	PFHxSaAm
3-(N-(2-carboxyethyl)- trideca-perfluorobutylsulfonamido)-N,N-dimethylpropan-1-aminium	PFBSaAmA
3-(N-(2-carboxyethyl)- trideca-perfluoropropylsulfonamido)-N,N-dimethylpropan-1-aminium	PFPeSaAmA
3-(N-(2-carboxyethyl)- trideca-perfluorohexylsulfonamido)-N,N-dimethylpropan-1-aminium	PFHxSaAmA
3-(N-(2-carboxyethyl)- trideca-perfluorooctylsulfonamido)-N,N-dimethylpropan-1-aminium	PFHxSaAmA

Table A.3.2) Analyte precursor ions, product ions, and compound-dependant acquisition parameters. [a] Telomerization-based analytes listed.

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	CE (V)	CV (V)
Fluorotelomer Thioamido Sulfonates (FtTAoS)				
4-2 FtTAoS	486	**135**	38	62
		80	56	
6-2 FtTAoS	586	**135**	40	64
		80	64	
8-2 FtTAoS	686	**135**	44	78
		80	68	
Fluorotelomer Thio Hydroxy Ammonium ($FtTHN^+$)				
6-2 $FtTHN^+$	496	**79**	44	54
		393	34	
Fluorotelomer Sulfonamido Betaines (FtSaB)				
6-2 FtSaB	571	**58**	38	78
		104	30	
8-2 FtSaB	671	**58**	40	80
		104	32	
10-2 FtSaB	771	**58**	44	96
		104	36	
12-2 FtSaB	871	**58**	48	100
		104	38	
Fluorotelomer Sulfamido Amines (FtSaAm)				
6-2 FtSaAm	513	**58**	44	60
		86	34	
8-2 FtSaAm	613	**58**	48	64
		86	38	
Fluorotelomer Betaines (FtB)				
5-1-2 FtB	432	**58**	38	60
		74	40	
7-1-2 FtB	532	**58**	40	72
		74	44	
9-1-3 FtB	632	**58**	42	78
		74	52	
5-3 FtB	432	**58**	38	60
		104	38	
7-3 FtB	514	**58**	40	72
		104	42	
9-3 FtB	614	**58**	42	78
		104	50	
Fluorotelomer Sulfonates (FtS)				
4-2 FtS	327	**81**	26	42
		307	19	
6-2 FtS	427	**81**	28	46
		407	22	
8-2 FtS	527	**81**	32	50
		507	25	

[a] CE (collision energy) and CV (cone voltage)

Table A.3.2) (Continued) Electrochemical fluorination-based analytes listed.

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	CE (V)	CV (V)
Perfluoroalkyl Sulfonamido Amines (PFSaAm)				
PFBSaAm	385	**85** 58	28 44	50
PFPeSaAm	435	**85** 58	30 44	52
PFHxSaAm	485	**85** 58	33 44	54
PFOSaAm	485	**85** 58	35 45	56
Perfluoroalkyl Sulfonamide Amino Carboxylates (PFSaAmA)				
PFBSaAmA	457	**85** 70	28 50	48
PFPeSaAmA	507	**85** 70	30 50	52
PFHxSaAmA	557	**85** 70	33 50	54
PFOSaAmA	657	**85** 70	35 52	56
Perfluoroalkyl Sulfonates (PFS)				
PFBS	299	**80** 99	32 26	50
PFHxS	399	**80** 99	36 28	58
PFHpS	449	**80** 99	46 32	64
PFOS	499	**80** 99	46 34	70
PFDS	599	**80** 99	52 36	76
Perfluoroalkyl Carboxylates (PFA)				
PFBA	213	**169** NA	8 NA	20
PFPeA	263	**219** NA	8 NA	20
PFHxA	313	**269** 119	8 22	20
PFHpA	363	**319** 169	8 14	20
PFOA	413	**369** 169	8 18	20
PFNA	463	**419** 169	8 18	22
PFDA	513	**469** 269	10 18	22
PFUdA	563	**519** 169	10 22	22
PFDoA	613	569 **169**	10 24	22
PFTrDA	663	**619** 169	12 26	24
PFTeDA	713	**669** 169	12 26	24

Table A.3.3) Range, number of points, R2, and internal standard for each quantitative and semi-quantitative analyte's calibration curve; and the calibration curve used for each qualitative analyte. Telomerization-based analytes listed.

	Calibration Range (ng/L)	Number of Points	R^2	Internal Standard
4:2 FtTAoS	Calculated Using 6:2 FtTAoS Calibration			$[^{13}C_2]$-PFHxA
6:2 FtTAoS	10 to 1,500	5	> 0.99	$[^{13}C_2]$-PFHxA
8:2 FtTAoS	Calculated Using 6:2 FtTAoS Calibration			$[^{13}C_2]$-PFHxA
6:2 FtTHN$^+$	15 to 2,250	5	> 0.99	$[^{13}C_4]$-PFOS
6:2 FtSaB	100 to 15,000	6	> 0.99	$[^{13}C_4]$-PFOS
8:2 FtSaB	Calculated Using 6:2 FtSaB Calibration			$[^{13}C_4]$-PFOS
10:2 FtSaB	Calculated Using 6:2 FtSaB Calibration			$[^{13}C_4]$-PFOS
12:2 FtSaB	Calculated Using 6:2 FtSaB Calibration			$[^{13}C_4]$-PFOS
6:2 FtSaAm	240 to 24,000	5	> 0.98	$[^{13}C_4]$-PFOS
8:2 FtSaAm	Calculated Using 6:2 FtSaAm Calibration			$[^{13}C_4]$-PFOS
5:1:2 FtB	58 to 5,800	5	> 0.98	$[^{13}C_4]$-PFOS
7:1:2 FtB	40 to 6,000	5	> 0.98	$[^{13}C_4]$-PFOS
9:1:2 FtB	32 to 3,200	5	> 0.98	$[^{13}C_4]$-PFOS
5:3 FtB	15 to 1,500	5	> 0.98	$[^{13}C_4]$-PFBA
7:3 FtB	30 to 3,000	5	> 0.98	$[^{13}C_4]$-PFBA
9:3 FtB	27 to 810	4	> 0.98	$[^{13}C_4]$-PFBA
4:2 FtS	5 to 750	5	> 0.99	$[^{18}O_2]$-PFHxS
6:2 FtS	5 to 750	5	> 0.99	$[^{18}O_2]$-PFHxS
8:2 FtS	5 to 750	5	> 0.99	$[^{18}O_2]$-PFHxS

Table A.3.3) (Continued) Electrochemical fluorination-based analytes listed.

	Calibration Range (ng/L)	Number of Points	R^2	Internal Standard
PFBSaAm	Calculated Using PFOS Calibration			$[^{13}C_4]$-PFOS
PFPeSaAm	Calculated Using PFOS Calibration			$[^{13}C_4]$-PFOS
PFHxSaAm	Calculated Using PFOS Calibration			$[^{13}C_4]$-PFOS
PFOSAm	Calculated Using PFOS Calibration			$[^{13}C_4]$-PFOS
PFBSaAmA	Calculated Using PFOS Calibration			$[^{13}C_4]$-PFOS
PFPeSaAmA	Calculated Using PFOS Calibration			$[^{13}C_4]$-PFOS
PFHxSaAmA	Calculated Using PFOS Calibration			$[^{13}C_4]$-PFOS
PFOSAmA	Calculated Using PFOS Calibration			$[^{13}C_4]$-PFOS
PFBS	5 to 10,000	6	> 0.99	$[^{18}O_2]$-PFHxS
PFHxS	5 to 10,000	6	> 0.99	$[^{18}O_2]$-PFHxS
PFHpS	5 to 10,000	6	> 0.99	$[^{13}C_4]$-PFOS
PFOS	5 to 10,000	6	> 0.99	$[^{13}C_4]$-PFOS
PFDS	5 to 10,000	6	> 0.99	$[^{13}C_4]$-PFOS
PFBA	5 to 10,000	6	> 0.99	$[^{13}C_4]$-PFBA
PFPeA	5 to 10,000	6	> 0.99	$[^{13}C_4]$-PFBA
PFHxA	5 to 10,000	6	> 0.99	$[^{13}C_2]$-PFHxA
PFHpA	5 to 10,000	6	> 0.99	$[^{13}C_2]$-PFHxA
PFOA	5 to 10,000	6	> 0.99	$[^{13}C_4]$-PFOA
PFNA	5 to 10,000	6	> 0.99	$[^{13}C_5]$-PFNA
PFDA	5 to 10,000	6	> 0.99	$[^{13}C_2]$-PFDA
PFUdA	5 to 10,000	6	> 0.99	$[^{13}C_2]$-PFUdA
PFDoA	5 to 10,000	6	> 0.99	$[^{13}C_2]$-PFDoA
PFTrA	5 to 10,000	6	> 0.99	$[^{13}C_2]$-PFDoA
PFTeA	5 to 10,000	6	> 0.99	$[^{13}C_2]$-PFDoA

Table A.3.4) Percent absolute extraction efficiency (% AEE) (n = 5, ± 95% CI) of the method for quantitative (Qn) and semi-quantitative (Sq) analytes. [a]

Analyte	% AEE	% 95 CI	Analyte	% AEE	% 95 CI
6:2 FtTAoS (Sq)	99	8.0	PFBS (Qn)	92	3.9
6:2 $FtTHN^{+}$ (Sq)	95	6.8	PFHxS (Qn)	98	8.2
			PFHpS (Qn)	93	4.9
6:2 FtSaB (Sq)	93	7.9	PFOS (Qn)	92	3.4
6:2 FtSaAm (Sq)	97	15	PFDS (Qn)	92	2.5
5:1:2 FtB (Sq)	98	5.8	PFBA (Qn)	93	7.0
7:1:2 FtB (Sq)	90	5.5	PFPeA (Qn)	93	2.4
9:1:2 FtB (Sq)	93	5.7	PFHxA (Qn)	94	5.2
			PFHpA (Qn)	98	11
5:3 FtB (Sq)	97	14	PFOA (Qn)	96	4.5
7:3 FtB (Sq)	88	10	PFNA (Qn)	96	5.1
9:3 FtB (Sq)	87	8.3	PFDA (Qn)	95	5.8
			PFUdA (Qn)	89	3.4
4:2 FtS (Qn)	87	13	PFDoA (Qn)	90	2.9
6:2 FtS (Qn)	97	11	PFTrA (Qn)	91	2.7
8:2 FtS (Qn)	93	7.2	PFTeA (Qn)	93	2.8

[a] Determined at concentrations of between 50 and 450 ng/L

Table A.3.5) Concentrations (mg/L) of newly-identified and legacy perfluorinated chemicals in undiluted telomerization-based aqueous film-forming foam mixtures from different manufacturers. Foams are diluted to approximately 3 % to 6 % before application.

		National Foam	Chemguard	Ansul	Buckeye Fire Equipment	Angus	Fire Service Plus
	Formulation Year	2003	2010	2005	2009	2002	NR[a]
		mg/L	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**
Newly-identified PFCs	**4:2 FtTAoS**[c]	ND	ND	26	ND	25	ND
	6:2 FtTAoS	ND	11,000	6,100	ND	4,900	ND
	8:2 FtTAoS[c]	ND	24	1,100	ND	170	ND
	6:2 FtTHN$^+$	ND	ND	ND	ND	2,200	ND
	6:2 FtSaB	4,600	ND	ND	ND	ND	4,800
	8:2 FtSaB[d]	540	ND	ND	ND	ND	1,800
	10:2 FtSaB[d]	450	ND	ND	ND	ND	830
	12:2 FtSaB[d]	210	ND	ND	ND	ND	430
	6:2 FtSaAm	2,100	ND	ND	ND	ND	3,400
	8:2 FtSaAm[e]	450	ND	ND	ND	ND	720
	5:1:2 FtB	ND	ND	ND	2,000	ND	ND
	7:1:2 FtB	ND	ND	ND	4,700	ND	ND
	9:1:2 FtB	ND	ND	ND	1,900	ND	ND
	5:3 FtB	ND	ND	ND	530	ND	ND
	7:3 FtB	ND	ND	ND	610	ND	ND
	9:3 FtB	ND	ND	ND	430	ND	ND
Legacy-PFCs	**4:2 FtS**	ND	ND	ND	ND	ND	ND
	6:2 FtS	42	ND	ND	ND	ND	53
	8:2 FtS	19	ND	ND	ND	ND	56
Ratios	**6:2 FtTAoS /4:2 FtTAoS**	NA[b]	NA[b]	235	NA[b]	196	NA[b]

[a] Not recorded (NR). [b] Not Applicable. Calculated assuming equal molar ratios to [c] 6:2 FtTAoS, [d] 6:2 FtSaB, and [e] 6:2 FtSaAm (see Chapter 4). Electrochemical fluorination-based perfluorinated chemicals (e.g. PFOS) were not detected.

Table A.3.6) Concentrations (mg/L) of newly-identified and legacy perfluorinated chemicals in undiluted electrochemical fluorination-based aqueous film-forming foams from 3M over different years. Prefluorocarboxylates above C8 (PFOA) were not detected. Foams are diluted to approximately 3 % to 6 % before application.

		AFFF Year					
		1989	**1989**	**1993**	**1993**	**1998**	**2001**
		mg/L	mg/L	ng/L	ng/L	ng/L	ng/L
Newly-Identified PFCs	**PFBSaAm** [a]	2.4	1.9	83	100,	95	61
	PFPeSaAm [a]	4.7	1.1	71	97	89	62
	PFHxSaAm [a]	120	120	470	660	690	550
	PFOSaAm [a]	2.0	0.4	24	17	24	15
	PFBSaAmA [a]	ND	ND	160	130	110	130
	PFPeSaAmA [a]	ND	ND	100	71	86	110
	PFHxSaAmA [a]	ND	ND	630	520	530	800
	PFOSaAmA [a]	ND	ND	13	14	16	13
Legacy PFCs	**PFBS**	240	240	160	110	150	190
	PFHxS	1,100	1,000	780	520	670	800
	PFHpS	160	140	75	50	63	97
	PFOS	6,000	5,800	4,600	3,500	3,900	5,200
	PFDS	7.9	10	6.9	3.5	3.6	9.5
	PFBA	15	14	9.4	10	17	20
	PFPeA	22	23	15	15	19	34
	PFHxA	160	150	88	100	110	160
	PFHpA	33	36	21	14	22	34
	PFOA	84	77	58	54	60	120
Ratios	**PFS/PFA** [b]	24	24	29	22	21	17
	Legacy/Newly-Identified	61	61	3.7	2.7	3.1	3.8
	PFOS/PFHxS	5.5	5.8	5.9	6.7	5.8	6.5

[a] Calculated assuming equal molar response to PFOS (see Chapter 4). [b] Total concentrations of perfluoroalkyl sulfonates (PFS)/ perfluoroalkyl carboxylates (PFA). Telomerization-based perfluorinated chemicals (e.g. 6:2 FtTAoS) were not detected.

Table A.3.7) The percent representativeness of quantitative and semi-quantitative analytes in a subsample (n = 5, ± 95% CI) determined at a spiked concentrations (Conc.).

Analyte	Representativeness (%)	± 95 CI (%)	Conc. (ng/L)
6-2 FtTAoS	69	5	250
6-2 $FtTHN^{+}$	62	2.5	250
6-2 FtSaB	124	9.6	930
6-2 FtSaAm	82	11	760
5-1-2 FtB	126	12	600
7-1-2 FtB	108	8.2	1250
9-1-2 FtB	88	6.6	340
5-3 FtB	100	7.8	150
7-3 FtB	89	11	314
9-3 FtB	74	7.7	84
4-2 FtS	106	8.1	400
6-2 FtS	102	4.9	400
8-2 FtS	99	8.1	400
PFBS	100	4.1	400
PFHxS	102	2.9	400
PFHpS	97	2.8	400
PFOS	101	1.5	400
PFDS	84	3.4	400
PFBA	96	1.6	400
PFPeA	99	3.4	400
PFHxA	96	1.5	400
PFHpA	95	3.5	400
PFOA	97	1.6	400
PFNA	95	4.7	400
PFDA	94	0.9	400
PFUdA	86	4.3	400
PFDoA	76	2.8	400
PFTrA	78	2.5	400
PFTeA	81	1.6	400