Determination of the accumulation of chiral pharmaceuticals (venlafaxine and *O*-desmethylvenlafaxine) in rainbow darters (*Etheostoma caeruleum*)

by

Sarah Jane Kowalczyk

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Author’s Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Pharmaceuticals are widespread contaminants of concern that enter the aquatic environment mainly via wastewater effluent. Over 50% of common pharmaceuticals are chiral, and this is notable due to the potential impact of chirality on the distribution, fate, and toxicity of these compounds. Historically, chirality has been overlooked when completing environmental risk assessments. The chiral antidepressant venlafaxine (VEN) and its major metabolite *O*-desmethylvenlafaxine (desVEN) are pseudo-persistent through wastewater treatment and are not removed completely, being detected in surface waters globally at levels greater than 2.0 µg/L. At these concentrations, VEN and desVEN have been shown to impact the behavior, metabolism, population structures, and other biological responses of aquatic biota in receiving environments. The possible enantioselective bioaccumulation of each enantiomer of VEN (R and S) and desVEN (R and S) needs further investigation, as they are normally treated as racemic mixtures.

An optimized and unbiased extraction method for individual enantiomers (R and S) of VEN and desVEN was developed. The extraction method and all subsequent sample cleanup was evaluated for the recoveries of VEN and desVEN enantiomers and possible enantiomeric bias. Accelerated solvent extraction (ASE) using acidified acetonitrile (1% formic acid by volume) was determined to be an acceptable extraction method with regards to the recoveries and chromatography for all VEN and desVEN enantiomers (performing better in comparison to ultrasonic solvent extraction, USE). The selection and mass of a fat retainer included in extraction cells for on-line extract cleanup and subsequent sample cleanup protocols were also optimized to reduce matrix effects (e.g., ion suppression) associated with co-eluates during liquid chromatography tandem mass spectrometry (LC-MS/MS). The addition of 2.5 g of neutral aluminum oxide during ASE had no enantioselective effects on extraction but resulted in better recoveries for all enantiomers of VEN and desVEN. Subsequent extract cleanup via solid-phase extraction (SPE) using Hydrophilic-Lipophilic-Balanced (HLB) cartridges had the highest recoveries in comparison to other SPE cartridges, liquid-liquid extraction, and QuEChERS, and no enantioselective effects were observed after analysis. Fish tissue mass up to 2.4 g and a final extract volume of 0.5 mL were chosen as the best compromise for a method with satisfactory sensitivity and minimal enantiomeric bias during extraction, while avoiding detrimental ion suppression for all VEN and desVEN enantiomers (with good absolute recovery, and extraction efficiency). The method detection limits (MDLs) for the final method ranged from 0.03 -

0.05 ng/g which is lower than or comparable to VEN extraction from fish tissues reported in other methods in the literature.

The validated extraction method was applied to an in-lab exposure of male rainbow darters (*Etheostoma caeruleum*) collected from a clean reference site in the Grand River, ON. The goal of this exposure was to determine if there was an enantioselective effect on the bioaccumulation of R-VEN and S-VEN in a sentinel small-bodied fish species exposed to a single enantiomer. The fish were exposed to 1 µg/L of R-VEN or S-VEN for up to 14 days, with samples being collected on days 0, 1, 4, and 14. S-VEN appeared to bioaccumulate in fish more than R-VEN, as S-VEN was significantly higher in fish tissue after 4 and 14 days of exposure. The metabolite S-desVEN was also found in fish exposed to S-VEN for 4 and 14 days, which suggests the metabolism of S-VEN into S-desVEN in fish, and subsequent accumulation and/or retention (there was no R- or S-desVEN detected in the water). In contrast, R-desVEN was not found in fish exposed to R-VEN at any time point. Subtle differences in bioaccumulation of VEN and desVEN enantiomers in fish were observed but further studies are needed to determine if there is an enantiomeric shift during bioaccumulation that would alter risk in wild fish.

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Dedication

I dedicate this thesis to my mom. I have held your words close to my heart over the last two years- I wish that you were here to see all my hard work pay off.

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List of Abbreviations

AHAB	Aquatic Habitats
AJS	Agilent Jet Stream
AR	Absolute recovery
ASE	Accelerated solvent extraction
AUPP	Animal utilization project proposal
CECs	Contaminants of emerging concern
CSP	Chiral stationary phase
CYP2D6	Cytochrome P450 2D6
d-	Deuterated
desVEN	*O*-desmethylvenlafaxine
desVEN-d6	*O*-desmethylvenlafaxine-d6
DO	Dissolved oxygen
EE	Extraction efficiency
EF	Enantiomeric fraction
EMR	Enhanced Matrix Removal
ER	Enantiomeric ratio
ESI	Electrospray ionization
HLB	Hydrophilic-lipophilic balanced
HPLC	High-performance liquid chromatography
LC	Liquid chromatograph
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLE	Liquid-liquid extraction
MAOIs	Monoamine oxidase inhibitors
MDL	Method detection limit
ME	Matrix effect
MRM	Multiple reaction mode
MS	Mass spectrometer
MS	Matrix spike
MS-222	Ethyl 3-aminobenzoate methanesulfonate
MWWTPs	Municipal wastewater treatment plants

ND	Non-detect
NET	Norepinephrine transporters
NSAIDs	Nonsteroidal anti-inflammatory drugs
OECD	Organisation for Economic Co-operation and Development
PCBs	Polychlorinated biphenyls
QA/QC	Quality assurance/quality control
QuEChERS	"Quick, Easy, Cheap, Effective, Rugged, Safe"
RBD	Rainbow darter
R-desVEN	R-*O*-desmethylvenlafaxine
RSD	Relative standard deviation
R-VEN	R-venlafaxine
SD	Standard deviation
S-desVEN	S-*O*-desmethylvenlafaxine
SERT	Serotonin transporters
SNRIs	Serotonin-norepinephrine reuptake inhibitors
SPE	Solid-phase extraction
SSRIs	Serotonin reuptake inhibitors
S-VEN	S-venlafaxine
TCAs	Tricyclic antidepressants
USE	Ultrasonic solvent extraction
VEN	Venlafaxine
VEN-d6	Venlafaxine-d6
WATER	Waterloo Aquatic Threats in Environmental Research
WWTP	Wastewater treatment plant

Chapter 1
Background

1.1 Pharmaceuticals in the environment

Pharmaceuticals and personal care products (PPCPs) typically enter the aquatic environment via the release of effluent from wastewater treatment plants (WWTPs) into surface waters. Pharmaceuticals are chemical compounds used for the treatment of human and animal diseases, while personal care products are products used for personal hygiene and cosmetic reasons such as moisturizers, makeup, hair products, deodorants, and toothpastes among others (Boxall et al., 2012). Classes of PPCPs that are commonly reported in municipal wastewater effluents globally include a wide variety of chemicals: antibiotics, antifungal/antimicrobial agents, nonsteroidal anti-inflammatory drugs (NSAIDs), anticonvulsants, antidepressants, beta-blockers, lipid regulating drugs, hormone contraceptives, UV filters from sunscreens, and stimulants (Tran et al., 2018).

PPCPs have been detected in the natural environment on every continent (Wilkinson et al., 2022) at relatively low concentrations in the ng/L to µg/L range (Fernandes et al., 2020; Lajeunesse et al., 2012; Metcalfe et al., 2010; Schultz et al., 2010). Pharmaceuticals are ingested, metabolized, and then excreted in urine and feces by humans, entering the sewer system and ultimately WWTPs as both the unchanged parent compound and the products of their metabolism. Pharmaceuticals used by humans are released from wastewater treatment plant outfalls into surface waters, although they can also enter terrestrial systems through landfill leachate (Yi et al., 2017) when municipal sludge is applied to farmland as fertilizer, or when effluent is used for irrigation (Kinney et al., 2006; Ternes et al., 2004). Exposure to PPCPs at wastewater outfalls is considered pseudo-persistent as they are continuously released into the environment resulting in continuous exposure to the biota downstream (Ebele et al., 2017; Petrie et al., 2015). Veterinary pharmaceuticals are usually released into the receiving environment either directly when used in aquaculture or on pasture animals, or indirectly via manure that is applied to agricultural lands (Boxall et al., 2003) but can also be found in municipal wastewaters.

Municipal wastewater is a known major source of surface water contamination, containing a wide variety of chemicals from residential, industrial, and agricultural sources. Wastewater undergoes treatment at WWTPs before it is released back into the aquatic environment as final effluents, including primary, secondary, and possible tertiary treatment. Primary treatment includes the removal

of large debris and suspended solids using methods such as screening, sedimentation, and floatation (Ramalho, 1977). Secondary treatment includes activated sludge processes, in which microorganisms break down organic materials. During these conventional biological treatment processes, there is the potential for the transformation and/or degradation of pharmaceutical contaminants. However, this degradation depends on the physicochemical structure of the compound and environmental conditions, including reactor configuration, retention times, and pH levels (J. Wang & Wang, 2016). Tertiary treatment includes further processes to remove residual contaminants that remain after secondary treatment. This can be additional biological, physical, or chemical processes, including carbon adsorption and advanced oxidation processes (AOPs) such as ozonation and UV photolysis (Eggen et al., 2014; J. Wang & Wang, 2016). Some pharmaceutical contaminants are resistant to degradation and are not effectively removed during wastewater treatment. Compounds including carbamazepine, diclofenac, and venlafaxine are generally resistant to degradation during treatment and have been found to remain in wastewater after secondary treatment (Rúa-Gómez & Püttmann, 2012; Tran & Gin, 2017). The demethylated metabolites may convert back into the parent compound during wastewater treatment (Calisto & Esteves, 2009; Lajeunesse et al., 2012). Additionally, glucuronide conjugate metabolites may undergo enzymatic cleavage during secondary treatment, converting them back into the parent compound as well (Bahlmann et al., 2014; Lindholm-Lehto et al., 2016).

Pharmaceuticals and metabolites that are not effectively removed during wastewater treatment ultimately enter the environment, where they retain bioactivity. Pharmaceuticals are designed to target specific biomolecules, such as proteins, to modify pathways and processes within organisms. Due to the conservation of mechanisms across different species, there is cause for concern when determining the potential effects of biologically active PPCPs on non-target organisms in the environment (Gunnarsson et al., 2008). The effects seen in non-target organisms can be specific or general, depending on the mechanism of action for the PPCP being studied and can even be seen at the low concentrations often found in the environment (Daughton & Ternes, 1999). Many different effects have been reported, including changes in physiology (Hodgson et al., 2020; Mehdi et al., 2022), behavior (Bisesi et al., 2014; McCallum et al., 2017), community structure (Aristone et al., 2022; Kidd et al., 2007; Mehdi et al., 2021), metabolic processes (Best et al., 2014; Du et al., 2019) including stress responses (Gauthier & Vijayan, 2020; Ings et al., 2011; Mehdi, 2017), and reproduction (Galus et al., 2013; Parrott & Blunt, 2005). Additionally, PPCPs are released into the

environment as part of complex mixtures that can lead to possible additive interactions (Ebele et al., 2017).

1.2 Venlafaxine and *O*-desmethylvenlafaxine

An increased pharmaceutical consumption has been noted worldwide, with a nearly 50% increase seen in countries that are a part of the Organisation for Economic Co-operation and Development (OECD) between 2011 and 2021 (OECD, 2023). Antidepressants are one of the most-prescribed groups of pharmaceuticals on the market globally (Magalhães et al., 2014), and have been since at least the 1990s (Dewan & Anand, 1999). Related to the high prescription frequency, the national rate of dispensing antidepressants in Canada rose 31.4% from January 2017 to December 2020 (Uthayakumar et al., 2022). Antidepressants are used in the treatment of major depressive disorder (Kennedy et al., 2016; Sansone & Sansone, 2014), and have also exhibited efficacy in the treatment of other psychiatric and non-psychiatric conditions as well (Karsnitz & Ward, 2011; Katzman et al., 2014; Mercier et al., 2013). They work as psychoactive pharmaceuticals by altering the levels and mechanisms of neurotransmitters, such as serotonin, norepinephrine, and dopamine in the brain (Caccia, 1998). The classification of antidepressants is based on their mechanism of action, including serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressants (TCAs), and monoamine oxidase inhibitors (MAOIs; Stahl, 1999).

Venlafaxine (VEN) specifically is one of the most-prescribed antidepressants, making up approximately 15% of all distributed antidepressants in Canada in 2020 (Uthayakumar et al., 2022). VEN acts as an SNRI by blocking the reabsorption of two neurotransmitters, serotonin and norepinephrine, from the synaptic cleft to prolong their effects within the central nervous system (Burnett & Dinan, 1998). VEN has a 30-fold higher affinity for serotonin and is a more potent serotonin reuptake inhibitor, compared to its affinity and reuptake inhibition for norepinephrine (Montgomery, 2008; Stahl et al., 2005). In humans, VEN is primarily metabolized in the liver via cytochrome P450 isoenzymes (Burnett & Dinan, 1998). The major metabolite formed during the metabolism of VEN by cytochrome P450 2D6 (CYP2D6) is *O*-desmethylvenlafaxine (desVEN) and the minor metabolite formed is *N*-desmethylvenlafaxine. There are additional minor metabolites that are also formed through further metabolism (Figure 1.1; Magalhães et al., 2014).

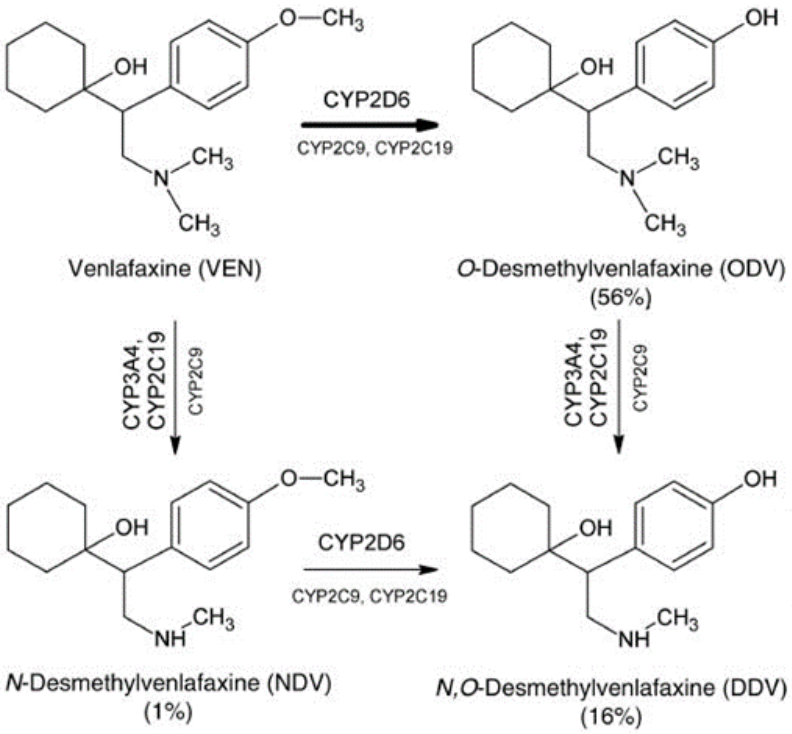

Figure 1.1 The main metabolic pathways of VEN in humans. Chemical structures of VEN and desVEN, along with other minor metabolites *N*-desmethylvenlafaxine and *N*,*O*-desmethylvenlafaxine, are shown. Adapted from Figure 1 in Magalhães et al. (2014).

1.3 Chirality

The fate and toxicity of VEN and other pharmaceuticals, in the environment and within organisms, is complicated due to the chiral nature of these compounds (Barclay et al., 2011; Kasprzyk-Hordern & Baker, 2012). A chiral molecule is defined as a compound that is optically active due to possessing at least one chiral center (e.g., an asymmetric carbon, as shown in Figure 1.2). The two resulting molecules are nonsuperimposable mirror images of each other and are called enantiomers (Kasprzyk-Hordern & Baker, 2012). VEN is a chiral molecule and has two enantiomers: R-VEN and S-VEN. desVEN is also chiral with two enantiomers: R-desVEN and S-desVEN (Figure 1.3).

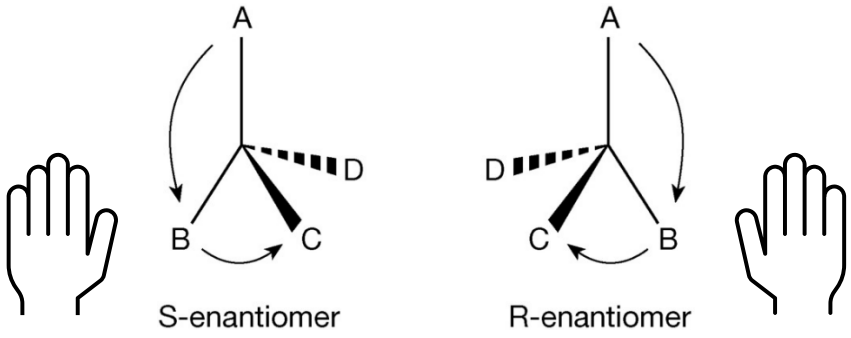

Figure 1.2 Demonstration of chirality, with two enantiomers (non-superimposable mirror images) in comparison to human hands. Diagram adapted from Burke & Henderson (2002).

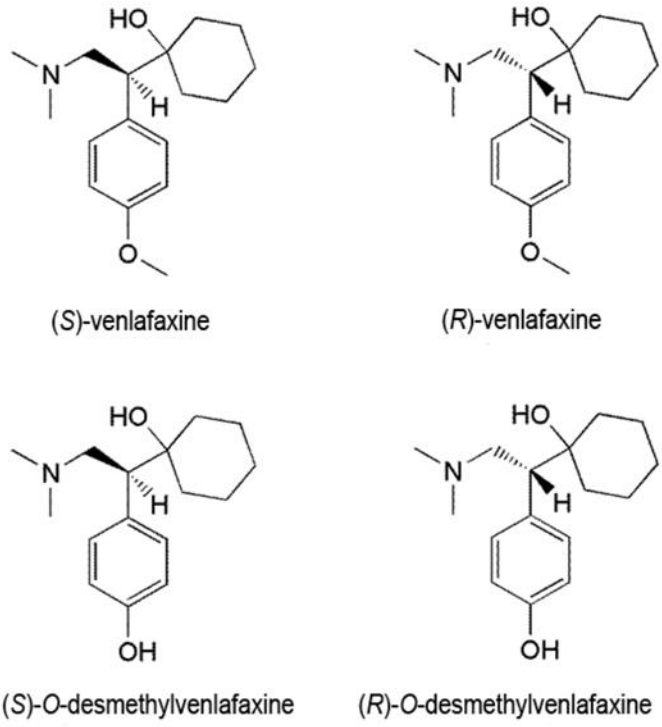

Figure 1.3 Chemical structures of S-VEN, R-VEN (top row) and S-desVEN, R-desVEN (bottom row). Adapted from Figure 5 in Vashistha et al. (2022).

Enantiomers have similar physiochemical properties, however the specific configuration around the chiral center can cause differing biological properties (Kasprzyk-Hordern & Baker, 2012). This difference in configuration can cause differing interactions between the two enantiomers with the

same receptors, enzymes, and other chiral molecules, leading to different, specific pharmacological effects (Barclay et al., 2011). For example, the two enantiomers of VEN interact with similar transporters, serotonin transporters (SERT) and norepinephrine transporters (NET), but R-VEN inhibits both serotonin and norepinephrine transporters while S-VEN mainly inhibits serotonin transporters (Silverstone et al., 1999). Due to their differing biological activity, the two forms of a chiral drug can have differing levels of toxicity (Barclay et al., 2011; Kasprzyk-Hordern & Baker, 2012). As a chiral compound is metabolized and excreted by an organism or passes through wastewater treatment, the enantiomeric composition of the drug may be altered (Kasprzyk-Hordern & Baker, 2012). One of the enantiomeric forms may be enriched, and the other depleted while undergoing these processes. This may lead to a different enantiomeric composition, activity, and toxicity within the receiving environment (Kasprzyk-Hordern & Baker, 2012).

Chirality is commonly defined with relative measurements of the enantiomeric composition of a mixture. There are two common conventions, enantiomeric ratio (ER) and enantiomeric fraction (EF). ER and EF can be calculated using Equation (1) and Equation (2) respectively, where E1 is the first eluted enantiomer, and E2 is the last eluted enantiomer (Arenas et al., 2022). If a mixture has a similar concentration of each enantiomer (i.e., the mixture is racemic), then ER = 1, or EF = 0.5. An ER of 0 or infinity or an EF of 0 or 1 corresponds to pure enantiomeric compounds.

$$\boldsymbol{ER} = [\boldsymbol{E1}]/[\boldsymbol{E2}] \qquad \text{Equation (1)}$$

$$\boldsymbol{EF} = [\boldsymbol{E1}]/([\boldsymbol{E1}]/[\boldsymbol{E2}]) \qquad \text{Equation (2)}$$

The enantiomeric composition can be affected by the method used to extract, clean-up or quantify the sample (Sanganyado et al., 2020) leading to a bias. The reliability of the EF value may be impacted by many factors related to the enantioselective analysis, such as matrix effects, or enantiomerization (the conversion of one enantiomer into another) due to high instrument temperatures (Arenas et al., 2022). These various factors must be accounted for during method development to avoid a bias in interpretation of environmental data.

1.3.1 Fate and impact of chiral pharmaceuticals

Many of the pharmaceuticals that enter the aquatic ecosystem via wastewater effluent are chiral compounds (Budău et al., 2017; Sanganyado et al., 2017). The type of treatment and physicochemical properties of contaminants impacts the removal rates of PPCPs during wastewater treatment in

WWTPs (Ebele et al., 2017; Petrie et al., 2015; Tran et al., 2018). Compounds such as venlafaxine have been shown to remain persistent and are not removed effectively during biological treatment (Rúa-Gómez & Püttmann, 2012; Tran & Gin, 2017). They are also considered pseudo-persistent as they are continuously released into the environment (Ebele et al., 2017; Petrie et al., 2015), and can be found at concentrations in surface waters that have been shown to affect non-target organisms (Lajeunesse et al., 2012; Metcalfe et al., 2010). Chiral PPCPs enter the sewer system along with their metabolites and may enter as racemic or non-racemic mixtures. The enantiomeric fraction of chiral PPCPs can shift as the compounds move through wastewater treatment and the environment due to biotic and abiotic processes. Chirality is still often overlooked, and these compounds are assumed to act as racemates. Pharmaceuticals that are chiral have been found to bioaccumulate in an enantioselective manner in aquatic species such as marine mollusks, crustaceans, and fish (Petrie & Moffat, 2022; Ruan et al., 2020). With the increasing prescription and use of antidepressants globally, it is increasingly important to study the environmental fate and effects of VEN and desVEN to better understand and ultimately mitigate their ecological impact. In addition, the importance of investigating the different enantiomers of chiral compounds separately, as there may be enantiospecific differences in the fate and effects of different enantiomers in the aquatic environment. With a robust, enantiomerically unbiased analytical method, the impact of chirality on toxicokinetic parameters can be assessed in a controlled lab environment.

1.4 Objectives and hypotheses

To improve assessment for the risk of chiral pharmaceuticals such as VEN, it is critical to have better (i.e., enantiomerically unbiased) methods for their measurement and an improved understanding of their fate and bioaccumulation. Although a chiral liquid chromatography tandem mass spectrometry (LC-MS/MS) analytical method was previously developed for chiral pharmaceuticals in water and wastewater (Jamal et al., 2020), this method has not been validated for fish tissue. Fish tissue is a complex matrix due to the presence of macromolecules such as lipids and proteins, which can co-extract with the target analytes and cause interferences during LC-MS/MS analysis (J. Wang & Gardinali, 2012). The first objective of the thesis was to develop and optimize an analytical method for the enantiomerically unbiased analysis of chiral VEN and desVEN (i.e., the R- and S-enantiomers) in fish tissue. A series of controlled experiments were conducted to optimize the extraction, cleanup, and analysis method for fish tissues. In particular, the methods considered

limitations to sample size, as small-bodied fish were of interest in field studies. For each experiment, the samples spiked with the standard were compared to the nominal concentration and enantiomeric composition of the injected standard.

Ho: The concentration and enantiomeric fraction found in extracted fish tissue is the same as the reference spiked standard sample.

The second objective was to apply the validated, enantiomerically unbiased analytical method to assess the possible enantioselectivity of the bioaccumulation in fish. Rainbow darters (*Etheostoma caeruleum)* were exposed to each enantiomer of VEN individually at a concentration of 1 µg/L and the water and fish tissue were quantified for the individual enantiomers of both VEN and its major metabolite desVEN.

Ho: The concentration of R- and S-VEN in fish will not differ after exposure to the same concentration in water (1 µg/L).

The secondary hypothesis tested was to confirm there was no enantiomerization of VEN during the exposure.

Ho: The other enantiomer (R/S) of VEN (or the metabolite, desVEN) in water exposures will not be detected in the fish tissue.

Chapter 2
Optimization of an extraction method for chiral venlafaxine and *O*-desmethylvenlafaxine

2.1 Introduction

Pharmaceuticals and personal care products (PPCPs) are a large group of contaminants of emerging concern, as some PPCPs are not effectively removed during wastewater treatment (Rúa-Gómez & Püttmann, 2012; Tran & Gin, 2017). PPCPs that are not removed during treatment are subsequently introduced into the aquatic environment, mainly via wastewater effluents that are discharged into surface waters. They are found in the aquatic environment globally (Wilkinson et al., 2022) at concentrations ranging from ng/L to µg/L (Fernandes et al., 2020; Lajeunesse et al., 2012; Metcalfe et al., 2010; Schultz et al., 2010). This has raised concern for aquatic biota living downstream of wastewater outfalls, since pharmaceuticals are biologically active compounds that have the potential to impact non-target organisms.

Many PPCPs that ultimately enter the environment are chiral compounds. A chiral compound is an optically active compound with at least one asymmetric carbon (i.e., a chiral center). This results in forms that are non-superimposable mirror images of each other, called enantiomers. Different enantiomeric forms of the same compound have the same physicochemical properties (except for their ability to rotate polarized light), however often have differing interactions within a chiral environment (Kasprzyk-Hordern, 2010; Nguyen et al., 2006). The biological systems in which these compounds act, are also chiral due to the three-dimensional nature of structures within them (e.g., antibodies, enzymes, and receptors; Kohler et al., 2000; Sanganyado et al., 2017). Enantiomer-specific binding, catalysis, and stabilization are examples of stereospecific interactions that can result in differing therapeutic properties, fate, or human health and environmental effects seen with enantiomers of the same compound (Brooks et al., 2011; Mehvar et al., 2002).

An understanding of the fate, distribution, and impacts of chiral compounds, and how their chirality changes these dynamics is important for the development of analytical methodology and risk assessment guidelines. Many chiral pharmaceuticals in current use are sold as racemates but can also be sold as single enantiomeric forms as well (Agranat et al., 2012; Kasprzyk-Hordern, 2010). Although chiral pharmaceuticals are often marketed as racemates, they do not always enter the

environment as racemates (i.e., there is not a 50:50 ratio of each enantiomer found in the environment). This may be due to chiral inversion that happens during human metabolism and excretion (Kasprzyk-Hordern, 2010), as well as other biotic processes like metabolism by microbes during wastewater treatment (Evans et al., 2017; Ruan et al., 2019). The implications of enantioselectivity in these processes are of particular interest, due to the possible enantiospecific fate and effects of individual enantiomeric forms of the same compound. There may be an under- or overestimation of the quantity and/or effects of chiral pharmaceuticals in the environment and on aquatic biota if studies are completed with the assumption that chiral compounds enter the environment as racemates (Kasprzyk-Hordern, 2010; Sanganyado et al., 2017). The true risk associated with a compound may be due to a specific enantiomeric form of the compound of interest (Brooks et al., 2011; Kasprzyk-Hordern, 2010).

There is a current gap in methodologies to quantitate the individual enantiomeric forms of compounds of interest, since chirality is often not considered when developing sample preparation, extraction, and analysis protocols. The extraction methods and cleanup protocols do not necessarily consider the chirality of the analytes of interest being extracted. It is therefore important to test each method and ensure there is no resulting enantiomeric enrichment in any of the steps.

Extraction and analysis of chiral compounds is an additional challenge. Two common extraction methods include ultrasonic solvent extraction (USE) and accelerated solvent extraction (ASE). USE is a traditional tissue extraction technique used for biological samples. In USE, cavitation bubbles are formed as acoustic waves travel through solvent. The bursting of these bubbles at the surface of fish tissue allows the solvent to pass through cellular membranes, extracting the compounds of interest (Frenkel et al., 1999). The main applications of USE include the extraction of pesticides from soil (Babić et al., 1998; Tor et al., 2006) and sediment (Vagi et al., 2007), and organic compounds from plant samples (Shirsath et al., 2012; Vilkhu et al., 2008) and biological samples (Schultz et al., 2010). USE has some advantages compared to other traditional extraction methods including the decreased volume of solvent needed, suitability to routine analysis, and being straightforward to use. The main disadvantages of USE are the difficulty of scalability and automation of USE protocols (Picó, 2013).

ASE is a solid-liquid extraction process that is an alternative to traditional tissue extraction techniques such as USE. During extraction, a higher temperature will increase the diffusion rates and capacity to solubilize analytes. This is done by weakening the interactions between the analytes and

its matrix. By combining high temperature and high pressure during extraction, the solvent is kept in its liquid state at temperatures that are above the theoretical boiling point (Sun et al., 2012). In addition, high pressures used during ASE increase extraction efficiency because the solvent is forced into areas of the sample that cannot normally be contacted when extracting under atmospheric pressure conditions (Richter et al., 1996). The main applications of ASE include the analysis of soil and sediment samples (Giergielewicz-Możajska et al., 2001), extraction of organic contaminants from plant, food, and biological samples (Carabias-Martínez et al., 2005), and the extraction of PPCPs from sewage sludge (Nieto et al., 2010). Some of the advantages of ASE over traditional methods include decreased use of solvents, less laboratory waste, the ability to have smaller sample sizes, high-throughput, and automation of the extraction (Sun et al., 2012). The ability to use smaller sample sizes is important when developing methods to extract tissue samples of small-bodied fish. Another advantage of ASE is the ability to include fat retaining compounds in the extraction cell for on-line sample cleanup. Fat retaining compounds are included in the extraction cell to adsorb lipids and other co-eluates, preventing the co-elution of these compounds in the final sample extract (Carabias-Martínez et al., 2005). The main disadvantage of this method is the expensive lab equipment that is required. Another disadvantage is extraction temperatures that are too high can decrease the selectivity of the method, leading to the degradation of thermolabile compounds of interest (Moreno et al., 2007). Therefore, it is crucial to find a temperature that improves extraction efficiency without negatively impacting analytes.

Before the fish extracts can be analyzed (e.g., LC-MS/MS) further cleanup is needed to remove co-extractives that can interfere with analysis or operation of the instrument. Unwanted compounds can be co-eluted with analytes of interest during the extraction of biological samples, such as macromolecules like proteins and lipids. Examples of methods that can be used for sample cleanup include solid phase extraction (SPE), gel permeation chromatography, liquid-liquid extraction (LLE), and freezing out lipid co-extractives (Peña-Herrera et al., 2019). SPE, LLE, and commercially available QuEChERS kits were tested in this study as potential sample cleanup protocols used after extraction for additional lipid removal. In SPE, compounds are separated from a liquid mixture due to their differing physical and chemical properties. The compounds of interest are retained on the cartridge and the contaminants are separated in the flow-through, or vice versa (Berrueta et al., 1995). LLE is an extraction method that depends on the varying solubility of an analyte in an aqueous sample vs. an organic solvent (Pena-Pereira et al., 2009). QuEChERS (“quick, easy, cheap, effective,

rugged, and safe") is completed with commercially available kits. The first step of a QuEChERS extraction protocol is a liquid-liquid partitioning between water and solvent, with the addition of salts. After extraction, a dispersive solid phase extraction (dSPE) step is completed to remove lipid contamination and excess water to improve the extraction of compounds of interest. It is necessary to remove these endogenous compounds, as their presence in the matrix can interfere with the ionization process and resulting signal of analytes during analysis, and result in matrix effects (J. Liu et al., 2023). Due to the presence of co-eluents, these matrix effects can cause ion suppression or enhancement, where the signal of the analyte is lower or higher, respectively (J. Liu et al., 2023). Sample cleanup can be done by adding materials to the extraction cell to start cleanup during extraction, and/or by using subsequent cleaning methods on the extracts to clean up the resulting eluate. Matrix effects can also be addressed with the final dilution of the concentrated sample extracts (Oldekop et al., 2014; Stahnke et al., 2012).

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is an analytical technique that can be applied for the analysis of chiral compounds due to its high sensitivity, selectivity, and capability to identify and quantify enantiomers in complex matrices (Evans & Kasprzyk-Hordern, 2014). Enantiomers have the same physicochemical properties (except their ability to rotate polarized light); and therefore, traditional separation during LC-MS/MS is not sufficient for the separation of the different enantiomeric forms of a chiral compound from one another during analysis (Evans & Kasprzyk-Hordern, 2014). The use of a chiral stationary phase (CSP) is crucial in the analysis of chiral compounds with LC-MS/MS. CSPs facilitate enantiomeric separation by incorporating chiral selectors onto the stationary phase that interact with each enantiomer differently (Lämmerhofer, 2010). Based on the differing, specific atomic arrangement of each enantiomer, one form will preferentially bind to the CSP, while the other form will bind weakly in comparison (Lämmerhofer, 2010). The enantiomeric forms of a chiral compound are separated via differing retention times during analysis, as the enantiomer that binds weakly will pass through the column faster and elute before the enantiomer that binds preferentially (Evans & Kasprzyk-Hordern, 2014). Examples of chiral selectors that have been used include macromolecular selectors, macrocyclic selectors and low-molecular mass selectors (Lämmerhofer, 2010). The macrocyclic glycopeptide selectors vancomycin and teicoplanin (e.g., in Chiral V and Chiral T chiral columns) are commonly used in the analysis of chiral pharmaceuticals in environmental samples, including surface and wastewater (Bagnall et al., 2012; Barclay et al., 2011; MacLeod et al., 2007; A. R. Ribeiro et al., 2012). Methods investigating

chiral pharmaceuticals in biological samples, including human plasma and blood, have also used vancomycin-bonded columns (Kingbäck et al., 2010; W. Liu et al., 2007). It is important to develop enantiomerically unbiased methods capable of independently analyzing the enantiomers of chiral compounds from environmental matrices, including fish tissues.

The objective of this study was to develop and characterize an analytical method for the unbiased quantification of the enantiomers of VEN and desVEN. Several common approaches to the extraction and cleanup of tissue samples were compared and optimized. Different extraction methods (accelerated solvent extraction [ASE] and ultrasonic solvent extraction [USE]), subsequent sample cleanup protocols (several SPE cartridges, liquid-liquid extraction, and QuEChERS), fat retainers (included for on-line sample cleanup during ASE), and sample size and volume (matrix effects) were compared in terms of recovery and enantiomeric selectivity. The enantiomeric fractions (EFs) of the compounds of interest will be evaluated to measure bias (or lack there of) present in the developed method protocols, to ensure that there is no enantioselectivity in any step. The range of what is considered a racemic EF for analytical methods was determined in a study completed by Kobližková et al. (2008) on chiral PCBs using the relative standard deviation (RSD) values for samples prepared with their methodology. This method of determining the range of EF values that is considered racemic has been applied in further studies of chiral PCBs (Carlsson et al., 2016), and Sanganyado et al. (2020) suggest using this methodology in their review of the application of EF values in environmental forensics for all classes of chiral pollutants including pharmaceuticals. The final method was characterized by evaluating the absolute recoveries, extraction efficiencies, and matrix effects, as well as determining the method detection limits for each analyte of interest.

2.2 Materials and methods

The optimization of the method included a series of experiments where tissue samples were spiked with a standard, and the recoveries (%) and enantiomeric fractions (EFs) of different treatments were compared. Initial selection of the extraction and cleanup conditions were based on preliminary studies and/or literature, and optimization was progressive as each step was tested. The goal of method development was to maximize the recovery of the target compounds and minimize matrix effects, while ensuring there was no enantiomeric bias at each step. As the species of interest in field studies was a small-bodied fish (rainbow darter; *Etheostoma caeruleum*), the final method sensitivity

(method detection limits; MDLs) using only a few grams of tissue was also an important consideration.

2.2.1 Materials, reagents, and solvents

Racemic standards for venlafaxine (VEN) and *O*-desmethylvenlafaxine (desVEN) were purchased from Sigma-Aldrich (Oakville, ON, CAN). Individual enantiomeric forms of VEN and desVEN were purchased from Toronto Research Chemicals Inc. (Toronto, ON, CAN). Corresponding deuterated standards for VEN and desVEN were used for the quantitation of the unlabeled compounds of interest during analysis. Venlafaxine-d6 (VEN-d6) and *O*-desmethylvenlafaxine-d6 (desVEN-d6) were purchased from CDN Isotopes (Pointe-Claire, QC, CAN). All solvents used for standard preparation, sample extraction, sample clean-up, and instrumental analysis were high-performance liquid chromatography (HPLC) grade or higher purchased from Fisher Scientific (Mississauga, ON, CAN).

2.2.2 Preparation of standard solutions

The stock solutions for each analyte were individually prepared at a concentration of 1 g/L in methanol. A racemic calibration curve with the concentrations of 0, 0.1, 0.5, 1, 5, 10, 25, 50, 75, 100, 200, 300, 400 µg/L was prepared using the stock solutions of each individual enantiomer. The internal standard solution of VEN-d6 and desVEN-d6 used for all samples was prepared at a concentration of 100 µg/L in methanol. All prepared solutions were stored in amber glass vials in a freezer at -20°C.

2.2.3 LC-MS/MS analysis

The chiral liquid chromatography tandem mass spectrometry (LC-MS/MS) method developed by Jamal et al. (2020) was used for the analysis of water samples and was adapted for the analysis of fish tissue samples. The analysis of VEN and desVEN was performed using an Agilent 1260 liquid chromatograph (LC) with a 6460 Triple Quad mass spectrometer (MS) system equipped with an Agilent Jet Stream (AJS) electrospray ionization (ESI) source in positive ionization mode. The column used for this method was an Agilent Poroshell 120 Chiral-V, 2.7 µm, 4.6 x 100 mm column, with a column temperature of 25°C. The isocratic mobile phase used for each 10 µL injection was methanol with 4 mM ammonium acetate and 0.005% formic acid, with a flow rate of 0.5 mL/min, and a total method run time of 20 min. Samples were analyzed in multiple reaction mode (MRM) with a cell accelerator voltage of 4 V. Compound-dependent parameters for VEN, desVEN, and deuterated

internal standards are shown in Table 2.1. Q1 represents the precursor ion mass and Q3 represents the product ion mass. The source parameters include gas temperature of 250°C, gas flow of 5 L/min, nebulizer of 45 psi, sheath gas temperature of 400°C, sheath gas flow of 12 L/min, capillary voltage of 3000 V, and nozzle voltage of 0 V.

Table 2.1 Compound-dependent liquid chromatography tandem mass spectrometry (LC-MS/MS) analytical method parameters for the analysis of venlafaxine (VEN), *O*-desmethylvenlafaxine (desVEN), and deuterated internal standards in water samples (adapted from Jamal et al. [2020]).

Compound	Q1 (m/z)	Q3 (m/z)	Fragmentor (V)	Collision energy (V)
VEN	278.2	121	78	28
	278.2	58.1	78	16
VEN-d6	284.2	121.1	86	28
	284.2	64.1	86	16
desVEN	264.2	58.1	86	16
	264.2	42.1	86	116
desVEN-d6	270.2	252.2	78	10
	270.2	64.1	78	18

The LC-MS/MS method was further optimized for analysis of fish samples to create a more sensitive, specific, and time-efficient analysis method. Switching from using MRM to dynamic MRM increases the sensitivity by shortening the window of time the mass spectrometer is using to identify the compounds of interest, allowing for more scans per second for each compound. The analytes of interest were eluted at earlier retention times, therefore the total method run time was reduced to 13 min from 20 min. Some compound-dependent parameters for VEN, desVEN, and deuterated internal standards including differences in the Q3 ion masses, fragmentor voltage, and collision energy voltage were further optimized for fish tissue (described in Table 2.2). The Q3 ion masses were changed to be more specific for VEN and desVEN to help mitigate interference caused by isomers with similar masses.

Table 2.2 Compound-dependent liquid chromatography tandem mass spectrometry (LC-MS/MS) analytical method parameters for the analysis of venlafaxine (VEN), *O*-desmethylvenlafaxine (desVEN), and deuterated internal standards in fish tissue samples.

Compound	Q1 (m/z)	Q3 (m/z)	Fragmentor (V)	Collision energy (V)
VEN	278.2	260.2	70	9
	278.2	58.1	70	17
VEN-d6	284.2	266.2	68	9
	284.2	64.2	68	21
desVEN	264.2	246.2	75	9
	264.2	58.1	75	21
desVEN-d6	270.2	252.2	75	10
	270.2	64.1	75	18

2.2.4 Sample preparation

Rainbow trout (*Oncorhynchus mykiss*) were purchased from Silvercreek Aquaculture and housed in the Waterloo Aquatic Threats in Environmental Research (WATER) facility. They were used and sacrificed according to AUPP 44775. There was no quantifiable VEN or desVEN in the rainbow trout muscle tissue used in experiments, as determined with quality assurance/quality control (QA/QC) samples spiked with deuterated internal standard and extracted in the same manner as experimental samples. Fish were euthanized with ethyl 3-aminobenzoate methane sulfonate (MS-222; Sigma-Aldrich) followed by spinal severance. The fish muscle was filleted and stored in plastic storage bags at -20°C. Prior to extraction, the muscle was ground to a fine powder using a mortar and pestle under liquid nitrogen, and aliquoted into 2.4 g samples. An aliquot weight of 2.4 g was chosen based on the average weight of adult rainbow darters (*Etheostoma caeruleum*) collected from previous studies in the Grand River (Dawe et al., 2024; Hicks et al., 2023; Tetreault et al., 2011). Aliquots were stored at -20°C until they were needed for experimentation.

2.2.5 Sample extraction

Based on previous preliminary studies (Kaur, 2021; Kowalczyk, 2022) two sample extraction methods, accelerated solvent extraction (ASE) and ultrasonic solvent extraction (USE), were assessed for the extraction of chiral VEN in fish tissue. All samples were prepared as described above before

various solvents conditions were tested for each extraction method. The final eluate was collected and evaporated to dryness under nitrogen using a Biotage TurboVap LV nitrogen evaporator with the water bath set to 40°C. With the optimal method, the final sample extract was reconstituted to a final volume of 0.5 mL with methanol containing 75 µg/L lorazepam and chloramphenicol (two compounds that are used for QA/QC, since they do not occur within the samples). The extracts were stored in 2 mL amber vials and kept at -20°C until further analysis.

The USE method used for this experiment was adapted from (Schultz et al., 2010). A VWR 2.8 L ultrasonic water bath was used for sonication of the samples. First, fish tissue aliquots were spiked with a deuterated internal standard to a final concentration of 20 ng/g, and a regular racemic VEN and desVEN standard to a final concentration of 20 ng/g. Next, 8 mL of cold extraction solvent was added to each sample before being placed in the ultrasonic bath to extract for 10 min. Once extraction was complete, the samples were centrifuged for 10 min, at 3500 rpm and -10°C. After centrifugation, the supernatant was directly applied to an EMR-Lipid SPE cartridge for subsequent sample cleanup, being careful to avoid transferring any solid fish tissues. USE was tested only during the extraction solvent experiments since ASE was determined to be the better extraction technique. All other method parameter optimization experiments were completed for ASE only.

There are many extraction parameters to be optimized for ASE to ensure good extraction of chiral compounds from fish tissue. ASE parameters were initially chosen based on a previous pilot study that adapted the method described by (Chu & Metcalfe, 2007). ASE was automated using a Thermo Scientific Dionex ASE 350 Accelerated Solvent Extractor system. Initial experiments (not shown) were completed to optimize the ASE extraction method in the laboratory using rainbow trout as the matrix. The final ASE parameters used for the following experiments were 1500 psi extraction pressure, 2 static extraction cycles, 300 s static time, 100 s purge, 75% flush volume, and 70°C extraction temperature.

Preparation of the ASE cells for extraction was important in method optimization experiments to ensure it was sensitive and not enantiomerically biased. Stainless steel cells with a volume of 10 mL were used for ASE to be able to hold the mass of the fish tissue and other included materials (i.e., diatomaceous earth filler and fat retainers) needed while minimizing the amount of solvent used. The ASE cells were prepared for extraction by adding cellulose filters, fat retainer, diatomaceous earth, and the fish tissue samples (Figure A 1). The fish tissue was spiked inside of the ASE cells with a

deuterated internal standard to a final concentration of 20 ng/g, and a regular racemic VEN and desVEN standard to a final concentration outlined for the experiment. A flowchart outlining the ASE protocol and the subsequent sample cleanup protocols followed for the optimized method can be found in Appendix A (Figure A 2). During extraction, the ASE eluate was collected in 40 mL glass vials. The eluate was evaporated to dryness under nitrogen. After evaporation, the samples were reconstituted to an appropriate volume with an appropriate solvent outlined for the subsequent cleanup method being tested. Samples were further cleaned up after ASE with Oasis hydrophilic-lipophilic balanced (HLB) solid-phase extraction (SPE) cartridges (6 cc, 500 mg, Waters Corporation) and the samples were reconstituted to 10 mL of MilliQ water with 1% methanol (v/v) at a pH of 9.7. The final eluate was collected and evaporated to dryness under nitrogen.

2.2.5.1 Extraction solvent

Extraction solvents were tested to determine which was the best to extract the compounds of interest, while not co-extracting other macromolecules that may cause matrix effects. The extraction solvents tested for both ASE and USE included methanol, acetonitrile, and 1:1 methanol:acetonitrile. The recoveries of the compounds of interest were assessed in this experiment by determining the recoveries (%) in comparison to a standard with a known concentration of each enantiomer of VEN and desVEN. The resulting chromatograms were also assessed for additional peaks and/or abnormal peak shapes caused by matrix effects. Additional peaks indicate the presence of another compound co-eluting at a similar retention time with the same ion mass. Sample cleanup after extraction was completed with Agilent Captiva Enhanced Matrix Removal (EMR) lipid solid-phase extraction (SPE) cartridges (6 cc, 600 mg). EMR-Lipid SPE cartridges were selected for subsequent sample cleanup based on a previous pilot study completed in the lab for the extraction of chiral VEN and desVEN from fish tissue samples of approximately 100 mg.

2.2.5.2 Acidified extraction solvent

Acidifying the extraction solvent (acetonitrile) was tested to determine if this improved recoveries (%) or mitigated enantiomeric selectivity between the extraction of the two enantiomeric forms of VEN and desVEN. Experiments were completed to compare the efficiency of extraction with acetonitrile vs acidified acetonitrile to select the best solvent conditions for ASE. Acetonitrile with 1% formic acid was made by adding 10 mL of formic acid to 990 mL acetonitrile and mixing well. Recoveries were determined by comparing the concentrations of each enantiomer extracted with ASE

to the concentrations of each enantiomer in a standard of a known concentration, determined with the LC-MS/MS method described in Section 2.2.3. Sample cleanup after extraction was completed with Oasis HLB SPE cartridges (6 cc, 500 mg, Waters Corporation), since they were determined to be the optimal cartridge for post-extraction sample cleanup in the experiment outlined in Section 2.2.6.2.

2.2.6 Lipid removal protocol optimization in ASE

Removal of lipids from the samples prior to analysis by LC-MS/MS was important for both the sample quality and quantitation of the analytes of interest, as well as the condition and continued operation of the instrument. The experiments completed to determine the optimal lipid removal protocols included on-line sample cleanup and subsequent cleanup protocols.

2.2.6.1 Fat retainer inclusion during ASE

Fat retaining powders were tested for their effectiveness at cleaning lipids from samples during ASE. The four fat retainers tested were acidic aluminum oxide (also called "alumina," which will be used going forward), neutral alumina, basic alumina and Florisil (synthetic magnesium-silicate; Sigma-Aldrich). These specific compounds were chosen for testing based on a previous study of the extraction of polychlorinated biphenyls (PCBs) from fat-containing samples using ASE (Björklund et al., 2001).

First, the effectiveness of each fat retainer was compared to determine the most suitable fat retainer to include in the optimized extraction method by comparing the recoveries (%) of VEN and desVEN enantiomers for each of the four fat retainers. Next, different masses of fat retainer used in the ASE cell were tested for neutral alumina: 1, 2.5, and 5 g. This was completed to determine the optimal mass to include such that the samples were sufficiently cleaned of lipids, however the analytes of interest were also not removed in the process. Samples were extracted with ASE (with different fat retainers added to the ASE cell) and then underwent cleanup with Oasis HLB SPE cartridges (6 cc, 500 mg, Waters Corporation).

2.2.6.2 Post-extraction sample cleanup

Additional sample cleanup techniques were also tested after ASE extraction, including solid-phase extraction (SPE), liquid-liquid extraction (LLE) using heptane solvent, and a QuEChERS ("Quick, Easy, Cheap, Effective, Rugged, Safe", Agilent) protocol. Three different SPE cartridges were tested: Oasis hydrophilic-lipophilic balanced (HLB; 6 cc, 500 mg, Waters Corporation), Supelclean ENVI-

Carb (6 cc, 500 mg, Sigma-Aldrich), and Captiva Enhanced Matrix Removal (EMR) lipid cartridges (6 cc, 600 mg, Agilent). The recoveries (%) for VEN and desVEN enantiomers were compared for each of the tested cleanup techniques.

Although EMR-Lipid SPE cartridges were shown to be effective for cleanup in previous studies when extracting chiral compounds from 100 mg of fish tissue (Kaur, 2021; Kowalczyk, 2022), the EMR-Lipid SPE cartridges were tested again in this study for the extraction of larger tissue weights (2.4 g). It was determined that Oasis HLB SPE cartridges had the best recoveries (%) for all compounds of interest in later optimization experiments (outlined in Section 2.2.6.2). For the earlier experiments outlined in Section 2.2.5.1, post-extraction sample cleanup was completed with EMR-Lipid SPE cartridges before it was determined that Oasis HLB SPE cartridges had the best recoveries when extracting chiral VEN and desVEN from larger tissue weights.

2.2.7 Evaluation of matrix effects

Compounds that co-elute with analytes of interest can alter the ionization efficiency for the analytes of interest, and cause either a loss in response (ion suppression) or an increase in response (ion enhancement) for the target compounds (Zhou et al., 2017). Therefore, experiments were completed to evaluate the matrix effects (%) to ensure they were not detrimental to the sensitivity of the method. The first experiment was completed to determine the fish tissue mass (g) to extract such that the recoveries were satisfactory (in the range of 80 to 120%). Preliminary experiments (Kowalczyk, 2022) were completed with fish tissue masses of 100 mg, and this does not represent the mass of a whole rainbow darter. Therefore, additional experiments were completed to determine the highest mass of fish able to be extracted with minimal matrix effects. Masses of 1 - 4.8g were optimized. The second experiment was completed to determine the optimal final sample extract volume (mL) such that the recovery values were satisfactory, and the sample was concentrated sufficiently to achieve lower method detection limits. Reconstitution volumes of 0.5 - 1 mL were optimized. A compromise must be met such that the extracts are sufficiently concentrated to be able to detect and quantitate the analytes of interest, while not impacting the sensitivity by concentrating unwanted interfering molecules leftover in the extract.

2.2.8 Method characterization

Characterization of the optimized method was completed by assessing parameters including the absolute recovery (AR), extraction efficiency (EE), matrix effects (ME), method detection limit (MDL), the inter-day precision (relative standard deviation, RSD), and the linear range at 9 concentration levels (0.1, 0.5, 1, 5, 10, 25, 50, 75, 100 µg/L).The enantiomeric bias of the method was also evaluated by determining the enantiomeric fractions (EFs) for VEN and desVEN. The parameters of the final ASE method included a 1500 psi extraction pressure, two static extraction cycles, 300 s static time, 100 s purge, 75% flush volume, 70°C extraction temperature, acetonitrile with 1% formic acid extraction solvent, 2.5 g neutral alumina fat retainer included in the extraction cell, up to 2.4 g of tissue, a final extract volume of 0.5 mL, and subsequent sample cleanup using an Oasis HLB cartridge (6 cc, 500 mg, Waters Corporation).

The AR, EE, and ME were calculated using the equations described by Peña-Herrera et al. (2019). Multiple fish tissue samples were extracted after being spiked at different stages: four samples spiked to a final concentration of 240 µg/L of VEN and desVEN before extraction and cleanup, four samples spiked to a final concentration of 240 µg/L of VEN and desVEN after extraction and cleanup, and four blank samples that were not spiked with regular VEN and desVEN. All samples were spiked with the same concentration of deuterated internal standard for QA/QC. These samples were compared to an injected standard at the same concentration of 240 µg/L of VEN and desVEN and deuterated internal standard in methanol. This was used to determine the AR, EE, and ME of the optimized method.

To determine the absolute recovery (AR), the peak areas for the analytes in fish extracts spiked before extraction and sample cleanup were compared to the peak areas for the analytes in fish extracts spiked after extraction and sample cleanup, according to Equation (3).

$$\boldsymbol{AR} = \frac{\boldsymbol{Peak\ Area_{Spiked\ Before}}}{\boldsymbol{Peak\ Area_{Spiked\ After}}} \qquad \text{Equation (3)}$$

The EE was determined by comparing the peak areas for the analytes in fish extracts spiked before extraction and sample cleanup and the peak areas for the analytes of an injected standard, according to Equation (4).

$$\boldsymbol{EE} = \frac{\boldsymbol{Peak\ Area_{Spiked\ Before}}}{\boldsymbol{Peak\ Area_{Standard}}} \qquad \text{Equation (4)}$$

The ME were evaluated by comparing the peak areas for the analytes in fish extracts spiked after extraction and sample cleanup and the peak areas for the analytes of an injected standard, according to Equation (5).

$$\boldsymbol{ME = \frac{Peak\ Area_{Spiked\ After}}{Peak\ Area_{Standard}}} \qquad \text{Equation (5)}$$

To determine the MDLs two analyte concentrations were tested: 0.1 µg/L and 0.8 µg/L. Seven clean fish tissue samples were spiked with all four analytes and the same concentration of deuterated internal standard, extracted with the optimized ASE method and sample cleanup methods and analyzed with the adapted LC/MS-MS method for fish tissue described above in Section 2.2.3. Equation (6) from the United States Environmental Protection Agency (2016) was used to calculate the MDL for each enantiomer of VEN and desVEN.

$$\boldsymbol{MDL = SD_{Spiked\ Aliquots} * t_{(n-1,1-\alpha)}} \qquad \text{Equation (6)}$$

Inter-day precision was assessed at three concentrations: low (0.2 ng/g), medium (10 ng/g), and high (30 ng/g). Six clean fish tissue samples were spiked with the enantiomers of VEN and desVEN for each concentration and a known concentration of deuterated internal standard on six different days. The relative standard deviation (RSD) for each analyte was determined for all three concentrations on all six extraction days using Equation (7).

$$\boldsymbol{RSD = \frac{SD_{Replicates}}{Average\ Concentration\ (ng/g)_{Replicates}} * 100\%} \qquad \text{Equation (7)}$$

The nominal accuracy (%) for each analyte was determined for all three concentrations on all six extraction days using Equation (8).

$$\boldsymbol{Accuracy = \frac{Calculated\ Concentration\ (ng/g)}{Nominal\ Concentration\ (ng/g)} * 100\%} \qquad \text{Equation (8)}$$

The linear range of VEN and desVEN when extracted with the optimized method was evaluated by extracting a standard curve of nine concentrations of the analytes of interest spiked in fish tissue. For each standard curve point, four clean fish tissue samples were spiked with 0.1 - 100 µg/L of the four analytes and a set concentration of the internal deuterated standard. The ratio of the analyte peak area to the internal deuterated standard peak area was determined and plotted against the analyte concentration for each of the enantiomers of VEN and desVEN. The coefficients of determination (R^2) were determined for each individual enantiomer using a linear regression.

2.2.8.1 Determination of the racemic enantiomeric fraction range

The racemic EF range of the method developed for this study was determined with the inter-day validation RSD values for VEN and desVEN. The expected variability of a normally distributed EF value is determined by setting the tolerance limits to the 0.005 - 0.995th percentile range of the Student's t-test, and using Equation (9). This was completed to account for any EF values that deviate from 0.5 due to the instrumentation and/or matrix effects to determine if it is a significant deviation from the theoretical racemic EF of 0.5.

$$\boldsymbol{Racemic\ Range = 0.5 \pm (3 * Experimental\ Range)} \qquad \text{Equation (9)}$$

2.2.9 Statistical analysis

Statistical analyses were completed using GraphPad Prism 9.3.1 for Windows (San Diego, CA, USA). The differences in recoveries and matrix effects (%) for each chiral compound and between enantiomers of VEN or desVEN within the same experimental treatment were determined by completing two-way analysis of variance (ANOVA) tests, with Tukey's post-hoc tests. The normality of the data was assessed with the Shapiro-Wilk's normality test, and the homogeneity of variance was assessed with the F-test for homogeneity of variance. For all statistical analyses, an alpha ($\boldsymbol{\alpha}$) value of 0.05 was used to compare the p-values. Figures present the data as the mean ± the standard deviation (SD).

2.3 Results

An enantiomerically unbiased ASE method with good recoveries for chiral VEN and desVEN in fish tissue was developed and characterized. Various experiments were conducted to optimize the extraction and ensure minimal enantiomeric bias. The extraction method, extraction solvent, inclusion of a fat retainer in extraction cells, subsequent sample cleanup protocols, maximum sample mass, and final extract volume were optimized.

2.3.1 Sample extraction and extraction solvent

Two different extraction methods were compared (ASE vs. USE) with three different solvents (methanol, acetonitrile, and 1:1 methanol:acetonitrile). For the USE experiments, acetonitrile had the highest recoveries (Figure 2.1). The recoveries of S-desVEN, R-VEN, and S-VEN were significantly higher when extracted with acetonitrile compared to the other solvents tested (two-way ANOVA and

Tukey's post-hoc, $p < 0.05$). The EF values for USE with acetonitrile also show enantioselectivity, with an EF value for VEN of 0.62 (demonstrating an enrichment of S-VEN), and an EF value for desVEN of 0.24 (demonstrating an enrichment of R-desVEN; Table 2.3).

ASE had the highest recoveries (%) for all four analytes in comparison to USE (Figure 2.1). For each solvent tested for ASE, there was a significant difference between the recoveries for R-desVEN and S-desVEN, demonstrating an enantiomeric selectivity in the extraction of chiral desVEN with the solvents tested in this experiment (two-way ANOVA and Tukey's post-hoc, $p < 0.05$). There was a significant difference between the recoveries for R-VEN and S-VEN when extracted with methanol using ASE, demonstrating enantiomeric selectivity in the ASE extraction of chiral VEN using methanol (two-way ANOVA and Tukey's post-hoc, $p = 0.0214$). In addition, the ASE method showed a slight enantiomeric bias since the EF values for VEN are greater than 0.5 (ranging between 0.53 and 0.64), demonstrating an enrichment of S-VEN, and the EF values for desVEN are less than 0.5 (ranging between 0.34 and 0.42), demonstrating an enrichment of R-desVEN (Table 2.3).

Based on the comparison of the recoveries of the analytes with ASE and USE, ASE was chosen as the extraction method for further optimization since it had the highest recoveries for all four analytes for all solvents. Acetonitrile was chosen as the solvent for further experiments since it had low variability as well as good chromatographic peaks (the peaks for all 4 analytes of interest for both ASE and USE are shown in Figure A 3 in Appendix A). For ASE with acetonitrile solvent, the following experiments focused on decreasing the enantioselective differences in recoveries between the two enantiomers of both VEN and desVEN.

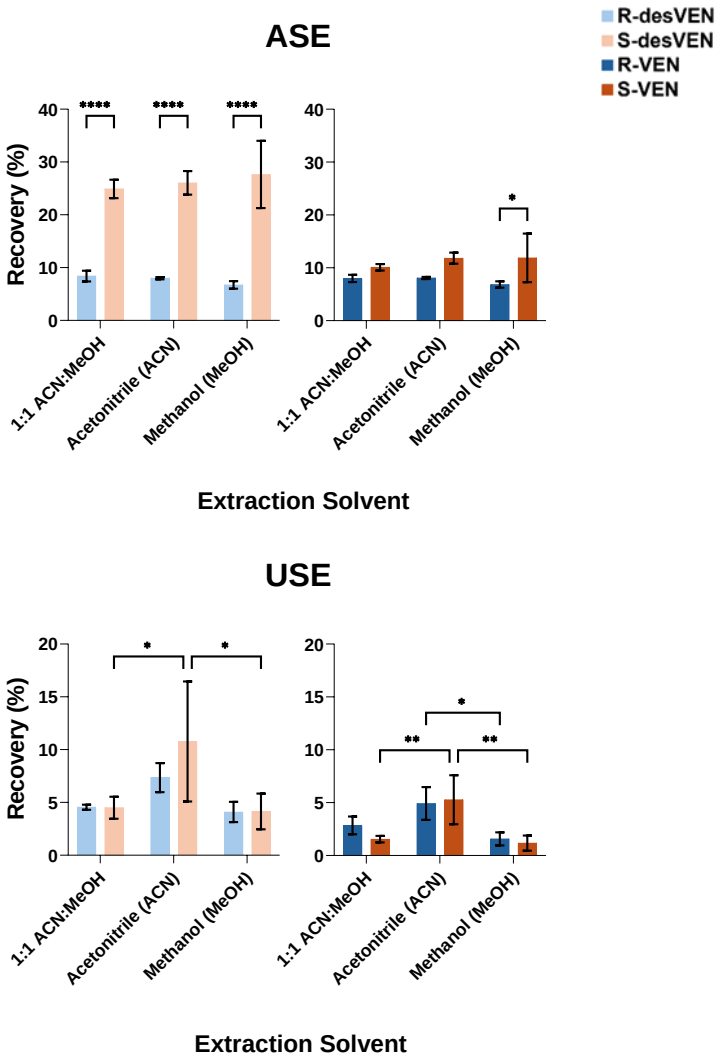

Figure 2.1 Optimization of extraction solvents for accelerated solvent extraction (ASE) and ultrasonic solvent extraction (USE) for the extraction of R-VEN, S-VEN, R-desVEN, and S-desVEN. Data are presented as a mean ± SD, compared using a two-way ANOVA. An asterisk represents a significant difference, determined by Tukey Test (n = 4), between the recovery (%) of an individual compound when extracted using different solvents (1:1 acetonitrile:methanol, acetonitrile, or methanol) or a difference between the recovery of enantiomeric forms (R- vs. S-enantiomer of VEN and/or desVEN) when extracted using the same solvent, using a specific extraction method (ASE or USE). * = $p < 0.05$; ** = $p < 0.01$, **** = $p < 0.0001$.

Table 2.3 Enantiomeric fraction (EF), standard deviation (SD), and relative standard deviation (RSD) values for chiral VEN and desVEN for ASE and USE extraction solvent optimization.

Extraction Method	Extraction Solvent	VEN			desVEN		
		EF	SD	RSD	EF	SD	RSD
ASE	1:1 Acetonitrile:methanol	0.59	0.02	3.6	0.42	0.01	2.7
	Acetonitrile	0.53	0.03	6.1	0.34	0.02	6.0
	Methanol	0.64	0.06	9.9	0.40	0.02	5.7
USE	1:1 Acetonitrile:methanol	0.42	0.10	23.0	0.22	0.10	43.8
	Acetonitrile	0.62	0.06	9.5	0.24	0.04	16.9
	Methanol	0.56	0.18	32.6	0.23	0.05	20.7

2.3.1.1 Acidified extraction solvent

Acetonitrile was compared to acetonitrile with 1% formic acid by volume in the ASE extraction to determine if acidifying the solvent would improve recoveries (%) and/or reduce the enantioselectivity of the extraction method. For all four analytes of interest, acidified acetonitrile with 1% formic acid by volume had higher recoveries (%) compared to acetonitrile that was not acidified (Figure 2.2), with significantly higher recoveries for R-desVEN and S-desVEN (two-way ANOVA and Tukey's post hoc, $p = 0.0003$ for both analytes). The enantiomeric fractions (EFs) for both acetonitrile and acidified acetonitrile were considered racemic for both VEN and desVEN and were 0.51 and 0.49 for VEN and desVEN, respectively (Table 2.4). No significant differences between the recoveries of the individual enantiomers of VEN (R-VEN vs. S-VEN) and the individual enantiomers of desVEN (R-desVEN vs. S-desVEN) were observed with acidified acetonitrile (two-way ANOVA and Tukey's post hoc, $p > 0.05$). Overall, this demonstrates there is no statistically significant enantioselective effect on the ASE method for the extraction of chiral VEN and desVEN with acetonitrile and acetonitrile with 1% formic acid. Therefore, acetonitrile with 1% formic acid by volume, the solvent with higher recoveries, was chosen for further method optimization.

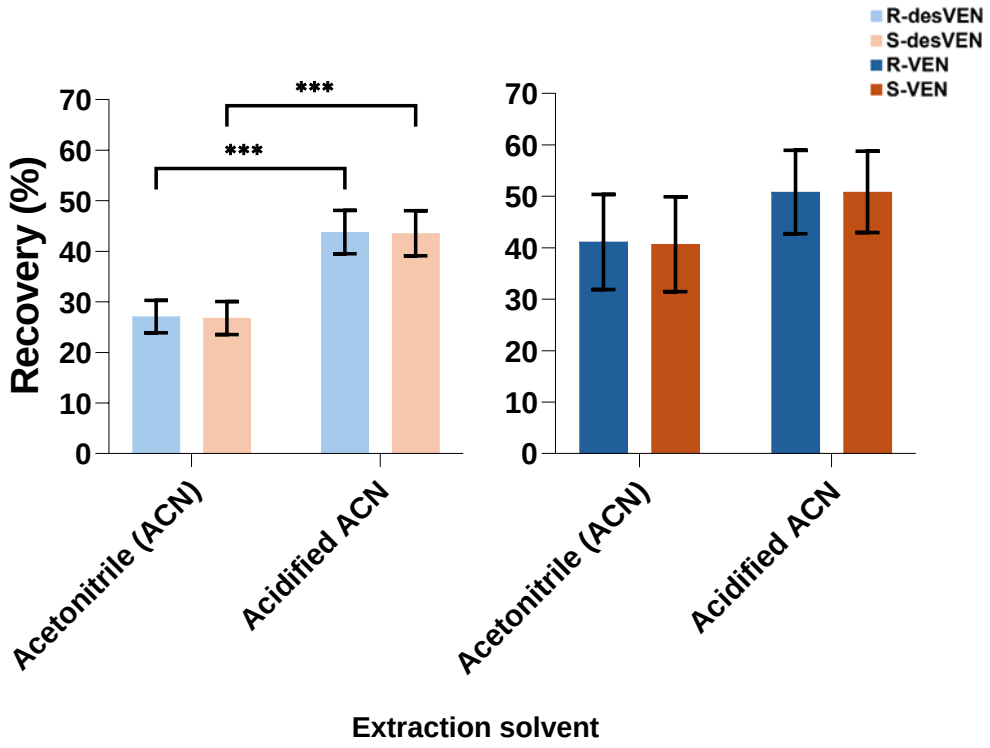

Figure 2.2 Evaluation of acetonitrile vs. acidified acetonitrile with 1% formic acid for the accelerated solvent extraction (ASE) of R-VEN, S-VEN, R-desVEN, and S-desVEN. Data are presented as a mean ± SD, compared using a two-way ANOVA. An asterisk represents a significant difference determined by the Tukey Test (n = 4), between the recovery (%) of an individual compound when extracted using acetonitrile and acetonitrile with 1% formic acid. *** = $p < 0.001$.

Table 2.4 Enantiomeric fraction (EF), standard deviation (SD), and relative standard deviation (RSD) values for chiral VEN and desVEN for extraction with acetonitrile and acidified acetonitrile with 1% formic acid.

Extraction Solvent	**VEN**			**desVEN**		
	EF	**SD**	**RSD**	**EF**	**SD**	**RSD**
Acetonitrile	0.51	0.003	0.59	0.49	0.004	0.77
Acetonitrile with 1% formic acid	0.51	0.001	0.29	0.49	0.003	0.56

2.3.2 Lipid removal protocol optimization in ASE

2.3.2.1 Fat retainer inclusion during ASE

In ASE extraction, the addition of fat retainers was tested for four materials: acidic alumina, neutral alumina, basic alumina and Florisil. The highest recoveries (%) were seen for all analytes when the alumina materials were included in the ASE extraction cells (80.1 - 90.8% for VEN enantiomers, 61.5 - 64.9% for desVEN enantiomers; Figure 2.3). The inclusion of Florisil resulted in the lowest recoveries (%) for all analytes (34.4 - 36.3% for VEN enantiomers, 40.3 - 42.9% for desVEN enantiomers). The recoveries of VEN and desVEN enantiomers were all significantly lower when Florisil was included compared to the recoveries when extractions were completed with the inclusion of the other three alumina materials during ASE (two-way ANOVA, Tukey's post-hoc test, $p < 0.0001$ for all comparisons).

The EFs for both VEN and desVEN are racemic for each treatment (i.e., the material tested), ranging between 0.49 – 0.52 for VEN and 0.49 – 0.50 for desVEN (Table 2.5). Neutral alumina was chosen for further optimization experiments due to having the highest recoveries of both VEN enantiomers, high recoveries of both desVEN enantiomers, and EFs for VEN and desVEN, 0.51 and 0.50 respectively. There were no significant differences between recoveries of the individual enantiomers of VEN and the individual enantiomers of desVEN for all four materials tested for on-line lipid cleanup during ASE (two-way ANOVA, $p > 0.05$ for all comparisons). Ultimately, this demonstrates that the addition of the tested fat retainers does not introduce any enantioselectivity to the extraction and cleanup of fish tissue samples, while greatly increasing the recoveries of VEN and desVEN. Neutral alumina was chosen for further method optimization since there were high, unbiased recoveries with low variance during the extraction of chiral VEN and desVEN when it was included.

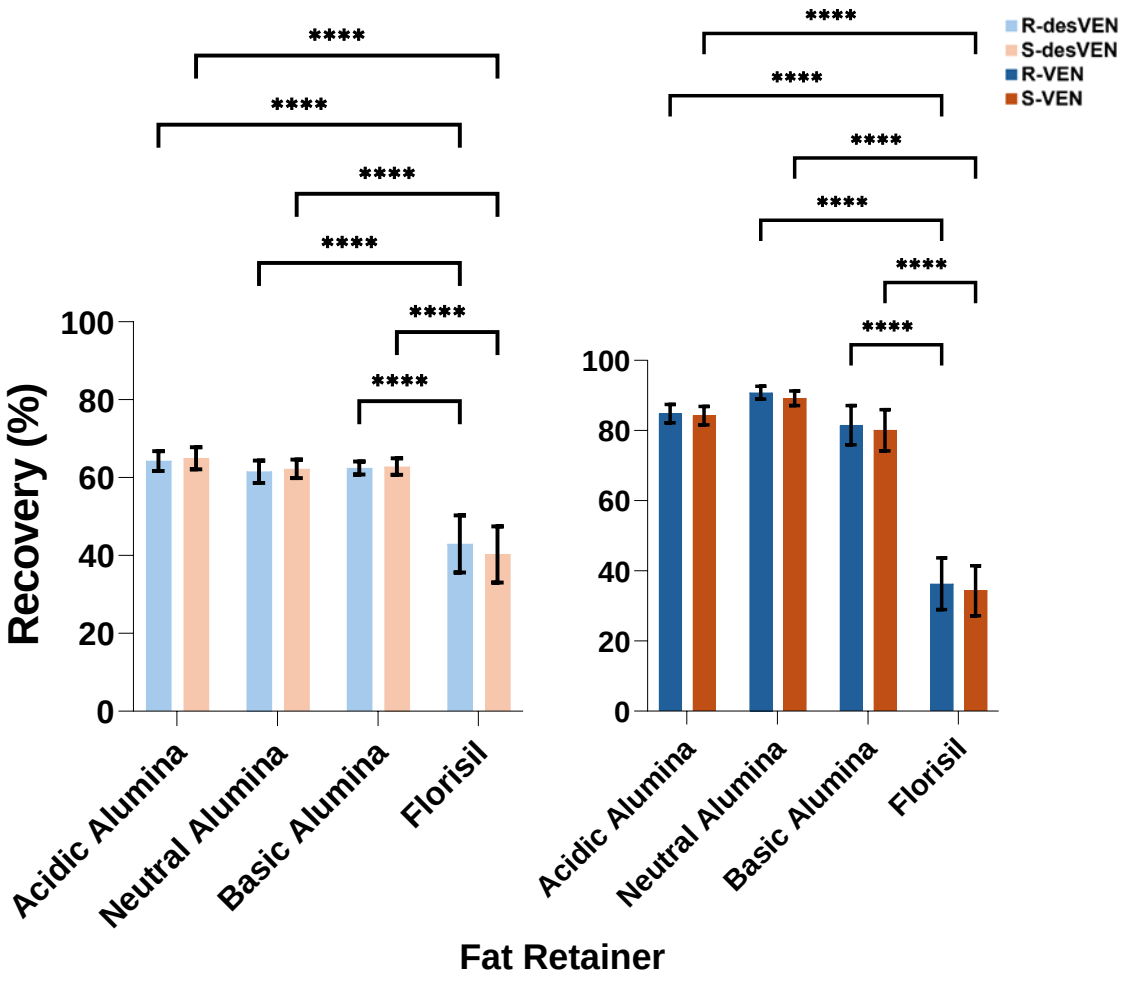

Figure 2.3 Evaluation of recoveries (%) of R-VEN, S-VEN, R-desVEN, and S-desVEN with the addition of fat retainers (acidic alumina, neutral alumina, basic alumina, and Florisil) to ASE extraction cells during extraction for on-line sample cleanup. Data are presented as mean ± SD compared using a two-way ANOVA. An asterisk represents a significant difference, determined by Tukey Test (n = 3-4), between the recovery (%) of enantiomers of VEN and desVEN when different fat retainers are included for on-line sample cleaning during ASE. **** = $p < 0.0001$.

Table 2.5 Enantiomeric fraction (EF), standard deviation (SD), and relative standard deviation (RSD) values for chiral VEN and desVEN with the addition of fat retainers to ASE extraction cells during extraction for on-line sample cleanup.

Fat Retainer	VEN			desVEN		
	EF	SD	RSD	EF	SD	RSD
Acidic alumina	0.52	0.003	0.6	0.49	0.006	1.3
Neutral alumina	0.51	0.005	0.9	0.50	0.001	0.2
Basic alumina	0.51	0.003	0.5	0.49	0.003	0.7
Florisil	0.49	0.004	0.8	0.49	0.004	0.9

2.3.2.2 Concentration of fat retainer

Once neutral alumina was chosen for further optimization, the mass (g) of fat retainer to be used during ASE was tested. The mass of neutral alumina with the highest recoveries (%) for all analytes was 2.5 g. Recoveries (%) of R- and S-VEN and R- and S-desVEN were 78.0%, 75.6%, 70.2%, and 67.8% respectively (Figure 2.4). For the enantiomers of VEN, the recoveries were significantly higher when 2.5 g of neutral alumina was included for on-line sample cleanup in comparison to 1.0 g of neutral alumina (two-way ANOVA, Tukey's post-hoc test, $p < 0.05$). For the enantiomers of desVEN, the recoveries were significantly higher when 2.5 g of neutral alumina was included in comparison to 1.0 g and 5.0 g of neutral alumina (two-way ANOVA, Tukey's post-hoc test, $p < 0.001$).

The EFs for VEN and desVEN are racemic for all masses of neutral alumina tested, ranging between 0.50 and 0.51 and 0.48 and 0.51 for the two compounds, respectively (Table 2.6). There were no significant differences between recoveries of the individual enantiomers of VEN or desVEN when 1.0, 2.5, or 5.0 g of neutral alumina was included during ASE (two-way ANOVA, $p > 0.05$ for all comparisons). These results demonstrate that the mass of neutral alumina included does not have an enantioselective effect on the ASE method for chiral VEN and desVEN.

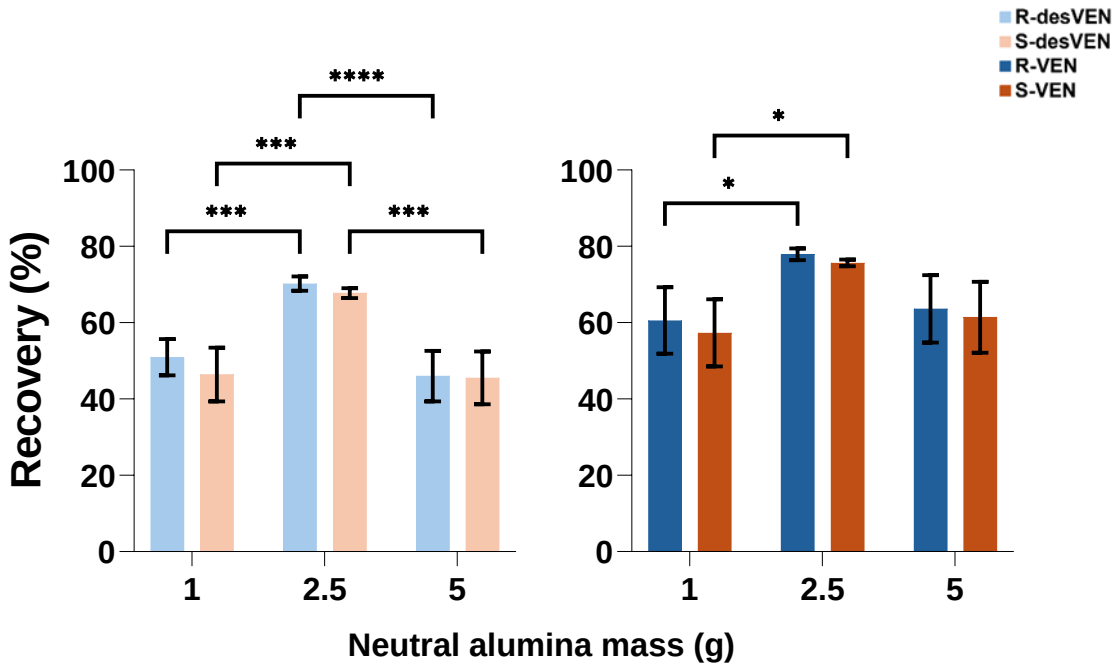

Figure 2.4 Evaluation of recoveries (%) of R-VEN, S-VEN, R-desVEN, and S-desVEN with the inclusion of different masses (g) of neutral alumina fat retainer to ASE extraction cells during extraction for on-line sample cleanup. Data are presented as mean ± SD compared using a two-way ANOVA. An asterisk represents a significant difference, determined by Tukey Test (n = 4), between the recovery (%) of an individual compound when different masses (g) of neutral alumina are included for on-line sample cleaning during ASE. * = $p < 0.05$, *** = $p < 0.001$, and **** = $p < 0.0001$.

Table 2.6 Enantiomeric fraction (EF), standard deviation (SD), and relative standard deviation (RSD) values for chiral VEN and desVEN with the inclusion of different masses (g) of neutral alumina fat retainer to ASE extraction cells during extraction for on-line sample cleanup.

Mass of neutral alumina (g)	**VEN**			**desVEN**		
	EF	**SD**	**RSD**	**EF**	**SD**	**RSD**
1	0.50	0.006	1.2	0.48	0.022	4.6
2.5	0.51	0.005	0.9	0.51	0.002	0.3
5	0.51	0.003	0.6	0.51	0.010	2.0

2.3.2.3 Post-extraction sample cleanup

Sample cleanup after ASE using solid-phase extraction, liquid-liquid extraction, and the commercial QuEChERS dispersive SPE kit were compared for their ability to increase analyte recoveries (%). Oasis HLB SPE cartridges had the highest recoveries for R-VEN, S-VEN, R-desVEN, and S-desVEN, which were 81.9%, 79.5%, 87.4%, and 86.6% respectively (Figure 2.5). In addition, the recoveries for all analytes were significantly higher when Oasis HLB SPE was used in comparison to all other cleanup methods tested (two-way ANOVA, Tukey's post hoc test, $p < 0.05$). Oasis HLB SPE had racemic EF values for both VEN and desVEN (0.51 and 0.50, respectively), and was the only cleanup method tested that was not enantioselective for both VEN and desVEN (Table 2.7). As a result, Oasis HLB SPE was chosen for the final optimized extraction method because it increases the recoveries for all analytes with no enantiomeric bias.

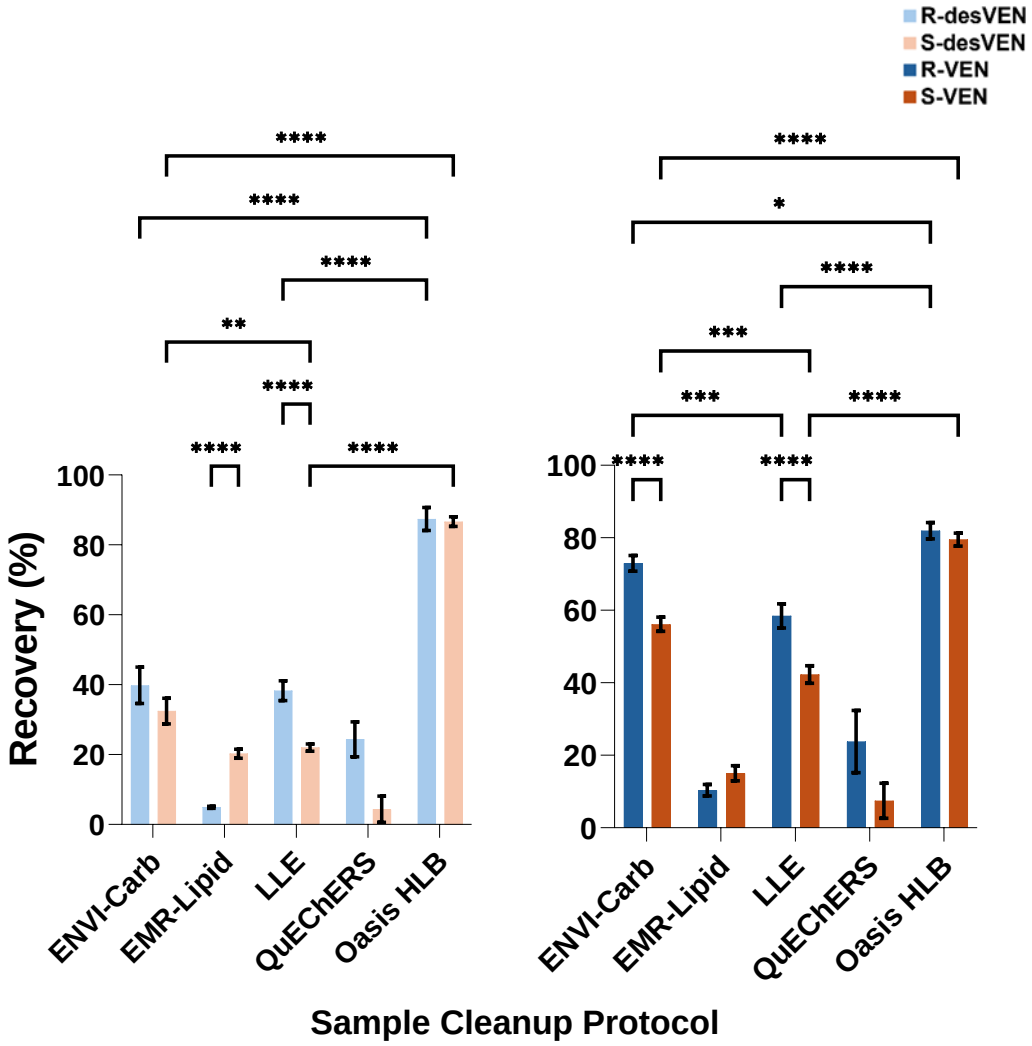

Figure 2.5 Optimization of sample cleanup protocols performed after ASE for R-VEN, S-VEN, R-desVEN, and S-desVEN. Data are presented as mean ± SD compared using a two-way ANOVA. An asterisk represents a significant difference, determined by Tukey Test (n = 4), between the recovery (%) of an individual compound when different sample cleanup protocols (ENVI-Carb SPE, EMR-Lipid SPE, LLE with heptane, QuEChERS, and Oasis HLB SPE) are used to remove excess lipids after ASE, or between R- and S-enantiomers of VEN and desVEN with one cleanup protocol. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$, **** = $p < 0.0001$.

Table 2.7 Enantiomeric fraction (EF), standard deviation (SD), and relative standard deviation (RSD) values for chiral VEN and desVEN for different sample cleanup protocols completed after ASE for the removal of excess lipids from the final ASE eluate.

Sample Cleanup Method	VEN			desVEN		
	EF	SD	RSD	EF	SD	RSD
EMR-Lipid SPE	0.62	0.02	3.0	0.50	0.02	3.4
ENVI-Carb SPE	0.59	0.01	1.6	0.45	0.01	1.2
Oasis HLB SPE	0.51	0.01	1.7	0.50	0.01	1.2
LLE with heptane	0.57	0.01	1.0	0.39	0.004	1.0
QuEChERS	0.51	0.05	10.3	0.25	0.08	32.6

2.3.3 Evaluation of matrix effects

Matrix effects can cause ion suppression of an analyte signal due to the extraction and fish matrix. The final concentration of the sample can also impact the amount of matrix effects. Matrix effects were evaluated by comparing the signal of a known concentration of an injected standard with the signal of an analyte in the extracted sample. The effects of the mass (g) of fish tissue extracted and the final reconstituted volume (mL) of the sample extract were determined. Some ion suppression was seen for all analytes in each experiment (i.e., the recovery of VEN and desVEN spiked into the sample was below the same concentration of a standard injected directly into the instrument).

When the mass of fish tissue increased and the reconstitution volume was constant, the matrix effects increased (Figure 2.6). For both VEN and desVEN, there were significant decreases in the recoveries as the mass of fish tissue increased (two-way ANOVA, Tukey's post hoc test, $p < 0.05$). This is likely due to the extraction of more co-extractives from larger masses of tissue, which were not completely removed during sample cleanup and became more concentrated in the final sample extract. Additionally, there were no significant changes in the magnitude of matrix effects seen with the different reconstitution volumes when a constant mass of fish tissue was extracted (Figure 2.7; two-way ANOVA, Tukey's post hoc test, $p > 0.05$ for all comparisons). The variability was highest at the lowest final reconstitution volume tested (0.25 mL; shown as ± SD error bars on each bar).

The EF values for chiral VEN and desVEN were determined to be racemic for both matrix effects experiments (Table 2.8 and Table 2.9 for the tissue mass and reconstitution volume experiments respectively). This result demonstrates there are no enantioselective effect on the matrix effects seen with the extraction of the individual enantiomers of chiral VEN and desVEN when extracting up to 4.8 g of fish tissue, or reconstituting the final sample extract to a final volume up to 1.0 mL The optimized tissue mass chosen for the final method was 2.4 g, since it demonstrates a good response for all analytes without detrimental matrix effects, without compromising the sensitivity of the method. A final extract volume of 0.5 mL was chosen to ensure the final extracts are concentrated enough to be able to quantitate all four analytes, with no detrimental matrix effects impacting the sensitivity of the method.

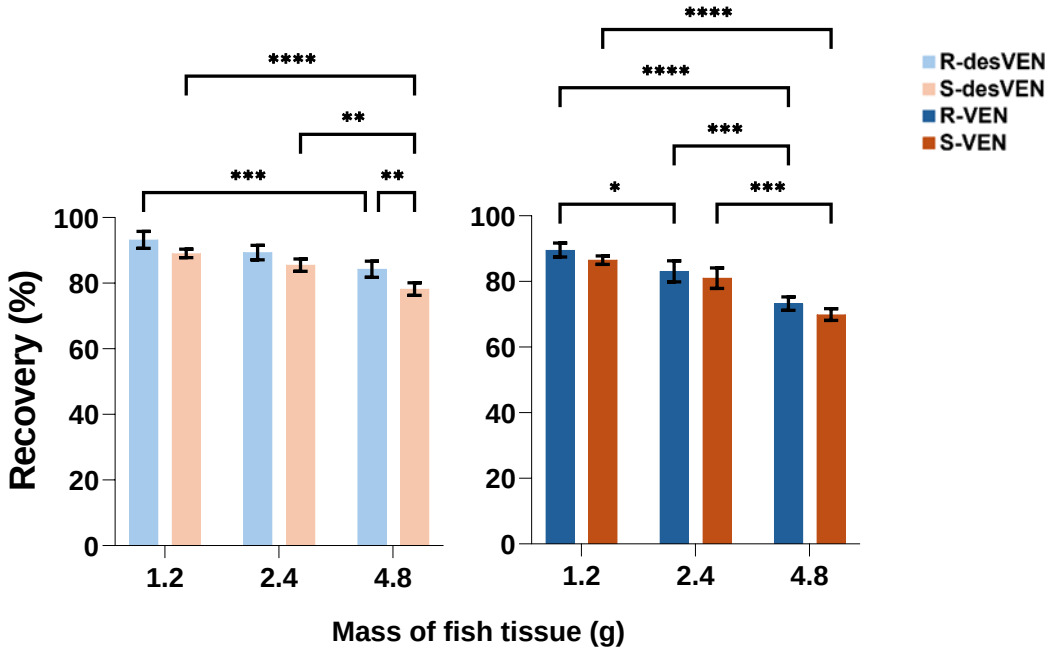

Figure 2.6 Determination of matrix effects observed for R-VEN, S-VEN, R-desVEN, and S-desVEN due to the mass of fish tissue (g) extracted with the optimized ASE method and subsequent sample cleanup protocol with a final sample volume of 0.5 mL. Data are presented as mean ± SD compared using a two-way ANOVA. An asterisk represents a significant difference, determined by Tukey Test (n = 4), between the matrix effects (%) of an individual compound when different masses (g) of fish tissue are extracted, or between R- and S-enantiomers of VEN and desVEN when the same mass of fish tissue is extracted. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

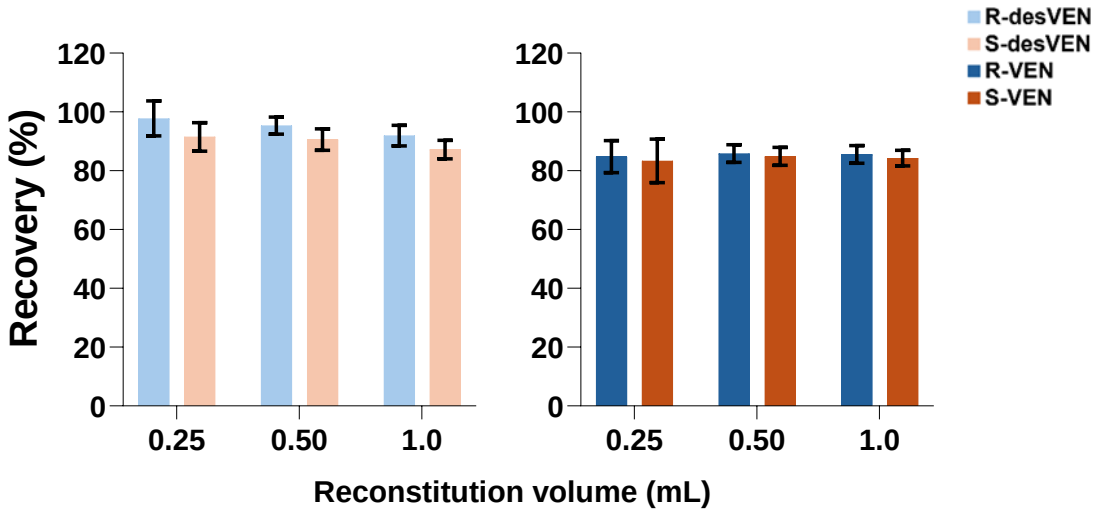

Figure 2.7 Determination of matrix effects (%) observed for R-VEN, S-VEN, R-desVEN, and S-desVEN due to the final sample extract reconstitution volume (mL) for the optimized ASE method and subsequent sample cleanup protocol when extracting 2.4 g of fish tissue. Data are presented as mean ± SD and were compared using a two-way ANOVA. No significant differences determined by Tukey Test (n = 4), between the matrix effects (%) of an individual compound when final extracts are reconstituted to different final volumes (mL), or between R- and S-enantiomers of VEN and desVEN when samples are reconstituted to the same final volume ($p > 0.05$).

Table 2.8 Enantiomeric fraction (EF), standard deviation (SD), and relative standard deviation (RSD) values for chiral VEN and desVEN when evaluating the matrix effects (%) observed for due to the mass of fish tissue (g) extracted with a final sample extract volume of 0.5 mL.

Mass of fish tissue (g)	**VEN**			**desVEN**		
	EF	**SD**	**RSD**	**EF**	**SD**	**RSD**
1.2	0.49	0.002	0.5	0.50	0.002	0.4
2.4	0.49	0.004	0.9	0.50	0.004	0.7
3.6	0.50	0.005	1.0	0.50	0.002	0.4

Table 2.9 Enantiomeric fraction (EF), standard deviation (SD), and relative standard deviation (RSD) values for chiral VEN and desVEN when evaluating the matrix effects (%) observed due to the final sample extract reconstitution volume (mL) when extracting 2.4 g of fish tissue.

Reconstitution volume (mL)	VEN			desVEN		
	EF	SD	RSD	EF	SD	RSD
0.25	0.48	0.007	1.4	0.51	0.011	2.2
0.5	0.48	0.007	1.5	0.51	0.001	0.2
1.0	0.48	0.006	1.4	0.50	0.007	1.4

2.3.4 Method characterization

The final method that was characterized in this study was ASE with 1500 psi extraction pressure, two static extraction cycles, 300 s static time, 100 s purge, 75% flush volume, 70°C extraction temperature, and acetonitrile with 1% formic acid extraction solvent. Neutral alumina was the fat retainer chosen for on-line sample cleanup. Up to 2.4 g of fish tissue can be extracted in each cell. The resulting ASE eluate underwent subsequent cleanup with Oasis HLB SPE. The final sample extract was reconstituted to a volume of 0.5 mL.

2.3.4.1 Validation parameters

The optimized method had coefficients of determination (R^2) for a standard curve (nine concentrations, 0.1 - 100 µg/L) for R-VEN, S-VEN, R-desVEN, and S-desVEN of 0.994, 0.996, 0.993, and 0.996, respectively (Table 2.10). The absolute recoveries (%) for R-VEN, S-VEN, R-desVEN, and S-desVEN ranged from 62.4 to 78.6%, with extraction efficiencies (%) of 52.6 to 66.0% (Table 2.10). Matrix effects (% suppression) values for all analytes ranged from -19.4 to -15.0% (Table 2.10).

Table 2.10 Validation parameters, including the evaluation of linearity (expressed as the coefficient of determination, or R^2 value), absolute recovery (AR; %), extraction efficiency (EE; %), and matrix effects (ME; % suppression) for extraction with the optimized ASE and sample cleanup protocol.

Compound	R^2	AR (n = 4)			EE (n = 4)			ME (n = 4)		
		%	SD	RSD	%	SD	RSD	%	SD	RSD
R-VEN	0.994	78.6	4.0	5.0	66.0	3.3	5.0	-18.5	2.3	12.4
S-VEN	0.996	74.3	3.6	4.8	59.2	2.8	4.8	-15.0	2.0	13.1
R-desVEN	0.993	74.0	2.1	2.8	60.4	1.7	2.8	-19.4	2.7	13.7
S-desVEN	0.996	62.4	1.9	3.1	52.6	1.6	3.1	-17.5	2.1	11.7

The inter-day recoveries (% nominal) were determined for all four analytes of interest at low (0.2 ng/g), mid (10 ng/g), and high (30 ng/g) concentrations and were greater than 88% for all compounds (Table 2.11). This is considered satisfactory (in the range of 70 - 80 to 120% [Schoenau, 2019; Tiwari & Tiwari, 2010]). In addition, the RSD values are also considered satisfactory (< 20% [Schoenau, 2019]).

Table 2.11 Inter-day precision analysis of the recovery (%) for low (0.2 ng/g), mid (10 ng/g), and high (30 ng/g) concentrations extracted with the optimized ASE and sample cleanup protocol. Six replicates were extracted once daily for six days (n = 36).

Compound	Low		Mid		High	
	Recovery (% nominal)	RSD	Recovery (% nominal)	RSD	Recovery (% nominal)	RSD
R-VEN	89.9	8.0	97.4	7.6	102.9	7.2
S-VEN	87.4	8.3	94.6	8.3	100.7	7.4
R-desVEN	91.4	9.2	98.5	8.0	103.9	8.0
S-desVEN	88.0	10.5	96.2	9.7	105.3	9.7

The EF values for both VEN and desVEN are racemic for each spiked concentration tested in the inter-day precision experiment completed for validation (Table 2.12). This demonstrates there is no enantioselectivity for VEN or desVEN when fish tissue samples were extracted with the method developed for this study, within the concentration range that was validated (0.2 ng/g – 30 ng/g).

Table 2.12 Enantiomeric fraction (EF), standard deviation (SD), and relative standard deviation (RSD) values in the inter-day precision analysis of the recovery for low (0.2 ng/g), mid (10 ng/g), and high (30 ng/g) concentrations for chiral VEN and desVEN extracted with the optimized ASE and sample cleanup protocol.

Concentration	**VEN**			**desVEN**		
	EF	**SD**	**RSD**	**EF**	**SD**	**RSD**
Low	0.49	0.015	3.0	0.49	0.014	3.0
Mid	0.49	0.005	1.0	0.49	0.008	1.6
High	0.48	0.005	1.1	0.48	0.005	1.0

2.3.4.2 Racemic enantiomeric fraction (EF) range

The determination of the racemic EF range for the final optimized method developed in this study was completed using the RSD (%) values for VEN and desVEN from the inter-day validation experiments. The VEN RSD values for the EF values of different spiking conditions (low, medium, high) are 3.0%, 1.0%, and 1.1% respectively. The desVEN RSD values for the EF values of the different spiking conditions (low, medium, high) are 3.0%, 1.6%, and 1.0% respectively (Table 2.12). The racemic EF range was determined with the greatest value of RSD determined for both VEN and desVEN (3.0% for both compounds). The experimental range can be calculated using the RSD value for VEN and desVEN and the racemic EF value of 0.5: The racemic EF range limits were then determined for the tolerance limits using Equation (9):

$$\text{Lower limit} = 0.5 - (3 * 0.015) = 0.455$$

$$\text{Upper limit} = 0.5 + (3 * 0.015) = 0.545$$

The range of racemic EF values for both VEN and desVEN with the method that was validated for this study is 0.455 - 0.545 since the RSD determined in method validation was 3.0%. The EF values determined throughout method optimization for each experiment have been evaluated with the

racemic EF range calculated above, and the observations are summarized in (Table 2.13). Through the series of optimization experiments completed for this study, enantioselectivity in the extraction of chiral VEN and desVEN was reduced. In the first optimization experiment for the extraction method and solvent choice, there was still enantioselectivity for the extraction of desVEN enantiomers. By the final method characterization experiments, all EF values were within the racemic range for both VEN and desVEN.

Table 2.13 Summary of EF values for optimization experiments within racemic range determined for the optimized extraction method (EF = 0.455 – 0.545) of VEN and desVEN from fish tissue.

Experiment	Table #	EF values within racemic range		EF values outside racemic range	
		VEN	desVEN	VEN	desVEN
Extraction solvent	Table 2.3	ASE: acetonitrile	n/a	ASE: 1:1 acetonitrile:methanol ASE: methanol USE: 1:1 acetonitrile:methanol USE: acetonitrile USE: methanol	ASE: 1:1 acetonitrile:methanol ASE: acetonitrile ASE: methanol USE: 1:1 acetonitrile:methanol USE: acetonitrile USE: methanol
Acidified extraction solvent	Table 2.4	All experiments	All experiments	n/a	n/a
Fat retainer	Table 2.5	All experiments	All experiments	n/a	n/a
Concentration of fat retainer	Table 2.6	All experiments	All experiments	n/a	n/a
Post-extraction sample cleanup	Table 2.7	Oasis HLB SPE QuEChERS	Oasis HLB SPE EMR-Lipid SPE	EMR-Lipid SPE ENVI-Carb SPE LLE	ENVI-Carb SPE LLE QuEChERS
Matrix effects: Mass of fish tissue	Table 2.8	All experiments	All experiments	n/a	n/a
Matrix effects: Reconstitution volume	Table 2.9	All experiments	All experiments	n/a	n/a
Characterization: Inter-day precision	Table 2.12	All experiments	All experiments	n/a	n/a

2.3.4.3 Method detection limits (MDLs)

Method detection limits (MDLs) were determined individually for each of the chiral chemicals in the optimized ASE method and subsequent sample cleanup protocol as part of the final method characterization. The MDLs were 0.04 ng/g, 0.05 ng/g, 0.03 ng/g, and 0.05 ng/g for R-VEN, S-VEN, R-desVEN, and S-desVEN respectively.

2.4 Discussion

Most extraction and sample cleanup methodologies described in the literature for pharmaceuticals do not consider chirality, even though chirality can be important in assessing the risk of environmental contaminants, such as venlafaxine and its metabolites (Stanley & Brooks, 2009). This is a relatively unexplored area of study, and there is a lack of robust, validated extraction methods for chiral pharmaceuticals in fish tissues which limits the ability to reliably assess the toxicokinetic parameters and risk these chemicals pose. Initial experiments showed ASE to be superior to USE based on having the highest recoveries, lower variability across samples, and chromatography with the best peak separation and shape for all four analytes (Figure A 3, Chromatograms A - D in Appendix A). Further refinement of the ASE method and sample cleanup protocol to remove co-extractives (e.g., lipids) from the final extracts reduced matrix effects to < 20%, increased the recoveries for all the compounds of interest to > 88%, and decreased the observed enantiomeric bias between the enantiomers.

Under the conditions used in this study, USE was determined to have very low recoveries for the individual enantiomers of VEN and desVEN. This contrasts with studies in the literature that had satisfactory recoveries for the extraction of VEN and its metabolites from fish with USE methods (Arnnok et al., 2017; Grabicova et al., 2017; Maulvault et al., 2018; Santos et al., 2020, 2023). However, the extraction methods used in the referenced studies do not consider the chirality of VEN and desVEN. The current study highlights the importance of considering chirality during method development, as there may be an enantioselective effect that cannot be observed when considering a chiral compound as a singular compound. A bias in the extraction of one enantiomeric form of a compound can lead to the over- or under-estimation of the concentration of enantiomers. This can lead to misinterpretation of the fate, bioaccumulation, and ultimately the risk of these chiral compounds. In addition, the chromatograms for samples extracted with USE (Chromatograms E – H in Figure A 3 in Appendix A) had less peak separation and shorter height in comparison to those of samples extracted with ASE (e.g., there are observable shoulders and/or splits on some peaks for the

samples and their internal standards extracted with USE). This demonstrates an issue for extracting enantiomers of chiral antidepressants when using the USE method.

Overall, the results of the current study emphasize that extraction methods can introduce a bias in the determination of the relative abundance of enantiomers of compounds such as VEN and desVEN in tissue samples. Chirality must be considered during method development, and enantiomeric bias must be mitigated and accounted for. Sample extracts need additional cleanup after ASE due to the presence of co-extractives, such as lipids, which may cause matrix effects and impact recoveries and effect instrumentation. Different types of ASE fat retainers have been shown to increase the recovery of compounds of interest (chiral VEN and desVEN). Similar results were found when extracting dioxins, furans, and PCBs from fat-containing samples with ASE (Björklund et al., 2001; Lavin & Hageman, 2012; Sporring et al., 2003). Under conditions used in this study, alumina fat retainers were shown to increase the recoveries of the enantiomers of VEN and desVEN from fish tissue when included for on-line sample cleanup during ASE. In addition, alumina fat retainers did not act in an enantioselective manner (i.e., there was no observed bias in the extraction of R- and S-enantiomers). Based on the available literature about the addition of fat retainers during ASE extraction for sample cleanup, this study may be the first to report the enantiomerically unbiased nature of alumina fat retainers for pharmaceuticals during ASE extraction (i.e., VEN and desVEN).

Through method characterization, the MDLs for R-VEN, S-VEN, R-desVEN, and S-desVEN were determined. The MDLs determined for each of the analytes were 0.04 ng/g, 0.05 ng/g, 0.03 ng/g, and 0.05 ng/g respectively, and these are comparable to MDL values in the studies of VEN in fish completed by Jin et al. (2023) and Ruan et al. (2020). In the study completed by Jin et al. (2023), the MDLs and matrix-spiked recovery values of the method are stated for racemic VEN (i.e.., individual values for R-VEN and S-VEN are not reported). However, when comparing the MDLs for VEN determined for the method developed in this study and the method used by Jin et al. (2023), the MDLs are comparable (i.e., 0.03 ng/g in whole fish for VEN in their method, compared to 0.04 ng/g and 0.05 ng/g for R- and S-VEN in this method). In addition, the MDLs of R-desVEN and S-desVEN from the method developed for this study are comparable to the MDLs for the major metabolites found in their study (i.e., 0.02 ng/g in whole fish for *N*-desmethylvenlafaxine in their method, compared to 0.03 ng/g and 0.05 ng/g for R- and S-desVEN in this method). The MDLs for chiral VEN in the study completed by Ruan et al. (2020) are comparable to those determined in this study and in the study completed by Jin et al. (2023). No major metabolites were included in the study

completed by Ruan et al. (2020), therefore MDLs and recoveries of desVEN cannot be compared and commented on.

The recoveries determined in the inter-day experiment for this study (outlined in Table 2.11) are higher for each of the enantiomers of VEN, ranging from 89.8 – 102.9% for R-VEN and 87.4 – 100.7% for S-VEN, in comparison to the 81.7% matrix-spiked recovery for VEN for whole fish samples reported by Jin et al. (2023). They did not study *O*-desmethylvenlafaxine in their work, as they determined other metabolites of VEN (i.e., *N*, *O*-didesmethylvenlafaxine and *N*-desmethylvenlafaxine) were major metabolites. Recoveries of the desVEN enantiomers determined for this study are higher in comparison to the recovery of *N*-desmethylvenlafaxine from whole fish determined by Jin et al. (2023). Once again, the matrix-spiked recoveries are higher for both enantiomers of VEN in the method developed for this study in comparison to the matrix-spiked recoveries determined by Ruan et al. (2020), which range from 83-86% and 80-85% for R-VEN and S-VEN respectively. The comparable MDLs and higher recoveries for the extraction of chiral VEN and desVEN developed in this study demonstrate that enantioselectivity can be mitigated, and recoveries can be improved even when considering chirality.

2.5 Conclusions

In this study, an enantiomerically unbiased ASE method for chiral VEN and desVEN in fish tissue was developed. The final optimized method has high recovery (> 88%), minimal matrix effects (< 20%) and is not enantiomerically biased. The method selected uses accelerated solvent extraction (ASE) at 1500 psi extraction pressure, two static extraction cycles, 300 s static time, 100 s purge, 75% flush volume, 70°C extraction temperature, and acetonitrile with 1% formic acid extraction solvent. In the extraction cell, 2.5 g neutral alumina fat retainer was included, and up to 2.4 g of fish tissue was extracted and reconstituted to a final extract volume of 0.5 mL. The subsequent sample cleanup protocol chosen was completed using Oasis HLB cartridges (6 cc, 500 mg), by introducing the ASE extracts to the cartridges, collecting the eluate containing the analytes of interest, and reconstituting the extracts to a final volume of 0.5 mL following the protocol outlined in Appendix A, Figure A 2.

Developing a method for extracting both compounds while also considering their chirality is important due to *O*-desVEN being the major metabolite found in the aquatic environment along with VEN, sometimes at higher concentrations than VEN itself. When evaluating any enantioselective processes (e.g., bioaccumulation of different enantiomers), the analytical method that is applied

should also be validated specifically for each enantiomer, such that enantioselective effects at different steps of the sample preparation, extraction, and analysis can be determined and mitigated and the concentration of individual enantiomers can be evaluated accurately.

A thorough validation of methods needs to be undertaken for environmental matrices such as surface waters and biota exposed to complex mixtures like wastewater effluent. There can be additional compounds present in these environmental mixtures (e.g., wastewater effluents) that may be identified during analysis. Over the course of this project, it was identified that S-desVEN has the exact same structure as an isomer of tramadol when ionized during LC-MS/MS analysis. Since tramadol is often found at low levels in municipal wastewater, the method developed in this study requires additional modification for the separation and quantitation of S-desVEN in wastewater or environmental samples, due to the possible presence of this tramadol isomer peak. Although the method developed has high recoveries, is sensitive, and is enantiomerically unbiased for VEN and desVEN, further method development is necessary to separate and quantify these isomers in environmental samples, especially where tramadol is a potential contaminant. However, under controlled conditions this method can be applied to determine the enantiomeric composition of relatively small fish tissue samples.

Chapter 3
In-lab bioaccumulation exposure of rainbow darter to chiral venlafaxine

3.1 Introduction

Many bioactive contaminants of emerging concern (CECs) are not effectively removed from wastewater as it undergoes treatment, including pharmaceuticals and personal care products (PPCPs; Ellis, 2006; Ternes et al., 2004). These compounds are minimally transformed and/or degraded during treatment, and therefore remain in the final effluent that gets released into the receiving aquatic environment (Lishman et al., 2006; Srikanthan, 2019). Commonly prescribed antidepressants and their metabolites are a group of CECs that are poorly removed during treatment that can be found downstream of wastewater treatment plant outfalls. This includes venlafaxine (VEN) and its major metabolite *O*-desmethylvenlafaxine (desVEN; Kasprzyk-Hordern & Baker, 2012; Metcalfe et al., 2010) that are selective serotonin and norepinephrine reuptake inhibitors (SNRIs) prescribed for the treatment of depression and anxiety disorders (Sansone & Sansone, 2014).

With a noted increase in antidepressant prescription and consumption (Magalhães et al., 2014; OECD, 2023), specifically for VEN (Uthayakumar et al., 2022), this raises concerns with regard to their release into the aquatic environment via wastewater effluent. VEN has been found at concentrations in the range of ng/L to μg/L in receiving waters globally (Wilkinson et al., 2022), and in Canadian municipal wastewaters specifically (Arlos et al., 2015; Couperus et al., 2016; Gauvreau et al., 2022; Metcalfe et al., 2010). In studies conducted in the Grand River watershed, these compounds can be found many kilometers downstream of municipal wastewater outfalls and can be detected in surface waters (Arlos et al., 2015; Srikanthan, 2019), tissues of fish (Togunde et al., 2012; S. Wang et al., 2011), and mollusks (De Solla et al., 2016). Despite major upgrades to WWTPs located in the Grand River watershed, the treatment is still not effective in completely removing VEN and its degradation products such as desVEN, and therefore these compounds are still present in surface waters (Couperus et al., 2016; Gauvreau et al., 2022; Srikanthan, 2019). It remains uncertain if exposure to these compounds represents a continued risk to organisms and the environment.

The chirality of VEN and desVEN further complicates their fate in the environment and the assessment of their exposure and risk to organisms (Barclay et al., 2011; Kasprzyk-Hordern & Baker,

2012). Enantiomers have similar physiochemical properties; however, their biological properties can differ due to the specific configuration of the molecule around the chiral center (Kasprzyk-Hordern & Baker, 2012). The interactions of the enantiomers with receptors, enzymes, and other chiral molecules can differ, and they may mediate different, specific pharmacological effects (Barclay et al., 2011). For example, the S-enantiomer of VEN has been found to more selectively inhibit serotonin reuptake, whereas the R-enantiomer is a potent inhibitor for both serotonin and norepinephrine reuptake (Silverstone et al., 1999). In addition, the metabolism of VEN into its major metabolite desVEN via the cytochrome P450 (CYP) enzyme CYP2D6 has been shown to be stereoselective towards the R-enantiomer in humans (Eap et al., 2003).

The enantiomeric composition of the drug may also be altered as it is metabolized and excreted by an organism, or as it passes through wastewater treatment and/or enters the aquatic environment where it is also subjected to more biodegradation processes (Kasprzyk-Hordern & Baker, 2012). One of the enantiomeric forms may be enriched while the other is depleted as it undergoes these processes, leading to a different enantiomeric composition, activity, and risk (Stanley & Brooks, 2009). Studies of chiral pharmaceuticals in wastewater influents and/or effluents have found non-racemic enantiomeric fraction (EF) values for multiple classes of pharmaceutical contaminants (e.g., NSAIDs [Khan et al., 2014], beta-blockers [Bagnall et al., 2012; Evans et al., 2015; Vazquez-Roig et al., 2014], and antidepressants [Bagnall et al., 2012; Evans et al., 2015]). The EF of a chiral compound may also be changed as it undergoes wastewater treatment or other biotic transformation processes once it enters the receiving environment. In a study by Khan et al. (2014), for both ibuprofen and naproxen (examples of chiral NSAIDs) there was enantioselective degradation of the S-enantiomer of both compounds during wastewater treatment. In three studies (Bagnall et al., 2012; Evans et al., 2015; Vazquez-Roig et al., 2014) both propranolol and atenolol (examples of chiral beta-blockers) showed varied EF values in effluent, with S- or R-enrichment depending on the treatment process. The two studies completed by Bagnall et al. (2012) and Evans et al. (2015) found different EF values for the chiral antidepressant venlafaxine in effluent. Bagnall et al. (2012) found a slightly R-enriched EF value, whereas Evans et al. (2015) found a racemic EF value. This highlights the complexity of enantioselective degradation across different classes of chiral pharmaceuticals in influent and effluent. Further research is needed to better understand the mechanisms and factors impacting biological treatment (e.g., microbe activity; [Gasser et al., 2012]) that may lead to enantioselective degradation.

The presence of these compounds in effluents released into the environment can lead to their bioaccumulation in non-target organisms downstream. Bioaccumulation is the process in which organisms absorb and retain chemical compounds that are present in their environment via water, air, and/or diet. Bioconcentration factor (BCF) is a metric used to assess bioaccumulation of a chemical in an organism relative to the ambient water concentration specifically via respiratory or dermal routes (Arnot & Gobas, 2006). A compound is designated as likely to bioaccumulate if it has a BCF value between 500 - 2000, depending on the regulatory guidelines that are used (Gimeno et al., 2024). The BCF can be influenced by the physical and chemical properties of the compound of interest. The main factor used to estimate BCF values is the lipophilicity, or the octanol/water partition coefficient (K_{ow}). It is generally observed that the bioconcentration of a lipophilic compound will increase as the $\log K_{ow}$ increases, up to a value of 6 (Arnot & Gobas, 2006). However, recent studies have determined that using only the K_{ow} may lead to an underestimation of the bioconcentration of pharmaceuticals (Kowalska et al., 2021), and lipophilicity is not the best predictor of the BCF for some neuroactive pharmaceuticals (Duarte et al., 2022).

Chirality has also been shown to alter the bioaccumulation of chiral compounds, leading to the enantioselective accumulation of specific enantiomers. In a lab exposure of rainbow trout (*Oncorhynchus mykiss*) to chiral organochlorine compounds, it was found that the fish enantioselectively eliminated specific isomers of compounds over the depuration phase (Wong et al., 2002). Enantioselective analysis has also been completed on European flounder (*Platichthys flesus*) and common dab (*Limanda limanda*) collected from the Clyde Estuary in Scotland (Petrie & Moffat, 2022), as well as15 species of fish collected from Hong Kong waters (Ruan et al., 2020). Petrie & Moffat (2022) found that fish enantioselectively accumulated fluoxetine, citalopram, and venlafaxine in their muscle and livers. Ruan et al. (2020) found enantiomeric shifts in the concentrations of metoprolol enantiomers found in fish and organisms from lower trophic levels.

The enantiomeric composition of chemicals as they enter the environment is not well studied or applied when conducting risk assessments, however, and it may be an important factor to consider when assessing the overall risk (Sanganyado et al., 2017). Also, the potential effect of chirality on the uptake and bioaccumulation of these chiral compounds has not been widely studied despite it having potential to alter the risk to exposed organisms in the receiving environments. Both biotic and abiotic factors have been shown to change the enantiomeric fraction of contaminants, which complicates the prediction of their fate and risk in the environment (A. R. Ribeiro et al., 2020; Stanley & Brooks,

2009). It is important to measure the concentration of these chiral compounds individually in environmental matrices including fish tissues since it allows for a more specific assessment of exposure to individual enantiomeric forms that can influence risk.

Although studies have been hampered by a lack of validated methods for measuring chiral compounds in fish tissues, this gap was specifically addressed in Chapter 2 of this study. A validated sensitive and enantiomerically unbiased method for both water and fish tissue can be applied to better characterize VEN and desVEN in fish tissues under controlled conditions to determine if there is enantiomeric shift during bioaccumulation. The aim of the current study was to examine the bioaccumulation of the enantiomers of VEN and its major metabolite desVEN in a small-bodied freshwater fish under controlled lab conditions to determine if their bioaccumulation is an enantioselective process.

3.2 Materials and methods

3.2.1 Fish collection

In July 2022, male rainbow darters (RBD) were collected from the Grand River upstream of Grand Valley, ON. This collection site was chosen since it was located upstream of major urban areas or municipal wastewater treatment plants (MWWTPs). Although this site is assumed to be a relatively clean reference site, some upstream contamination from human activities is possible (e.g., septic systems). Only males were collected and used for the exposure to reduce variability that may be caused by possible sex differences. The fish were collected using backpack electrofishing under the University of Waterloo Animal Utilization Protocol (AUPP 44777). After collection, fish were held in aerated buckets until they could be sexed and only male fish transported in coolers back to the Waterloo Aquatic Threats in Environmental Research (WATER) facility at the University of Waterloo. The RBD were housed in an Aquatic Habitats (AHAB) system (Pentair, USA) for two weeks at 19°C to acclimate and allow any depuration of contaminants before being separated into the exposure tanks. The overhead lights for the room were on a timer such that fish were exposed to 12 h of light daily between 7 a.m. and 7 p.m. Fish were fed frozen bloodworms once daily.

3.2.2 Exposure setup

Male RBD were exposed to 1 µg/L of the individual venlafaxine (VEN) enantiomers (R or S) or no VEN (Control) for 14 days in a 24-h static renewal exposure. All exposure tanks used were 20 L glass

aquariums. Six male RBD were randomly placed into each tank after the two-week acclimation period in the AHAB system. The fish acclimated in the tanks for four days before the exposure was started. The fish used in the study had a mean body weight of 3.0 ± 0.8 g and a mean length of 6.3 ± 0.5 cm. The exposure tanks were randomly placed into four large water troughs (as shown in Figure 3.1) to maintain a steady water temperature of approximately 19°C over the exposure period. The exposure water was replaced daily with a 60% static water change. On the first day of the exposure, 225 µL of 80 mg/L R-VEN or S-VEN dissolved in MilliQ water was spiked into the exposure tanks. After each daily static water change, the exposure tanks were spiked with 135 µL of 80 mg/L R-VEN or S-VEN to keep the concentration of VEN in the R- and S-VEN tanks consistent at 1 µg/L for all 14 days. Fish were fed bloodworms once daily after water quality measurements were collected and the subsequent static water change was completed. Three Control tanks were sampled on day 0. Three replicate fish tanks were sampled after 1, 4, and 14 days of exposure for each exposure condition. Two Chemistry Control tanks with no fish were maintained over the exposure experiment to ensure there was no cross-contamination between the tanks with different exposure conditions. This exposure was completed under the University of Waterloo Animal Utilization Protocol (AUPP 44775).

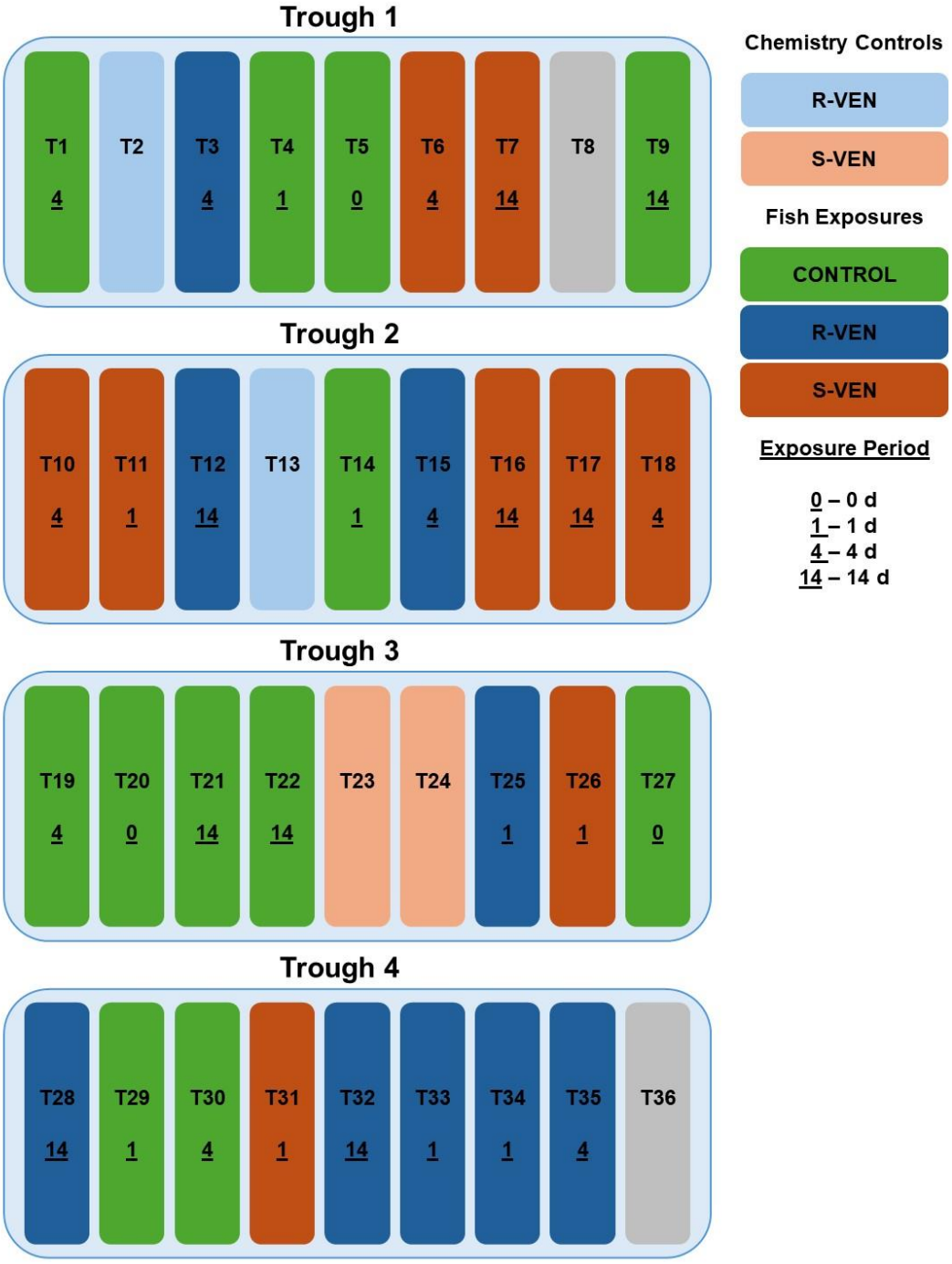

Figure 3.1 Randomized fish tank setup used for the 14-day venlafaxine (VEN) exposure.

3.2.3 Water quality and chemistry analysis

Water quality parameters were measured daily to ensure the maintenance of good water quality and ensure fish health. The water temperature (ºC) and dissolved oxygen (DO; mg/L) content were measured using an OxyGuard Handy Polaris Portable DO Meter (Farum, Denmark), and the pH, nitrate (mg/L), nitrite (mg/L), and ammonia (mg/L) content were measured using a Freshwater Master Test Kit from API Fishcare (Chalfont, PA, USA).

Tank water samples were collected after static renewal and spiking on days 0, 1, 4, and 14 to measure venlafaxine levels. One 100 mL tank water sample was collected from each tank, spiked with deuterated internal standard, and extracted immediately after collection. QA/QC samples composed of 100 mL MilliQ water were spiked with regular and deuterated internal standard and extracted alongside the tank water samples. All water samples were adjusted to a pH of 2, and extracted using solid-phase extraction (SPE) with Bond Elut Plexa cartridges (6 cc, 500 mg, Agilent) using the methods described by Arlos et al. (2015). The eluate was evaporated under nitrogen gas before being reconstituted with 500 µL methanol containing 75 µg/L lorazepam and chloramphenicol internal standards. The extracts were stored in 2 mL amber vials at -20°C until they could undergo LC-MS/MS analysis using the chiral analytical method for water samples by Jamal et al. (2020) described in Section 2.2.3.

3.2.4 Fish sampling

Fish from three tank replicates for each exposure condition were sampled on days 0, 1, 4, and 14. The fish were stunned with a quick blow to the head before being sacrificed via spinal severance. The fish were sexed, and length and weight measurements were taken. Fish were dissected and the brain, gills, liver, and gonads were removed for studies conducted by other researchers/students. The fish carcasses (i.e., without these specific organs) were stored in Whirl-Pak bags and frozen at -20°C for later analysis using the optimized tissue method developed in Chapter 2.

3.2.5 Fish bioaccumulation

The VEN and desVEN concentration in the fish carcasses was quantitated to evaluate the bioaccumulation of individual VEN and desVEN enantiomers in RBD over a 14-day exposure period. The fish were extracted using the optimized extraction method developed in Chapter 2. Three QA/QC samples were extracted with each batch of exposure samples; two matrix spike samples (MS1 and

MS2) and one blank (Blank). For the matrix spike samples, uncontaminated rainbow trout (*Oncorhynchus mykiss*) muscle tissue was homogenized and spiked with regular and deuterated internal standards. The blank samples were prepared in the same manner, however they were only spiked with deuterated internal standard. Extracts were stored in 2 mL amber vials at -20°C until they were analyzed with the adapted LC-MS/MS method for fish tissue described above in Section 2.2.3.

3.2.6 Bioconcentration factor (BCF)

The bioconcentration factor (BCF) for all fish samples was determined by comparing the concentration of the specific enantiomer of VEN in the fish to the concentration of the specific enantiomer of VEN in the tank water with Equation (10).

$$\boldsymbol{BCF} = \frac{[\boldsymbol{VEN\ enantiomer}]_{\boldsymbol{Fish}}}{[\boldsymbol{VEN\ enantiomer}]_{\boldsymbol{Water}}} \qquad \text{Equation (10)}$$

Although the BCF has units of L/kg, it is usually expressed without units based on the assumption that the density of fish tissue is approximately equal to the density of water.

3.2.7 Statistical analysis

Statistical analyses were completed using GraphPad Prism 9.3.1 for Windows. The differences in concentrations (µg/L) of R-VEN and S-VEN in the water of each exposure tank over the 14-day exposure were determined by completing one-way ANOVAs, with Tukey's post-hoc tests. The differences in concentrations (ng/g) of individual VEN enantiomers in the fish from each tank replicate, for each exposure length and each exposure condition, were determined by completing one-way ANOVAs, with Tukey's post-hoc tests. The differences in concentrations (ng/g) of individual VEN enantiomers in the fish for the two exposure conditions (R-VEN vs. S-VEN) at each exposure time (0, 1, 4, and 14 d) were compared with a two-way ANOVA and Tukey's post-hoc tests. For all statistical analyses, the normality of the data was assessed with the Shapiro-Wilk's normality test, and the homogeneity of variance was assessed with the F-test for homogeneity of variance. In addition, an alpha ($\boldsymbol{\alpha}$) value of 0.05 was used to compare the p-values, and p-values are considered significant if less than $\boldsymbol{\alpha}$ for all statistical analyses. Figures present the data as the mean ± the standard deviation (SD) or the standard error of the mean (SEM).

3.3 Results

3.3.1 Water quality and chemistry analysis

Water quality parameters were measured each day of the 14-day exposure period for all tanks that contained fish before the static water renewal took place. The average water temperature of the tanks was 19.2 ± 0.3°C, average DO was 7.8 ± 0.3 mg/L, and average pH was 7.4 ± 0.2. There were no significant differences between exposure tanks for temperature, dissolved oxygen, or pH. Over the entire exposure period there was an absence of, or very low concentrations of nitrate and ammonia measured in all tanks, indicating the water quality was good and did not impact the health of the fish. Nitrate was only present in the exposure tanks on days 10, 11, and 14, and the average concentration was 5 mg/L, 2.5 mg/L, and 2.5 mg/L for each sampling date respectively. Ammonia was only detectable in the exposure tanks on days 0, 3, 7, and 8, and the average concentration was 0.1 mg/L, 0.25 mg/L, 0.25 mg/L, and 0.25 mg/L for each sampling date respectively. There was no nitrite measured in any tank over the 14-day exposure period.

All exposure tanks with either R-VEN or S-VEN were spiked to a nominal concentration of 1 µg/L. The concentration of the exposure compounds in the tank water was confirmed using LC-MS/MS. VEN concentrations were approximately 1 µg/L as expected. During the exposure, R-VEN concentrations ranged from 0.88 – 1.05 µg/L in the R-VEN tanks and S-VEN concentrations ranged from 0.84 – 1.21 µg/L in the S-VEN tanks (Figure 3.2). The concentrations of R-VEN and S-VEN in the tank water did not significantly differ between tanks for each exposure condition across sampling dates (two-way ANOVA, Tukey's post hoc test, $p > 0.05$).

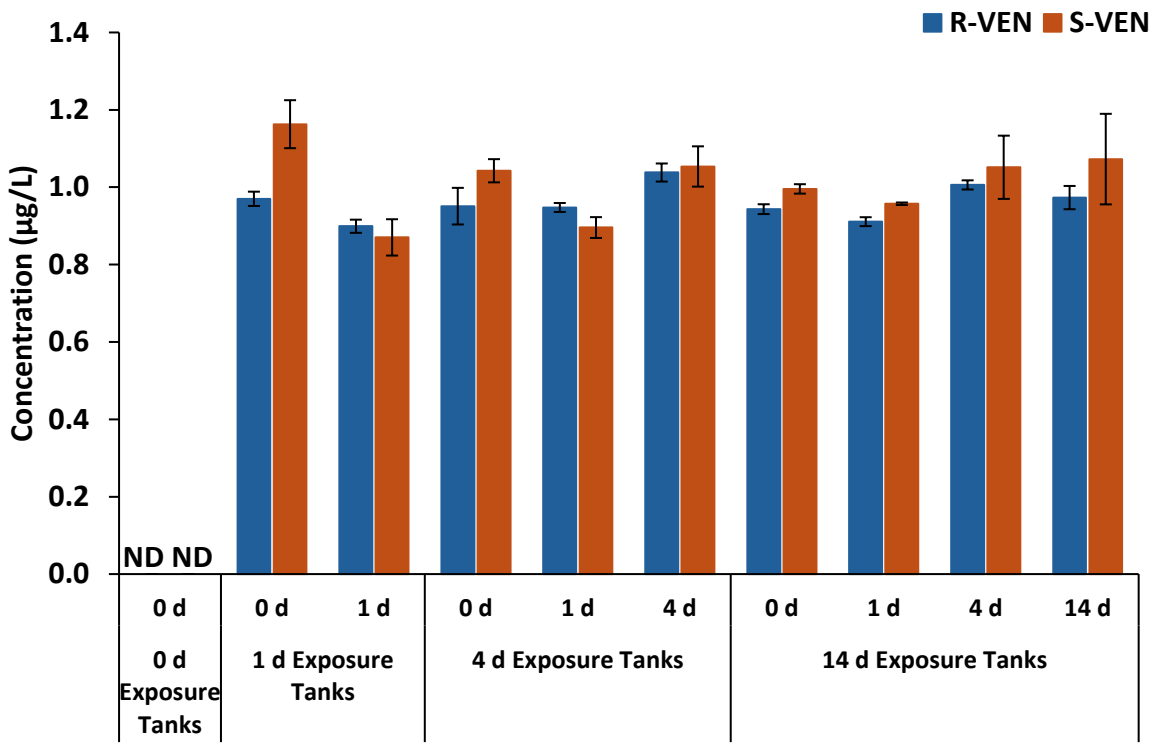

Figure 3.2 Concentration (µg/L) of VEN in tank water over 14-day period. Data is presented as mean ± SD, and the means determined for tank replicates of a specific exposure time points (1 d, 4 d, or 14 d exposure tanks) collected on a specific sampling date (0, 1, 4, or 14 d after the exposure started) are compared using a two-way ANOVA and Tukey post-hoc comparison test. No significant differences were determined by Tukey post-hoc test for the concentration (µg/L) of VEN in the tank water for all tank replicates on all sampling dates.

In all tanks spiked with 1 µg/L R- or S-VEN, only the original enantiomer was found in the water. Over the course of the 14-day exposure (with 60% daily static renewal), there was no desVEN quantitated in any of the exposure tank water at any timepoints (method detection limit = 0.15 µg/L). This suggests that VEN was likely not transformed into desVEN and released into the tank water at a quantifiable amount, via the fish, or other biotic and/or abiotic processes. Alternatively, desVEN may have been rapidly removed from the water such that it remained below the detection limits over the 14-day exposure period.

3.3.2 Fish bioaccumulation

The concentration of all four chiral compounds was measured in the whole fish bodies with the liver, gonad, gill, and brain dissected out. All fish sampled from Control tanks did not have any detectable VEN or desVEN. In all fish exposed to 1 µg/L R-VEN for 1, 4, and 14 days, only R-VEN was found after extraction. On sampling days 1, 4, and 14, fish exposed to 1 µg/L R-VEN had an average of 0.465, 0.726, and 0.754 ng/g R-VEN respectively. R-desVEN was not quantitated in any fish samples at any timepoint. Therefore, the fish either did not metabolize R-VEN into the major metabolite R-desVEN, or it was rapidly depurated below a quantifiable level (note that no desVEN was detected in any of the exposure tank water).

On sampling days 1, 4, and 14, fish exposed to 1 µg/L S-VEN had an average 0.700, 1.42, and 2.11 ng/g S-VEN found after analysis respectively. On sampling days 4 and 14, fish exposed to 1 µg/L S-VEN had an average of 0.142 and 0.383 ng/g S-desVEN detected in their tissues respectively (Figure 3.3). Due to the presence of S-desVEN in the fish extract but not in the associated fish tank water, it can be inferred that the fish metabolized some of the S-VEN into the major metabolite S-desVEN. There were statistically significant increases in the concentrations of S-VEN accumulated in fish exposed for 1 d vs. 4 d, 1 d vs. 14 d, and 4 d vs. 14 d (two-way ANOVA, Tukey's post hoc test, $p < 0.0001$).

There were also significantly higher concentrations of S-VEN in fish exposed to S-VEN compared to the concentrations of R-VEN in fish exposed to R-VEN for 4 d and 14 d (two-way ANOVA, Tukey's post hoc test, $p < 0.0001$; Figure 3.4). This demonstrates that there is a difference in the bioaccumulation of S-VEN relative to R-VEN after at least 4 d of exposure (1 µg/L).

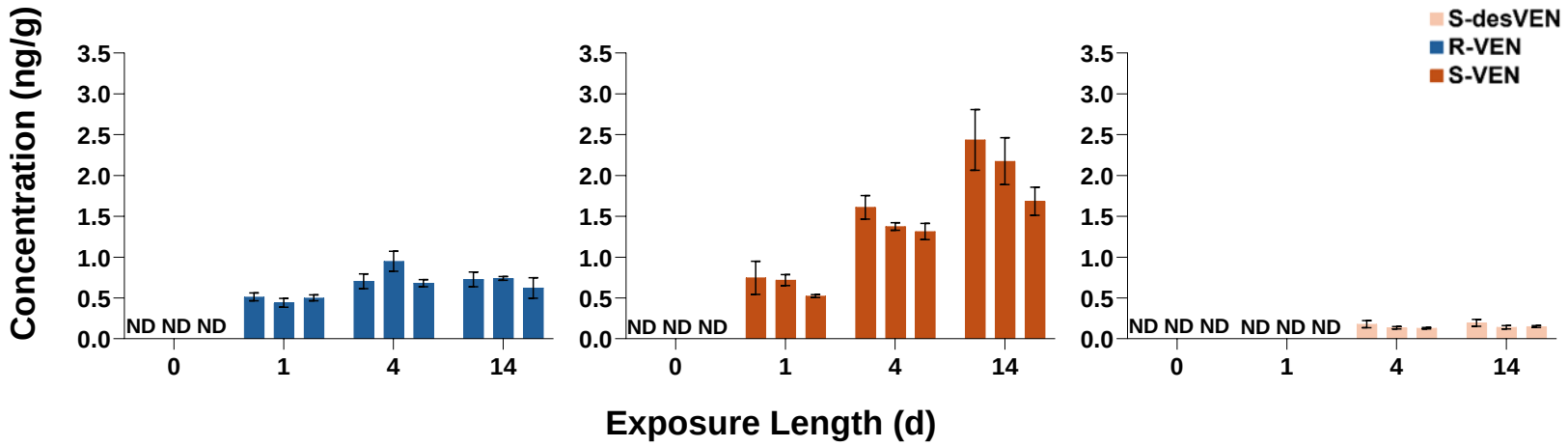

Figure 3.3 Concentration (ng/g) of R-VEN, S-VEN, and S-desVEN in rainbow darters over 14-day exposure. Data is presented as mean ± SEM (n = 4-6 per exposure condition, per tank replicate, per exposure length) and compared with a one-way ANOVA and Tukey post-hoc comparison test. No significant differences were determined by Tukey post-hoc test for the mean concentration (ng/g) of an individual compound (R-VEN, S-VEN, or S-desVEN) found in fish samples between the three tank replicates for a specific exposure condition and exposure length ($\alpha = 0.05$, $p > 0.05$).

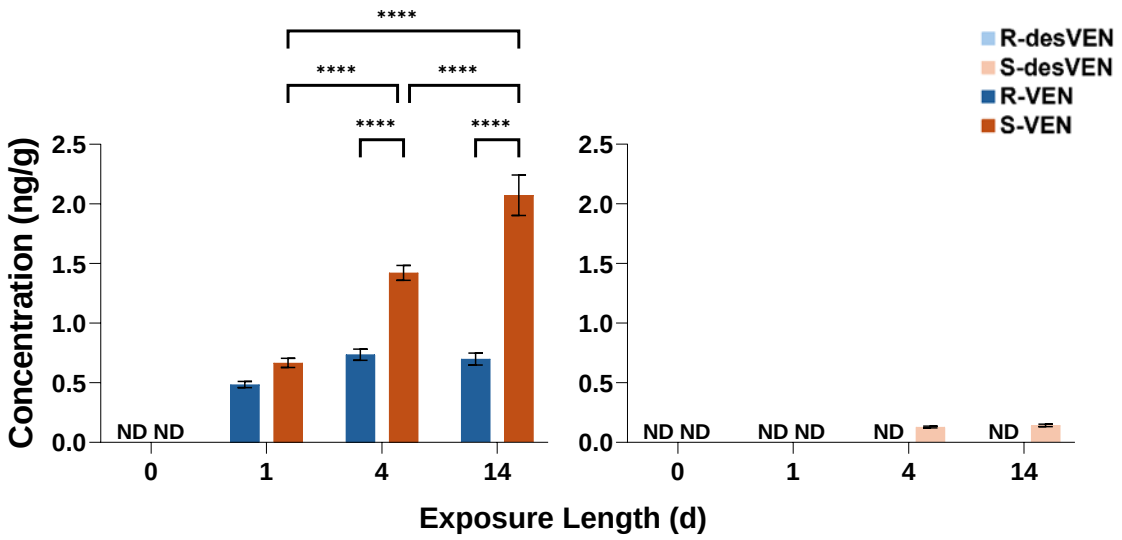

Figure 3.4 Concentration (ng/g) of R-VEN, S-VEN, R-desVEN, and S-desVEN in rainbow darters over 14-day exposure. Data is presented as mean ± SEM per exposure condition, per exposure length, and compared with a two-way ANOVA. An asterisk represents a significant difference, determined by Tukey Test (n = 16 - 18), between the concentration (ng/g) of R-VEN and S-VEN in rainbow darters exposed to individual enantiomers of VEN for different lengths of time (1, 4, or 14 d). **** = $p < 0.0001$.

3.3.3 Bioconcentration factor (BCF)

The bioconcentration factor (BCF) was determined for each exposure condition and exposure length. By correcting for the BCF, any bias due to variability in the concentration of VEN in the tanks was accounted for. The patterns seen for the BCF values were similar to the patterns seen for the concentrations of VEN enantiomers in fish tissue. This was expected since there were no significant differences in the concentrations of VEN between tank replicates on any of the sampling dates. The R-VEN BCF plateaued after 4 days of exposure (Figure 3.5), with the concentration of R-VEN in the fish less than the concentration of R-VEN in the tank water. There were no statistical differences between the BCF values for R-VEN over the 14-d exposure period (two-way ANOVA, Tukey's post hoc test, $p > 0.05$). For S-VEN, the BCF did not plateau after the full 14 d, reaching a BCF of 1.96 on the last sampling date (Figure 3.5). There were statistical differences among the BCF values for S-VEN over the 14-d exposure period. Fish were still accumulating the S-VEN up to the 14 d timepoint, contrasting the plateau pattern seen for R-VEN (two-way ANOVA, Tukey's post hoc test, $p < 0.001$). The BCF values for S-VEN were significantly higher than the BCF values for R-VEN in fish exposed for 4 d and 14 d (two-way ANOVA, Tukey's post hoc test, $p < 0.0001$). This demonstrates an

enantioselectivity in the accumulation of VEN enantiomers after at least 4 d of exposure, with a preference for S-VEN over R-VEN.

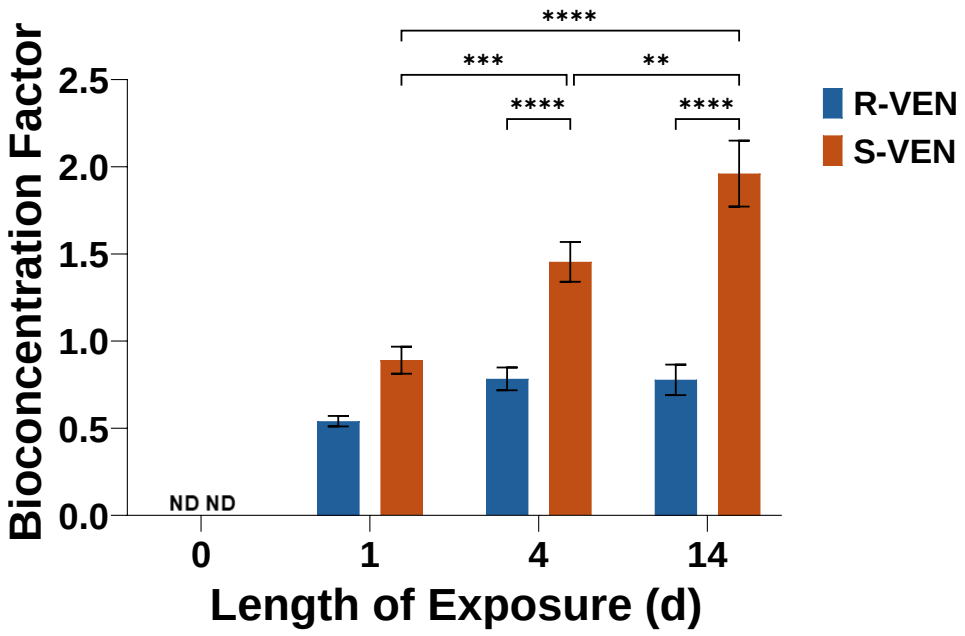

Figure 3.5 Bioconcentration factor (BCF) of R-VEN and S-VEN in rainbow darters over 14-day exposure. Data is presented as mean ± SEM per exposure condition, per exposure length and is compared with a two-way ANOVA. An asterisk represents a significant difference, determined by Tukey Test (n = 16 - 18), between the BCFs of R-VEN and S-VEN in rainbow darters exposed to individual enantiomers of VEN for different lengths of time (1, 4, or 14 d). ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

3.4 Discussion

In the 14-day exposure, both the R- and S-enantiomers of VEN bioaccumulated in the rainbow darters. In addition, S-VEN accumulated approximately twice as much compared to R-VEN after 14 days of exposure. The observed accumulation of VEN is corroborated by the results found by Jin et al. (2023) in their study of the enantioselective uptake and depuration of chiral VEN in medaka (*Oryzias melastigma*), as they also found that S-VEN has a higher bioaccumulation potential when medaka were exposed in a lab setting. These results demonstrate the enantioselectivity of the accumulation of the S-enantiomer in a controlled setting where no confounding factors are present (e.g., other pharmaceutical compounds found in complex matrices such as wastewater effluent). In contrast, a field study conducted by Ruan et al. (2020), found that marine fish in Hong Kong had apparent selective accumulation of R-VEN. However, these findings cannot be directly compared, as

in the field study the exact concentrations of the two VEN enantiomers in water were not reported. In their previous study, Ruan et al. (2019) found that R-VEN was enriched in influent, effluent, and sludge samples collected from the same geographical region the marine biota were collected from in the later study on the uptake of chiral pharmaceuticals. The higher concentration of R-VEN in fish in their study may therefore be due to its enrichment in the environment in which the samples were collected. This also demonstrates the importance quantitating compounds of interest within the relevant environmental matrices such that results can be validated, and comparisons can be made.

In the rainbow darters exposed to S-VEN, the major metabolite S-desVEN was also found in the fish tissues after fish were exposed for 4 and 14 days, whereas R-desVEN was not quantitated in the fish exposed to R-VEN at any time point. Qu et al. (2018, 2019) studied the uptake, distribution, and metabolism of R- and S-VEN in loach (*Misgurnus anguillicaudatus*) when co-exposed to microplastics. In their first published study, Qu et al. (2018) exposed the loach to racemic VEN in a simulated aquatic microcosm with added sediment, duckweed (*Lemna minor*), and microplastics and found both R- and S-desVEN in fish livers after exposure. However, Qu et al. (2018) found higher levels of S-desVEN in the livers of fish as indicated by the S-enriched, non-racemic EF value, demonstrating the enantioselectivity for the bioaccumulation of S-desVEN, similar to the results determined in this study with fish carcasses. In their second published study, Qu et al. (2019) exposed loach to racemic VEN in empty glass tanks. They found similar results to their first study, with both R- and S-desVEN metabolites present in the fish livers after exposure, however there were higher levels of S-desVEN as indicated by an S enriched EF. Both studies support enantioselective uptake of VEN and possible enantioselective differences in metabolism leading to increased relative concentrations of the S-enantiomer of desVEN in fish.

Lab exposures to fish have also been completed for other chiral antidepressants, such as fluoxetine. Fluoxetine is a selective serotonin reuptake inhibitor (SSRI), and also inhibits the reuptake of serotonin (Caccia, 1998). The enantioselectivity for the S-enantiomer of a chiral antidepressant was also reported by Cui et al. (2021) in their zebrafish (*Danio rerio*) exposure to fluoxetine, where they reported a higher BCF value for the S-fluoxetine in comparison to R-fluoxetine. VEN and fluoxetine are only two chiral antidepressants of many, however, this demonstrates a pattern of enantioselectivity in the accumulation of chiral antidepressants with similar biological mechanisms. In addition, the absence of enantiomerization (i.e., the conversion of one enantiomer into another) seen in singular enantiomer exposures was corroborated in the exposure completed by Cui et al.

(2021). The lack of quantifiable enantiomerization is important to note when determining the risk of a chiral compound, especially if one enantiomer has been shown to be more toxic than the other. The results found by Cui et al. (2021) with regards to the metabolite of fluoxetine, norfluoxetine, contrasts what was found in the VEN exposure with regards to its metabolite desVEN. For desVEN, the S-enantiomer was found in the fish after 4 d of exposure, whereas the R-enantiomer was not found in the fish even after 14 d of exposure; Cui et al. (2021) found both the R- and S-enantiomers of norfluoxetine in the fish after exposure to the individual enantiomers of the parent compound fluoxetine starting at 3 d of exposure. Also, the concentrations of both enantiomers of norfluoxetine were higher than the parent compound fluoxetine starting at 5 d of exposure, which contrasts with the much lower levels of S-desVEN compared to S-VEN found after 14 d of exposure. The higher levels of metabolites may be explained by the longer exposure period used for their experiment. However, the differences may also be due to the differing physicochemical characteristics of the compounds, which may lead to more accumulation and/or metabolism in comparison. Further research is needed to better understand how antidepressants, including SSRIs like fluoxetine and SNRIs like venlafaxine, are acting upon systems within the fish and are ultimately metabolized and/or excreted (Ferreira et al., 2023).

Further investigation into the possible differences in the enantioselective uptake of chiral pharmaceuticals in different fish species are warranted to better understand underlying mechanisms of bioaccumulation. Species-specific factors in the uptake of pharmaceuticals have been described previously (McCallum et al., 2017), and it may also be important in the uptake of the individual enantiomers of chiral pharmaceuticals. In the current study, neither the R- nor S-enantiomer of VEN was detected in fish exposed to the other enantiomer. This suggests that the enantiomers are not rapidly transformed into the other enantiomer in fish. The appearance of the S-enantiomer of desVEN in fish may be related to several possible factors, including the higher concentration (BCF) of S-VEN in the fish, higher retention of S-desVEN, or lower removal rates for S-desVEN from tissue. The mechanisms leading to this difference deserve further study.

The BCF values determined in this study were higher for S-VEN in comparison to R-VEN (i.e., 1.96 compared to 0.78), and this pattern is corroborated by the work completed by Jin et al. (2023). In their study, Jin et al. (2023) determined the BCF values of R-VEN and S-VEN in whole fish samples were 0.985 and 5.59 respectively. Although the BCFs are low, the pattern of bioaccumulation in the current study is similar to Jin et al. (2023). Lin et al. (2022) exposed zebrafish to 1 μg/L of racemic

VEN for 30 days and determined BCFs for the brain, gills, gut, and liver. The BCFs were all approximately 20. In addition, they did not consider chirality in their work, therefore comparisons cannot be made regarding enantiomeric compositions. The comparison of BCF values found in both rainbow darter and zebrafish demonstrates the possibility of species-specific accumulation of pharmaceuticals such as VEN for fish. Across different regulatory bodies in different countries, BCF values between 500 – 2000 are the cutoffs for designating a compound that is likely to bioaccumulate and raise additional concerns for chemical exposure (Gimeno et al., 2024). The BCF values determined for the enantiomers of VEN in this study (0.78 for R-VEN and 1.96 for S-VEN) and the BCF values determined across the literature (e.g., 0.985 for R-VEN and 5.59 for S-VEN in the study completed by Jin et al. [2023]) are all low, indicating that VEN is not likely to bioaccumulate to high levels within fish. The difference between the BCF values for the R- and S-enantiomers of VEN does however demonstrate an enantioselective difference in the accumulation of VEN. Although the difference is small, it may be important to evaluate different enantiomeric forms of chiral contaminants individually to ensure there is no bias in the regulatory decisions made for chiral contaminants of emerging concern. It is also important to consider the risk of metabolites which may also be present. This can result in larger impacts if the metabolites have similar mechanisms of action to the parent compound.

Tissue-specific studies have been completed for VEN in larger fish species exposed to larger concentrations and found tissue-specific accumulation (Grabicova et al., 2014; Lajeunesse et al., 2011). A study on tissue-specific accumulation of VEN in rainbow trout determined that VEN was found in the brain, liver, and plasma, with the highest levels in the liver (Grabicova et al., 2014). In another study (Lajeunesse et al., 2011), the accumulation of VEN in brook trout (*Salvelinus fontinalis)* was evaluated and VEN was quantified in liver, brain, and muscle in fish exposed to effluent, with the highest levels found in the liver as well. In both studies, the BCF values for VEN in all of the different fish tissues were higher than the BCF values determined in this study. The BCFs determined by Grabicova et al. (2014) and Lajeunesse et al. (2011) were highest in the liver. The second highest BCF values for both studies were found in the brains of fish. This is important to note for comparisons, as the fish collected from the exposure in this study had their livers and brains removed for other analyses. This is a limitation, since some of the accumulated VEN enantiomers may have been in the organs that were removed and was not accounted for in the BCF values determined for R- and S-VEN. As both literature studies did not incorporate chirality into the

experimental design, no comparisons can be made regarding enantiomeric compositions. Determination of tissue specific bioaccumulation can be limited by the detection limits and available mass of tissue. It may be difficult to complete tissue specific studies in a small fish of interest, such as the rainbow darter, without further method development to lower the detection limits. Future studies on the distribution of chiral compounds in fish tissues, especially the brain, would also be very informative. These compounds are expected to cause changes to behavior which may be mediated by effects in the brain (Salahinejad et al., 2022), and could be enantioselective.

Overall, the enantioselective accumulation and metabolism of VEN in fish shown in the present study demonstrates that future studies to determine if there are enantiospecific effects is a crucial next step in studying chiral compounds. Unfortunately, there are few studies investigating the enantioselective toxicity of chiral drugs in aquatic organisms. In studies that have been completed, species-specific effects have been shown after exposure to the chiral antidepressants fluoxetine and VEN. For example, in the study completed by Stanley et al. (2007), S-fluoxetine had a larger adverse effect on the growth and feeding behavior in fathead minnows (*Pimephales promelas*) compared to R-fluoxetine. In that same study, there were no observable enantiospecific differences in the standardized immobilization and reproduction experiments conducted with *Daphnia magna* exposed to fluoxetine. In the study completed by O. Ribeiro et al. (2022), R-VEN exposure resulted in a significantly higher percent of malformations of zebrafish embryos in comparison to S-VEN exposure at the same concentrations. In that same study, there were no enantiospecific differences seen in the fecundity of *D. magna* exposed to VEN. In comparing the two studies described above (O. Ribeiro et al., 2022; Stanley et al., 2007), it is clear that the effects of each chiral compound must be evaluated individually, even if they are in the same pharmaceutical class and have similar but different biological mechanisms. It is notable to make the comparison that in the exposure completed for the current study, S-VEN bioaccumulated more than R-VEN in the fish, however in the study completed by O. Ribeiro et al. (2022), more malformations were seen after exposure to R-VEN. This divergence highlights the need for evaluations of enantioselectivity when assessing chiral compounds for their bioaccumulation potential as well as their effects, as not to over- or underestimate them and their relation to each other.

3.5 Conclusions

An enantioselective effect was observed on the accumulation and the metabolism of VEN enantiomers in a controlled exposure with rainbow darter. The magnitude of accumulation of the two enantiomers of VEN was different, with S-VEN accumulating approximately twice as much as R-VEN after 14 days of exposure. In addition to accumulating S-VEN, the fish exposed to S-VEN also metabolized and ultimately accumulated S-desVEN at a level that was quantifiable. In contrast, the fish exposed to R-VEN were not found to metabolize and/or accumulate R-desVEN at levels above the detection limit. As R- and S-VEN may accumulate and metabolize differently, they potentially have slightly different effects in fish. This may be important in the assessment of their environmental risk, especially since VEN is also considered pseudo-persistent since it is continuously released into the environment (Ebele et al., 2017; Petrie et al., 2015).

Chapter 4
Conclusions and recommendations

4.1 Conclusions

An accelerated solvent extraction method for chiral VEN and desVEN was optimized and validated through a series of experiments completed in this study. Method development was important in this case to ensure the method was not enantiomerically selective, while also having high accuracy and sensitivity. The optimized and validated method for the extraction of chiral VEN and desVEN from fish tissue was applied to rainbow darters exposed to the individual enantiomers of VEN in a controlled lab exposure. This study has demonstrated the differences in the accumulation and potential metabolism of R- and S-VEN in fish exposed to the compounds for different lengths of time over a 14-d period. The key findings include 1) an increase in the accumulation of S-VEN as compared to R-VEN at the 14-d timepoint, and 2) the presence of the major metabolite S-desVEN at a level that can be quantitated in fish exposed to S-VEN for at least 4 d, whereas the major metabolite R-desVEN could not be quantitated in fish exposed to R-VEN over the whole 14-d exposure period. The differences of the bioaccumulation of both the parent and metabolite compounds in this study demonstrates that there are potential differences in how the enantiomers of VEN accumulate and/or are metabolized in fish.

These findings are important in their application to environmental risk assessments, which historically did not consider the chirality of CECs such as antidepressants, like VEN and desVEN. Through this exposure, chirality was shown to affect the way that VEN was accumulated and/or metabolized in a non-target organism. This demonstrates the importance of incorporating chirality into the development of quantitative analytical methods and the environmental risk assessments of chiral compounds, as not to over- or underestimate the possible concentration and/or effects of chiral compounds. The different enantiomeric forms of chiral compounds must be effectively separated in the methodology used such that each enantiomeric form can be accurately quantified during analysis.

In future field-based studies, all enantiomers of the compounds being studied should be included in analyses. The enantioselective behavior of these compounds during wastewater treatment, or any other abiotic or biotic processes they are subjected to after they are released into the environment via effluent, is also important context for studies on their accumulation and/or metabolism in a river

system. With VEN and desVEN, there may be differences seen between concentrations and potential bioaccumulation of the individual R- and S-enantiomers detected in wild fish collected downstream of wastewater outfalls. In a wild river system, the fish will be exposed to both enantiomers of VEN and/or desVEN simultaneously. There may be an enantioselective effect where one enantiomer is more readily bioaccumulated, metabolized, or degraded in comparison to another enantiomeric form. There may also be potential interactions seen between compounds of interest and other compounds found in the environment, especially in the case of wastewater effluent which is known to contain many other contaminants.

Each environmental matrix needs to be assessed carefully, and the analytical method validated. A preliminary study completed alongside this work has shown tramadol is present in real environmental samples (e.g., municipal wastewater) and can potential interfere with the signal for S-desVEN within the method developed in this study. Tramadol was not separated from S-desVEN in the current method as it has similar chemical structure, identical ionic mass and retention time when analyzed with the LC-MS/MS protocol presented.

4.2 Recommendations

Future depuration studies completed in a controlled lab setting are necessary for further understanding the bioaccumulation and metabolic degradation processes for the individual enantiomers of VEN and desVEN. A depuration rate could be determined through future experiments, and further insight into the bioaccumulation and possible enantioselectivity of this process could be determined. In the exposure completed for this study, only the uptake was considered. The patterns observed through the results would be further supported by a better understanding of the depuration rate, especially with regards to R-VEN, which was found at concentrations lower than the water concentration in this study, and R-desVEN, which was not found at a level that could be quantified in this study. Also, a longer exposure period may provide more insight into the accumulation and depuration of the enantiomers of VEN. The exposure completed for this study was only 14 d long. The concentration of S-VEN found in fish tissue did not plateau after 14 d, and may have continued to increase. A longer exposure period may have also been required to find R-desVEN at concentrations that could be quantitated with the analytical method used in this study. Future studies can also be completed with exposure conditions that include the individual enantiomers, as well as racemic and/or non-racemic mixtures of the enantiomers. In such a study, the enantiomeric fraction of samples exposed to

mixtures of enantiomers can be calculated to determine if there is an enantiomeric shift over time. If a shift is seen, there may be enantioselectivity in the bioaccumulation and/or metabolism.

In addition, many other chiral compounds that are found in the aquatic environment and impact non-target organisms are candidates for further study, including other antidepressants, their metabolic products, and other pharmaceuticals. Further understanding of these processes for individual compounds at a laboratory-scale is a step in starting to understand how these chiral compounds may interact in a more complex mixture. The enantioselectivity of the bioaccumulation and metabolic processes shown in the exposure completed highlight the importance of considering chirality in future studies for all chiral compounds.

Chirality should also be considered in future field-based studies of chiral compounds found in the environment. Chiral compounds are subject to different abiotic and biotic processes before they reach the aquatic environment and impact non-target organisms, and thus do not always enter the environment as a racemic mixture. Future studies of the enantioselective impacts on the enantiomeric fraction of chiral compounds are required to understand what is entering the aquatic environment and impacting aquatic biota. Once the enantiomeric fraction is determined, inferences can be made about potential impacts on organisms and extrapolated to the field.

Future studies of desVEN should be of interest since it is also found in wastewater effluents, and has been quantitated at higher concentrations than its parent compound VEN downstream of WWTPs (Gauvreau et al., 2022; Srikanthan, 2019). This may be a combination of human metabolism of VEN into desVEN and subsequent excretion into the sewer system, or metabolism within the WWTP or aquatic environment itself. In this case, fish in the receiving environment may be exposed to desVEN via both their own metabolism of VEN and the desVEN present from wastewater effluents. Experiments of the exposure to and bioaccumulation of individual enantiomers of desVEN itself in fish should also be completed to determine if these processes are enantiomerically selective for desVEN as well. The pattern of bioaccumulation can then be compared to the enantioselectivity seen with the bioaccumulation of chiral VEN as was demonstrated through this study. Further studies on the effects of desVEN on non-target organisms can also be completed and compared to VEN since they both have similar mechanisms of action and may have additive effects.

Unexpected results may arise in field-based studies since samples collected are complex mixtures (e.g., wastewater effluent). Complex mixtures contain many compounds, some of which may interfere

with the analytes of interest within a certain analytical method. Specifically, when working with chiral compounds, isomeric compounds (i.e., compounds with the same chemical formula and mass but have a distinct spatial arrangement) are the first type of compound to rule out as possible interferences when collecting and analyzing data. Through work completed for this study, tramadol was determined to likely be an isomer of S-desVEN in the LC-MS/MS method used (i.e., the fragment ion of tramadol may have the same fragment ion mass and retention time as the fragment ion of S-desVEN monitored in this method). In the current study, water samples were run with the analytical method developed by Jamal et al. (2020) but were not rerun with an adapted method as there was no tramadol in tank water. Future studies using this or similar methods may be confounded with the presence of tramadol. In particular, signal enhancement of S-desVEN in the field might be confounded by tramadol that is often found in municipal wastewaters. More method development must be completed to separate tramadol from S-desVEN in the current method before it can be reliably used in field studies, especially those exposed to municipal wastewaters.

Continued optimization and development of analytical methods required to address future lab- and field-based studies is another key part of better understanding and defining the role of chirality and its impact on how chiral compounds move through the environment and impact non-target organisms. If lower method detection limits are required for a specific compound and/or type of sample, a more sensitive instrument may be necessary. Larger tissue masses can also be used if method detection limits cannot be lowered and/or the analyte of interest is found at very low concentrations. However, as demonstrated through the method development completed in Chapter 2, the possible matrix effects that may result from a larger sample mass must be evaluated and mitigated. In addition, any enantiomeric bias must be addressed throughout method development to ensure that the final validated method can accurately quantitate the individual enantiomers of compounds of interest.

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Appendix A

Extraction method development supplementary information

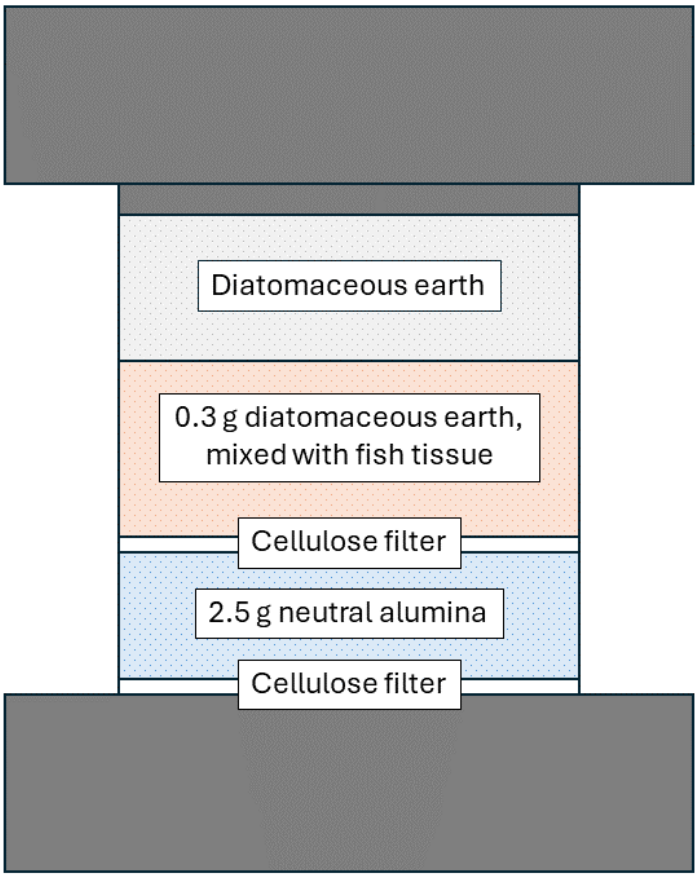

Figure A 1 Diagram of how accelerated solvent extraction (ASE) cells were filled for sample extraction.

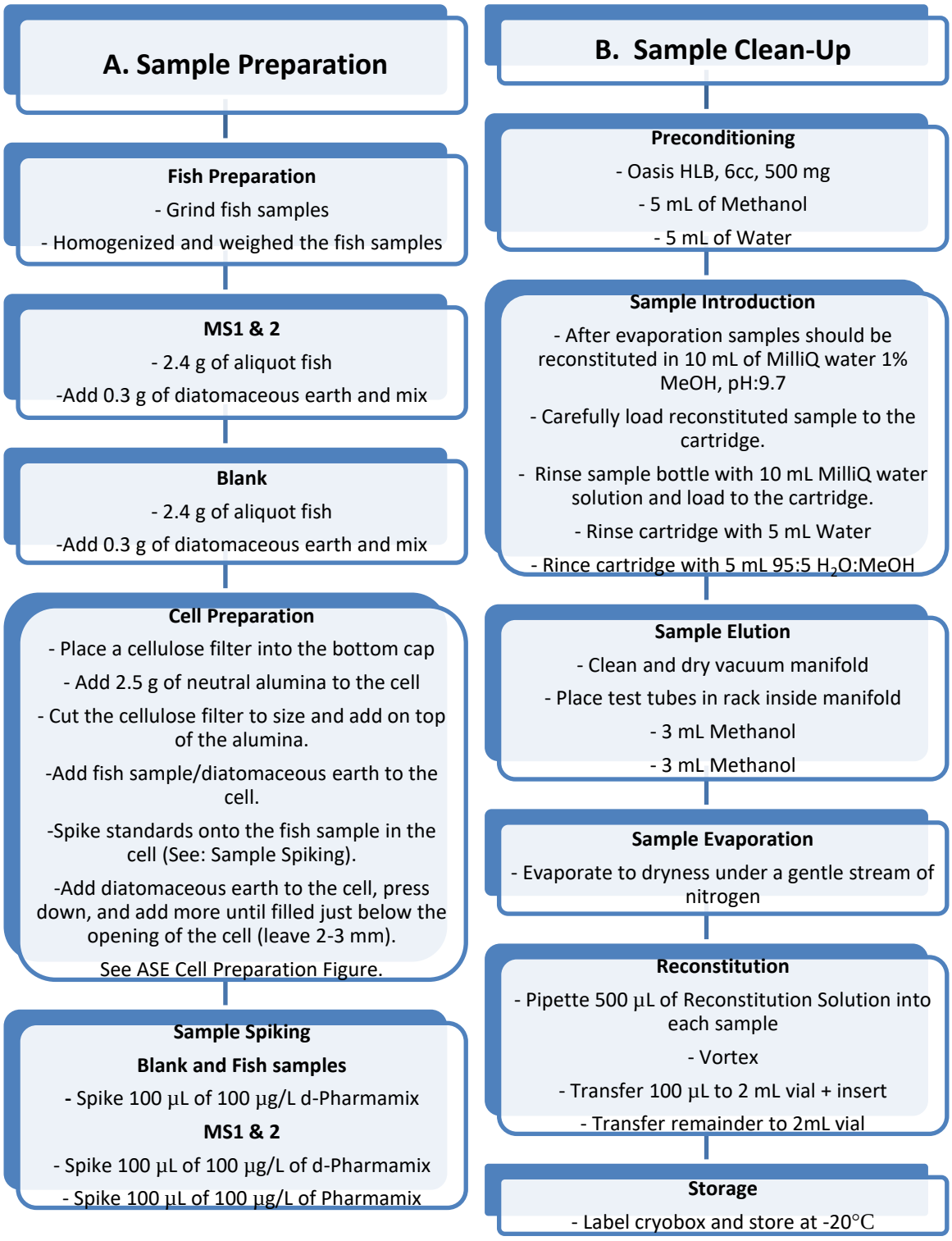

Figure A 2 ASE extraction cell preparation and sample cleanup protocol flowchart.

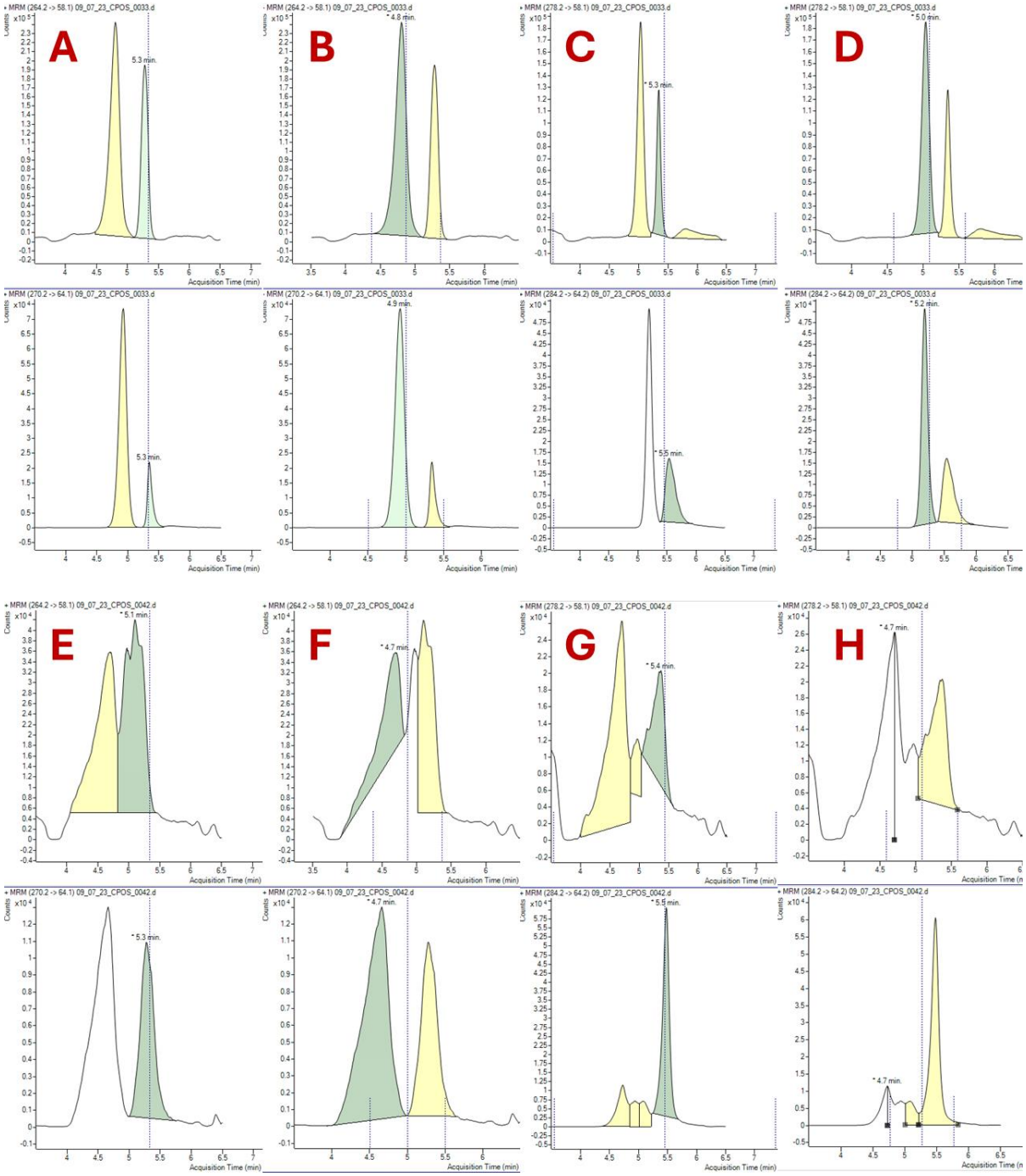

Figure A 3 Chromatograms for A) R-desVEN when extracted with ASE, B) S-desVEN when extracted with ASE, C) R-VEN when extracted with ASE, D) S-VEN when extracted with ASE, E) R-desVEN when extracted with USE, F) S-desVEN when extracted with USE, G) R-VEN when extracted with USE, and H) S-VEN when extracted with USE. The top chromatogram is the regular analyte peak, and the bottom chromatogram is the deuterated internal standard peak.

Table A 1 Two-way ANOVA Tukey comparison test for optimizing extraction solvent for accelerated solvent extraction (ASE) for the extraction of R-VEN, S-VEN, R-desVEN, and S-desVEN.

Treatment 1			Treatment 2					
Compound	Extraction Solvent	Extraction Method	Compound	Extraction Solvent	Extraction Method	Below threshold?	Summary	Adjusted P Value
R-desVEN	1:1 Acetonitrile:methanol	ASE	S-desVEN	1:1 Acetonitrile:methanol	ASE	Yes	****	<0.0001
R-desVEN	1:1 Acetonitrile:methanol	ASE	R-desVEN	Acetonitrile	ASE	No	ns	>0.9999
R-desVEN	1:1 Acetonitrile:methanol	ASE	S-desVEN	Acetonitrile	ASE	Yes	****	<0.0001
R-desVEN	1:1 Acetonitrile:methanol	ASE	R-desVEN	Methanol	ASE	No	ns	0.96
R-desVEN	1:1 Acetonitrile:methanol	ASE	S-desVEN	Methanol	ASE	Yes	****	<0.0001
S-desVEN	1:1 Acetonitrile:methanol	ASE	R-desVEN	Acetonitrile	ASE	Yes	****	<0.0001
S-desVEN	1:1 Acetonitrile:methanol	ASE	S-desVEN	Acetonitrile	ASE	No	ns	0.9923
S-desVEN	1:1 Acetonitrile:methanol	ASE	R-desVEN	Methanol	ASE	Yes	****	<0.0001
S-desVEN	1:1 Acetonitrile:methanol	ASE	S-desVEN	Methanol	ASE	No	ns	0.7569
R-desVEN	Acetonitrile	ASE	S-desVEN	Acetonitrile	ASE	Yes	****	<0.0001
R-desVEN	Acetonitrile	ASE	R-desVEN	Methanol	ASE	No	ns	0.9877
R-desVEN	Acetonitrile	ASE	S-desVEN	Methanol	ASE	Yes	****	<0.0001
S-desVEN	Acetonitrile	ASE	R-desVEN	Methanol	ASE	Yes	****	<0.0001
S-desVEN	Acetonitrile	ASE	S-desVEN	Methanol	ASE	No	ns	0.967
R-desVEN	Methanol	ASE	S-desVEN	Methanol	ASE	Yes	****	<0.0001
R-VEN	1:1 Acetonitrile:methanol	ASE	S-VEN	1:1 Acetonitrile:methanol	ASE	No	ns	0.6668
R-VEN	1:1 Acetonitrile:methanol	ASE	R-VEN	Acetonitrile	ASE	No	ns	>0.9999
R-VEN	1:1 Acetonitrile:methanol	ASE	S-VEN	Acetonitrile	ASE	No	ns	0.1156
R-VEN	1:1 Acetonitrile:methanol	ASE	R-VEN	Methanol	ASE	No	ns	0.9593
R-VEN	1:1 Acetonitrile:methanol	ASE	S-VEN	Methanol	ASE	No	ns	0.1083
S-VEN	1:1 Acetonitrile:methanol	ASE	R-VEN	Acetonitrile	ASE	No	ns	0.6983
S-VEN	1:1 Acetonitrile:methanol	ASE	S-VEN	Acetonitrile	ASE	No	ns	0.8145
S-VEN	1:1 Acetonitrile:methanol	ASE	R-VEN	Methanol	ASE	No	ns	0.2342
S-VEN	1:1 Acetonitrile:methanol	ASE	S-VEN	Methanol	ASE	No	ns	0.7966
R-VEN	Acetonitrile	ASE	S-VEN	Acetonitrile	ASE	No	ns	0.1274
R-VEN	Acetonitrile	ASE	R-VEN	Methanol	ASE	No	ns	0.9473
R-VEN	Acetonitrile	ASE	S-VEN	Methanol	ASE	No	ns	0.1194
S-VEN	Acetonitrile	ASE	R-VEN	Methanol	ASE	Yes	*	0.0231
S-VEN	Acetonitrile	ASE	S-VEN	Methanol	ASE	No	ns	>0.9999
R-VEN	Methanol	ASE	S-VEN	Methanol	ASE	Yes	*	0.0214

*Note: * = $p < 0.05$, **** = $p < 0.0001$, and ns = non-significant.

Table A 2 Two-way ANOVA Tukey comparison test for optimizing extraction solvent for ultrasonic solvent extraction (USE) for the extraction of R-VEN, S-VEN, R-desVEN, and S-desVEN.

Treatment 1			Treatment 2					
Compound	Extraction Solvent	Extraction Method	Compound	Extraction Solvent	Extraction Method	Below threshold?	Summary	Adjusted P Value
R-desVEN	1:1 Acetonitrile:methanol	USE	S-desVEN	1:1 Acetonitrile:methanol	USE	No	ns	>0.9999
R-desVEN	1:1 Acetonitrile:methanol	USE	R-desVEN	Acetonitrile	USE	No	ns	0.6382
R-desVEN	1:1 Acetonitrile:methanol	USE	S-desVEN	Acetonitrile	USE	Yes	*	0.029
R-desVEN	1:1 Acetonitrile:methanol	USE	R-desVEN	Methanol	USE	No	ns	0.9998
R-desVEN	1:1 Acetonitrile:methanol	USE	S-desVEN	Methanol	USE	No	ns	>0.9999
S-desVEN	1:1 Acetonitrile:methanol	USE	R-desVEN	Acetonitrile	USE	No	ns	0.6216
S-desVEN	1:1 Acetonitrile:methanol	USE	S-desVEN	Acetonitrile	USE	Yes	*	0.0275
S-desVEN	1:1 Acetonitrile:methanol	USE	R-desVEN	Methanol	USE	No	ns	>0.9999
S-desVEN	1:1 Acetonitrile:methanol	USE	S-desVEN	Methanol	USE	No	ns	>0.9999
R-desVEN	Acetonitrile	USE	S-desVEN	Acetonitrile	USE	No	ns	0.4347
R-desVEN	Acetonitrile	USE	R-desVEN	Methanol	USE	No	ns	0.4896
R-desVEN	Acetonitrile	USE	S-desVEN	Methanol	USE	No	ns	0.5058
S-desVEN	Acetonitrile	USE	R-desVEN	Methanol	USE	Yes	*	0.0174
S-desVEN	Acetonitrile	USE	S-desVEN	Methanol	USE	Yes	*	0.0184
R-desVEN	Methanol	USE	S-desVEN	Methanol	USE	No	ns	>0.9999
R-VEN	1:1 Acetonitrile:methanol	USE	S-VEN	1:1 Acetonitrile:methanol	USE	No	ns	0.6901
R-VEN	1:1 Acetonitrile:methanol	USE	R-VEN	Acetonitrile	USE	No	ns	0.2306
R-VEN	1:1 Acetonitrile:methanol	USE	S-VEN	Acetonitrile	USE	No	ns	0.1173
R-VEN	1:1 Acetonitrile:methanol	USE	R-VEN	Methanol	USE	No	ns	0.7065
R-VEN	1:1 Acetonitrile:methanol	USE	S-VEN	Methanol	USE	No	ns	0.4412
S-VEN	1:1 Acetonitrile:methanol	USE	R-VEN	Acetonitrile	USE	Yes	*	0.0141
S-VEN	1:1 Acetonitrile:methanol	USE	S-VEN	Acetonitrile	USE	Yes	**	0.0061
S-VEN	1:1 Acetonitrile:methanol	USE	R-VEN	Methanol	USE	No	ns	>0.9999
S-VEN	1:1 Acetonitrile:methanol	USE	S-VEN	Methanol	USE	No	ns	0.998
R-VEN	Acetonitrile	USE	S-VEN	Acetonitrile	USE	No	ns	0.9986
R-VEN	Acetonitrile	USE	R-VEN	Methanol	USE	Yes	*	0.0149
R-VEN	Acetonitrile	USE	S-VEN	Methanol	USE	Yes	**	0.0058
S-VEN	Acetonitrile	USE	R-VEN	Methanol	USE	Yes	**	0.0065
S-VEN	Acetonitrile	USE	S-VEN	Methanol	USE	Yes	**	0.0025
R-VEN	Methanol	USE	S-VEN	Methanol	USE	No	ns	0.9973

*Note: * = $p < 0.05$, ** = $p < 0.01$, and ns = non-significant.

Table A 3 Two-way ANOVA Tukey comparison test for evaluating acidified vs. not acidified acetonitrile extraction solvent for accelerated solvent extraction (ASE) of R-VEN, S-VEN, R-desVEN, and S-desVEN.

Treatment 1		Treatment 2		Below threshold?	Summary	Adjusted P Value
Compound	Extraction Solvent	Compound	Extraction Solvent			
R-desVEN	Acetonitrile	S-desVEN	Acetonitrile	No	ns	0.9997
R-desVEN	Acetonitrile	R-desVEN	Acidified acetonitrile	Yes	***	0.0003
R-desVEN	Acetonitrile	S-desVEN	Acidified acetonitrile	Yes	***	0.0003
S-desVEN	Acetonitrile	R-desVEN	Acidified acetonitrile	Yes	***	0.0002
S-desVEN	Acetonitrile	S-desVEN	Acidified acetonitrile	Yes	***	0.0003
R-desVEN	Acidified acetonitrile	S-desVEN	Acidified acetonitrile	No	ns	0.9997
R-VEN	Acetonitrile	S-VEN	Acetonitrile	No	ns	0.9999
R-VEN	Acetonitrile	R-VEN	Acidified acetonitrile	No	ns	0.4183
R-VEN	Acetonitrile	S-VEN	Acidified acetonitrile	No	ns	0.4183
S-VEN	Acetonitrile	R-VEN	Acidified acetonitrile	No	ns	0.3834
S-VEN	Acetonitrile	S-VEN	Acidified acetonitrile	No	ns	0.3834
R-VEN	Acidified acetonitrile	S-VEN	Acidified acetonitrile	No	ns	>0.9999

*Note: *** = $p < 0.001$ and ns = non-significant.

Table A 4 Two-way ANOVA Tukey comparison test for evaluating fat retainer inclusion for on-line sample cleanup during accelerated solvent extraction (ASE) of R-desVEN and S-desVEN.

Treatment 1		Treatment 2		Below threshold?	Summary	Adjusted P Value
Compound	Fat Retainer	Compound	Fat Retainer			
R-desVEN	Acidic Alumina	S-desVEN	Acidic Alumina	No	ns	>0.9999
R-desVEN	Acidic Alumina	R-desVEN	Neutral Alumina	No	ns	0.9893
R-desVEN	Acidic Alumina	S-desVEN	Neutral Alumina	No	ns	0.9982
R-desVEN	Acidic Alumina	R-desVEN	Basic Alumina	No	ns	0.9991
R-desVEN	Acidic Alumina	S-desVEN	Basic Alumina	No	ns	0.9998
R-desVEN	Acidic Alumina	R-desVEN	Florisil	Yes	****	<0.0001
R-desVEN	Acidic Alumina	S-desVEN	Florisil	Yes	****	<0.0001
S-desVEN	Acidic Alumina	R-desVEN	Neutral Alumina	No	ns	0.9641
S-desVEN	Acidic Alumina	S-desVEN	Neutral Alumina	No	ns	0.99
S-desVEN	Acidic Alumina	R-desVEN	Basic Alumina	No	ns	0.994
S-desVEN	Acidic Alumina	S-desVEN	Basic Alumina	No	ns	0.9978
S-desVEN	Acidic Alumina	R-desVEN	Florisil	Yes	****	<0.0001
S-desVEN	Acidic Alumina	S-desVEN	Florisil	Yes	****	<0.0001
R-desVEN	Neutral Alumina	S-desVEN	Neutral Alumina	No	ns	>0.9999
R-desVEN	Neutral Alumina	R-desVEN	Basic Alumina	No	ns	>0.9999
R-desVEN	Neutral Alumina	S-desVEN	Basic Alumina	No	ns	0.9998
R-desVEN	Neutral Alumina	R-desVEN	Florisil	Yes	****	<0.0001
R-desVEN	Neutral Alumina	S-desVEN	Florisil	Yes	****	<0.0001
S-desVEN	Neutral Alumina	R-desVEN	Basic Alumina	No	ns	>0.9999
S-desVEN	Neutral Alumina	S-desVEN	Basic Alumina	No	ns	>0.9999
S-desVEN	Neutral Alumina	R-desVEN	Florisil	Yes	****	<0.0001
S-desVEN	Neutral Alumina	S-desVEN	Florisil	Yes	****	<0.0001
R-desVEN	Basic Alumina	S-desVEN	Basic Alumina	No	ns	>0.9999
R-desVEN	Basic Alumina	R-desVEN	Florisil	Yes	****	<0.0001
R-desVEN	Basic Alumina	S-desVEN	Florisil	Yes	****	<0.0001
S-desVEN	Basic Alumina	R-desVEN	Florisil	Yes	****	<0.0001
S-desVEN	Basic Alumina	S-desVEN	Florisil	Yes	****	<0.0001
R-desVEN	Florisil	S-desVEN	Florisil	No	ns	0.9855

*Note: **** = $p < 0.0001$ and ns = non-significant.

Table A 5 Two-way ANOVA Tukey comparison test for evaluating fat retainer inclusion for on-line sample cleanup during accelerated solvent extraction (ASE) of R-VEN and S-VEN.

Treatment 1		Treatment 2		Below Threshold?	Summary	Adjusted P Value
Compound	Fat Retainer	Compound	Fat Retainer			
R-VEN	Acidic Alumina	S-VEN	Acidic Alumina	No	ns	>0.9999
R-VEN	Acidic Alumina	R-VEN	Neutral Alumina	No	ns	0.7803
R-VEN	Acidic Alumina	S-VEN	Neutral Alumina	No	ns	0.943
R-VEN	Acidic Alumina	R-VEN	Basic Alumina	No	ns	0.9877
R-VEN	Acidic Alumina	S-VEN	Basic Alumina	No	ns	0.9159
R-VEN	Acidic Alumina	R-VEN	Florisil	Yes	****	<0.0001
R-VEN	Acidic Alumina	S-VEN	Florisil	Yes	****	<0.0001
S-VEN	Acidic Alumina	R-VEN	Neutral Alumina	No	ns	0.6973
S-VEN	Acidic Alumina	S-VEN	Neutral Alumina	No	ns	0.8983
S-VEN	Acidic Alumina	R-VEN	Basic Alumina	No	ns	0.996
S-VEN	Acidic Alumina	S-VEN	Basic Alumina	No	ns	0.955
S-VEN	Acidic Alumina	R-VEN	Florisil	Yes	****	<0.0001
S-VEN	Acidic Alumina	S-VEN	Florisil	Yes	****	<0.0001
R-VEN	Neutral Alumina	S-VEN	Neutral Alumina	No	ns	0.9998
R-VEN	Neutral Alumina	R-VEN	Basic Alumina	No	ns	0.2165
R-VEN	Neutral Alumina	S-VEN	Basic Alumina	No	ns	0.1031
R-VEN	Neutral Alumina	R-VEN	Florisil	Yes	****	<0.0001
R-VEN	Neutral Alumina	S-VEN	Florisil	Yes	****	<0.0001
S-VEN	Neutral Alumina	R-VEN	Basic Alumina	No	ns	0.4236
S-VEN	Neutral Alumina	S-VEN	Basic Alumina	No	ns	0.2296
S-VEN	Neutral Alumina	R-VEN	Florisil	Yes	****	<0.0001
S-VEN	Neutral Alumina	S-VEN	Florisil	Yes	****	<0.0001
R-VEN	Basic Alumina	S-VEN	Basic Alumina	No	ns	0.9999
R-VEN	Basic Alumina	R-VEN	Florisil	Yes	****	<0.0001
R-VEN	Basic Alumina	S-VEN	Florisil	Yes	****	<0.0001
S-VEN	Basic Alumina	R-VEN	Florisil	Yes	****	<0.0001
S-VEN	Basic Alumina	S-VEN	Florisil	Yes	****	<0.0001
R-VEN	Florisil	S-VEN	Florisil	No	ns	0.9991

*Note: **** = $p < 0.0001$ and ns = non-significant.

Table A 6 Two-way ANOVA Tukey comparison test for evaluating mass of neutral alumina (g) included for on-line sample cleanup during accelerated solvent extraction (ASE) of R-VEN, S-VEN, R-desVEN, and S-desVEN.

Treatment 1		Treatment 2		Below threshold?	Summary	Adjusted P Value
Compound	Neutral Alumina Mass (g)	Compound	Neutral Alumina Mass (g)			
R-desVEN	1	S-desVEN	1	No	ns	0.8351
R-desVEN	1	R-desVEN	2.5	Yes	***	0.0008
R-desVEN	1	S-desVEN	2.5	Yes	**	0.0033
R-desVEN	1	R-desVEN	5	No	ns	0.7787
R-desVEN	1	S-desVEN	5	No	ns	0.7125
S-desVEN	1	R-desVEN	2.5	Yes	****	<0.0001
S-desVEN	1	S-desVEN	2.5	Yes	***	0.0003
S-desVEN	1	R-desVEN	5	No	ns	>0.9999
S-desVEN	1	S-desVEN	5	No	ns	0.9999
R-desVEN	2.5	S-desVEN	2.5	No	ns	0.9843
R-desVEN	2.5	R-desVEN	5	Yes	****	<0.0001
R-desVEN	2.5	S-desVEN	5	Yes	****	<0.0001
S-desVEN	2.5	R-desVEN	5	Yes	***	0.0002
S-desVEN	2.5	S-desVEN	5	Yes	***	0.0002
R-desVEN	5	S-desVEN	5	No	ns	>0.9999
R-VEN	1	S-VEN	1	No	ns	0.9873
R-VEN	1	R-VEN	2.5	Yes	*	0.0347
R-VEN	1	S-VEN	2.5	No	ns	0.0831
R-VEN	1	R-VEN	5	No	ns	0.9904
R-VEN	1	S-VEN	5	No	ns	>0.9999
S-VEN	1	R-VEN	2.5	Yes	**	0.0094
S-VEN	1	S-VEN	2.5	Yes	*	0.0239
S-VEN	1	R-VEN	5	No	ns	0.8224
S-VEN	1	S-VEN	5	No	ns	0.9652
R-VEN	2.5	S-VEN	2.5	No	ns	0.9974
R-VEN	2.5	R-VEN	5	No	ns	0.1089
R-VEN	2.5	S-VEN	5	Yes	*	0.0482
S-VEN	2.5	R-VEN	5	No	ns	0.2344
S-VEN	2.5	S-VEN	5	No	ns	0.1128
R-VEN	5	S-VEN	5	No	ns	0.9979

*Note: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, and ns = non-significant.

Table A 7 Two-way ANOVA Tukey comparison test for evaluating sample cleanup protocols for lipid removal after accelerated solvent extraction (ASE) of R-desVEN and S-desVEN.

Treatment 1		Treatment 2		Below threshold?	Summary	Adjusted P Value
Compound	Cleanup Protocol	Compound	Cleanup Protocol			
R-desVEN	ENVI-Carb	S-desVEN	ENVI-Carb	No	ns	0.0715
R-desVEN	ENVI-Carb	R-desVEN	EMR-Lipid	Yes	****	<0.0001
R-desVEN	ENVI-Carb	S-desVEN	EMR-Lipid	Yes	****	<0.0001
R-desVEN	ENVI-Carb	R-desVEN	LLE	No	ns	0.9995
R-desVEN	ENVI-Carb	S-desVEN	LLE	Yes	****	<0.0001
R-desVEN	ENVI-Carb	R-desVEN	QuEChERS	Yes	****	<0.0001
R-desVEN	ENVI-Carb	S-desVEN	QuEChERS	Yes	****	<0.0001
R-desVEN	ENVI-Carb	R-desVEN	Oasis HLB	Yes	****	<0.0001
R-desVEN	ENVI-Carb	S-desVEN	Oasis HLB	Yes	****	<0.0001
S-desVEN	ENVI-Carb	R-desVEN	EMR-Lipid	Yes	****	<0.0001
S-desVEN	ENVI-Carb	S-desVEN	EMR-Lipid	Yes	***	0.0003
S-desVEN	ENVI-Carb	R-desVEN	LLE	No	ns	0.2698
S-desVEN	ENVI-Carb	S-desVEN	LLE	Yes	**	0.0024
S-desVEN	ENVI-Carb	R-desVEN	QuEChERS	Yes	*	0.0352
S-desVEN	ENVI-Carb	S-desVEN	QuEChERS	Yes	****	<0.0001
S-desVEN	ENVI-Carb	R-desVEN	Oasis HLB	Yes	****	<0.0001
S-desVEN	ENVI-Carb	S-desVEN	Oasis HLB	Yes	****	<0.0001
R-desVEN	EMR-Lipid	S-desVEN	EMR-Lipid	Yes	****	<0.0001
R-desVEN	EMR-Lipid	R-desVEN	LLE	Yes	****	<0.0001
R-desVEN	EMR-Lipid	S-desVEN	LLE	Yes	****	<0.0001
R-desVEN	EMR-Lipid	R-desVEN	QuEChERS	Yes	****	<0.0001
R-desVEN	EMR-Lipid	S-desVEN	QuEChERS	No	ns	>0.9999
R-desVEN	EMR-Lipid	R-desVEN	Oasis HLB	Yes	****	<0.0001
R-desVEN	EMR-Lipid	S-desVEN	Oasis HLB	Yes	****	<0.0001
S-desVEN	EMR-Lipid	R-desVEN	LLE	Yes	****	<0.0001
S-desVEN	EMR-Lipid	S-desVEN	LLE	No	ns	0.9985
S-desVEN	EMR-Lipid	R-desVEN	QuEChERS	No	ns	0.7185
S-desVEN	EMR-Lipid	S-desVEN	QuEChERS	Yes	****	<0.0001
S-desVEN	EMR-Lipid	R-desVEN	Oasis HLB	Yes	****	<0.0001
S-desVEN	EMR-Lipid	S-desVEN	Oasis HLB	Yes	****	<0.0001
R-desVEN	LLE	S-desVEN	LLE	Yes	****	<0.0001
R-desVEN	LLE	R-desVEN	QuEChERS	Yes	****	<0.0001
R-desVEN	LLE	S-desVEN	QuEChERS	Yes	****	<0.0001
R-desVEN	LLE	R-desVEN	Oasis HLB	Yes	****	<0.0001
R-desVEN	LLE	S-desVEN	Oasis HLB	Yes	****	<0.0001
S-desVEN	LLE	R-desVEN	QuEChERS	No	ns	0.9863
S-desVEN	LLE	S-desVEN	QuEChERS	Yes	****	<0.0001
S-desVEN	LLE	R-desVEN	Oasis HLB	Yes	****	<0.0001
S-desVEN	LLE	S-desVEN	Oasis HLB	Yes	****	<0.0001
R-desVEN	QuEChERS	S-desVEN	QuEChERS	Yes	****	<0.0001
R-desVEN	QuEChERS	R-desVEN	Oasis HLB	Yes	****	<0.0001
R-desVEN	QuEChERS	S-desVEN	Oasis HLB	Yes	****	<0.0001
S-desVEN	QuEChERS	R-desVEN	Oasis HLB	Yes	****	<0.0001
S-desVEN	QuEChERS	S-desVEN	Oasis HLB	Yes	****	<0.0001
R-desVEN	Oasis HLB	S-desVEN	Oasis HLB	No	ns	>0.9999

*Note: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, and ns = non-significant.

Table A 8 Two-way ANOVA Tukey comparison test for evaluating sample cleanup protocols for lipid removal after accelerated solvent extraction (ASE) of R-VEN and S-VEN.

Treatment 1		Treatment 2		Below threshold?	Summary	Adjusted P Value
Compound	Cleanup Protocol	Compound	Cleanup Protocol			
R-VEN	ENVI-Carb	S-VEN	ENVI-Carb	Yes	****	<0.0001
R-VEN	ENVI-Carb	R-VEN	EMR-Lipid	Yes	****	<0.0001
R-VEN	ENVI-Carb	S-VEN	EMR-Lipid	Yes	****	<0.0001
R-VEN	ENVI-Carb	R-VEN	LLE	Yes	***	0.0002
R-VEN	ENVI-Carb	S-VEN	LLE	Yes	****	<0.0001
R-VEN	ENVI-Carb	R-VEN	QuEChERS	Yes	****	<0.0001
R-VEN	ENVI-Carb	S-VEN	QuEChERS	Yes	****	<0.0001
R-VEN	ENVI-Carb	R-VEN	Oasis HLB	Yes	*	0.0469
R-VEN	ENVI-Carb	S-VEN	Oasis HLB	No	ns	0.3057
S-VEN	ENVI-Carb	R-VEN	EMR-Lipid	Yes	****	<0.0001
S-VEN	ENVI-Carb	S-VEN	EMR-Lipid	Yes	****	<0.0001
S-VEN	ENVI-Carb	R-VEN	LLE	No	ns	0.9958
S-VEN	ENVI-Carb	S-VEN	LLE	Yes	***	0.0004
S-VEN	ENVI-Carb	R-VEN	QuEChERS	Yes	****	<0.0001
S-VEN	ENVI-Carb	S-VEN	QuEChERS	Yes	****	<0.0001
S-VEN	ENVI-Carb	R-VEN	Oasis HLB	Yes	****	<0.0001
S-VEN	ENVI-Carb	S-VEN	Oasis HLB	Yes	****	<0.0001
R-VEN	EMR-Lipid	S-VEN	EMR-Lipid	No	ns	0.7467
R-VEN	EMR-Lipid	R-VEN	LLE	Yes	****	<0.0001
R-VEN	EMR-Lipid	S-VEN	LLE	Yes	****	<0.0001
R-VEN	EMR-Lipid	R-VEN	QuEChERS	Yes	***	0.0006
R-VEN	EMR-Lipid	S-VEN	QuEChERS	No	ns	0.9804
R-VEN	EMR-Lipid	R-VEN	Oasis HLB	Yes	****	<0.0001
R-VEN	EMR-Lipid	S-VEN	Oasis HLB	Yes	****	<0.0001
S-VEN	EMR-Lipid	R-VEN	LLE	Yes	****	<0.0001
S-VEN	EMR-Lipid	S-VEN	LLE	Yes	****	<0.0001
S-VEN	EMR-Lipid	R-VEN	QuEChERS	No	ns	0.0571
S-VEN	EMR-Lipid	S-VEN	QuEChERS	No	ns	0.1568
S-VEN	EMR-Lipid	R-VEN	Oasis HLB	Yes	****	<0.0001
S-VEN	EMR-Lipid	S-VEN	Oasis HLB	Yes	****	<0.0001
R-VEN	LLE	S-VEN	LLE	Yes	****	<0.0001
R-VEN	LLE	R-VEN	QuEChERS	Yes	****	<0.0001
R-VEN	LLE	S-VEN	QuEChERS	Yes	****	<0.0001
R-VEN	LLE	R-VEN	Oasis HLB	Yes	****	<0.0001
R-VEN	LLE	S-VEN	Oasis HLB	Yes	****	<0.0001
S-VEN	LLE	R-VEN	QuEChERS	Yes	****	<0.0001
S-VEN	LLE	S-VEN	QuEChERS	Yes	****	<0.0001
S-VEN	LLE	R-VEN	Oasis HLB	Yes	****	<0.0001
S-VEN	LLE	S-VEN	Oasis HLB	Yes	****	<0.0001
R-VEN	QuEChERS	S-VEN	QuEChERS	Yes	****	<0.0001
R-VEN	QuEChERS	R-VEN	Oasis HLB	Yes	****	<0.0001
R-VEN	QuEChERS	S-VEN	Oasis HLB	Yes	****	<0.0001
S-VEN	QuEChERS	R-VEN	Oasis HLB	Yes	****	<0.0001
S-VEN	QuEChERS	S-VEN	Oasis HLB	Yes	****	<0.0001
R-VEN	Oasis HLB	S-VEN	Oasis HLB	No	ns	0.9939

*Note: * = $p < 0.05$, *** = $p < 0.001$, **** = $p < 0.0001$, and ns = non-significant.

Table A 9 Two-way ANOVA Tukey comparison test for evaluating matrix effects (%) caused by extracting different fish tissue masses (g) with accelerated solvent extraction (ASE) for R-VEN, S-VEN, R-desVEN, and S-desVEN.

Treatment 1		Treatment 2		Below threshold?	Summary	Adjusted P Value
Compound	Fish Tissue Mass (g)	Compound	Fish Tissue Mass (g)			
R-desVEN	1.2	S-desVEN	1.2	No	ns	0.116
R-desVEN	1.2	R-desVEN	2.4	No	ns	0.2213
R-desVEN	1.2	S-desVEN	2.4	Yes	**	0.0024
R-desVEN	1.2	R-desVEN	4.8	Yes	***	0.0002
R-desVEN	1.2	S-desVEN	4.8	Yes	****	<0.0001
S-desVEN	1.2	R-desVEN	2.4	No	ns	>0.9999
S-desVEN	1.2	S-desVEN	2.4	No	ns	0.2829
S-desVEN	1.2	R-desVEN	4.8	No	ns	0.0506
S-desVEN	1.2	S-desVEN	4.8	Yes	****	<0.0001
R-desVEN	2.4	S-desVEN	2.4	No	ns	0.2721
R-desVEN	2.4	R-desVEN	4.8	No	ns	0.0565
R-desVEN	2.4	S-desVEN	4.8	Yes	****	<0.0001
S-desVEN	2.4	R-desVEN	4.8	No	ns	0.9705
S-desVEN	2.4	S-desVEN	4.8	Yes	**	0.004
R-desVEN	4.8	S-desVEN	4.8	Yes	**	0.0099
R-VEN	1.2	S-VEN	1.2	No	ns	0.4236
R-VEN	1.2	R-VEN	2.4	Yes	*	0.0179
R-VEN	1.2	S-VEN	2.4	Yes	**	0.0016
R-VEN	1.2	R-VEN	4.8	Yes	****	<0.0001
R-VEN	1.2	S-VEN	4.8	Yes	****	<0.0001
S-VEN	1.2	R-VEN	2.4	No	ns	0.3996
S-VEN	1.2	S-VEN	2.4	No	ns	0.0535
S-VEN	1.2	R-VEN	4.8	Yes	****	<0.0001
S-VEN	1.2	S-VEN	4.8	Yes	****	<0.0001
R-VEN	2.4	S-VEN	2.4	No	ns	0.8585
R-VEN	2.4	R-VEN	4.8	Yes	***	0.0004
R-VEN	2.4	S-VEN	4.8	Yes	****	<0.0001
S-VEN	2.4	R-VEN	4.8	Yes	**	0.0041
S-VEN	2.4	S-VEN	4.8	Yes	***	0.0001
R-VEN	4.8	S-VEN	4.8	No	ns	0.3368

*Note: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, and ns = non-significant.

Table A 10 Two-way ANOVA Tukey comparison test for evaluating matrix effects (%) caused by a final sample extract volume (mL) for the accelerated solvent extraction (ASE) of R-VEN, S-VEN, R-desVEN, and S-desVEN.

Treatment 1		Treatment 2		Below threshold?	Summary	Adjusted P Value
Compound	Reconstitution Volume (mL)	Compound	Reconstitution Volume (mL)			
R-desVEN	0.25	S-desVEN	0.25	No	ns	0.3029
R-desVEN	0.25	R-desVEN	0.50	No	ns	0.954
R-desVEN	0.25	S-desVEN	0.50	No	ns	0.1884
R-desVEN	0.25	R-desVEN	1.0	No	ns	0.3729
R-desVEN	0.25	S-desVEN	1.0	Yes	*	0.0202
S-desVEN	0.25	R-desVEN	0.50	No	ns	0.7756
S-desVEN	0.25	S-desVEN	0.50	No	ns	0.9996
S-desVEN	0.25	R-desVEN	1.0	No	ns	>0.9999
S-desVEN	0.25	S-desVEN	1.0	No	ns	0.6891
R-desVEN	0.50	S-desVEN	0.50	No	ns	0.6023
R-desVEN	0.50	R-desVEN	1.0	No	ns	0.8472
R-desVEN	0.50	S-desVEN	1.0	No	ns	0.1078
S-desVEN	0.50	R-desVEN	1.0	No	ns	0.9974
S-desVEN	0.50	S-desVEN	1.0	No	ns	0.8472
R-desVEN	1.0	S-desVEN	1.0	No	ns	0.6023
R-VEN	0.25	S-VEN	0.25	No	ns	0.9967
R-VEN	0.25	R-VEN	0.50	No	ns	0.9994
R-VEN	0.25	S-VEN	0.50	No	ns	>0.9999
R-VEN	0.25	R-VEN	1.0	No	ns	0.9999
R-VEN	0.25	S-VEN	1.0	No	ns	>0.9999
S-VEN	0.25	R-VEN	0.50	No	ns	0.9652
S-VEN	0.25	S-VEN	0.50	No	ns	0.9962
S-VEN	0.25	R-VEN	1.0	No	ns	0.9799
S-VEN	0.25	S-VEN	1.0	No	ns	0.9997
R-VEN	0.50	S-VEN	0.50	No	ns	0.9996
R-VEN	0.50	R-VEN	1.0	No	ns	>0.9999
R-VEN	0.50	S-VEN	1.0	No	ns	0.9956
S-VEN	0.50	R-VEN	1.0	No	ns	>0.9999
S-VEN	0.50	S-VEN	1.0	No	ns	>0.9999
R-VEN	1.0	S-VEN	1.0	No	ns	0.9984

*Note: * = $p < 0.05$ and ns = non-significant.

Appendix B

In-lab exposure supplementary information

Table B 1 Two-way ANOVA Tukey comparison test for VEN concentration (µg/L) in tank water sampled over the 14-d exposure period.

Compound	Treatment 1 Tank Exposure Length (d)	Water Sampling Date (d)	Compound	Treatment 2 Tank Exposure Length (d)	Water Sampling Date (d)	Below Threshold?	Summary	Adjusted P Value
S-VEN	1	0	S-VEN	1	1	No	ns	0.0701
S-VEN	1	0	S-VEN	4	0	No	ns	0.9942
S-VEN	1	0	S-VEN	4	1	No	ns	0.1397
S-VEN	1	0	S-VEN	4	4	No	ns	0.9946
S-VEN	1	0	S-VEN	14	0	No	ns	0.8139
S-VEN	1	0	S-VEN	14	1	No	ns	0.5153
S-VEN	1	0	S-VEN	14	4	No	ns	0.9934
S-VEN	1	0	S-VEN	14	14	No	ns	0.9994
S-VEN	1	0	R-VEN	1	0	No	ns	0.6196
S-VEN	1	0	R-VEN	1	1	No	ns	0.1522
S-VEN	1	0	R-VEN	4	0	No	ns	>0.9999
S-VEN	1	0	R-VEN	4	1	No	ns	0.4357
S-VEN	1	0	R-VEN	4	4	No	ns	0.9796
S-VEN	1	0	R-VEN	14	0	No	ns	0.4053
S-VEN	1	0	R-VEN	14	1	No	ns	0.2043
S-VEN	1	0	R-VEN	14	4	No	ns	0.875
S-VEN	1	0	R-VEN	14	14	No	ns	0.6442
S-VEN	1	1	S-VEN	4	0	No	ns	0.7812
S-VEN	1	1	S-VEN	4	1	No	ns	>0.9999
S-VEN	1	1	S-VEN	4	4	No	ns	0.521
S-VEN	1	1	S-VEN	14	0	No	ns	0.9454
S-VEN	1	1	S-VEN	14	1	No	ns	0.9985
S-VEN	1	1	S-VEN	14	4	No	ns	0.5393
S-VEN	1	1	S-VEN	14	14	No	ns	0.3578
S-VEN	1	1	R-VEN	1	0	No	ns	0.9934
S-VEN	1	1	R-VEN	1	1	No	ns	>0.9999
S-VEN	1	1	R-VEN	4	0	No	ns	0.0653
S-VEN	1	1	R-VEN	4	1	No	ns	0.9997
S-VEN	1	1	R-VEN	4	4	No	ns	0.6648
S-VEN	1	1	R-VEN	14	0	No	ns	0.9998
S-VEN	1	1	R-VEN	14	1	No	ns	>0.9999
S-VEN	1	1	R-VEN	14	4	No	ns	0.9012
S-VEN	1	1	R-VEN	14	14	No	ns	0.991
S-VEN	4	0	S-VEN	4	1	No	ns	0.9218
S-VEN	4	0	S-VEN	4	4	No	ns	>0.9999
S-VEN	4	0	S-VEN	14	0	No	ns	>0.9999
S-VEN	4	0	S-VEN	14	1	No	ns	0.9997
S-VEN	4	0	S-VEN	14	4	No	ns	>0.9999
S-VEN	4	0	S-VEN	14	14	No	ns	>0.9999
S-VEN	4	0	R-VEN	1	0	No	ns	>0.9999
S-VEN	4	0	R-VEN	1	1	No	ns	0.9343

Table B 1 (continued).

S-VEN	4	0	R-VEN	4	0	No	ns	0.9993
S-VEN	4	0	R-VEN	4	1	No	ns	0.9989
S-VEN	4	0	R-VEN	4	4	No	ns	>0.9999
S-VEN	4	0	R-VEN	14	0	No	ns	0.9982
S-VEN	4	0	R-VEN	14	1	No	ns	0.9678
S-VEN	4	0	R-VEN	14	4	No	ns	>0.9999
S-VEN	4	0	R-VEN	14	14	No	ns	>0.9999
S-VEN	4	1	S-VEN	4	4	No	ns	0.7488
S-VEN	4	1	S-VEN	14	0	No	ns	0.9932
S-VEN	4	1	S-VEN	14	1	No	ns	>0.9999
S-VEN	4	1	S-VEN	14	4	No	ns	0.7652
S-VEN	4	1	S-VEN	14	14	No	ns	0.5793
S-VEN	4	1	R-VEN	1	0	No	ns	0.9998
S-VEN	4	1	R-VEN	1	1	No	ns	>0.9999
S-VEN	4	1	R-VEN	4	0	No	ns	0.1415
S-VEN	4	1	R-VEN	4	1	No	ns	>0.9999
S-VEN	4	1	R-VEN	4	4	No	ns	0.8634
S-VEN	4	1	R-VEN	14	0	No	ns	>0.9999
S-VEN	4	1	R-VEN	14	1	No	ns	>0.9999
S-VEN	4	1	R-VEN	14	4	No	ns	0.9819
S-VEN	4	1	R-VEN	14	14	No	ns	0.9996
S-VEN	4	4	S-VEN	14	0	No	ns	>0.9999
S-VEN	4	4	S-VEN	14	1	No	ns	0.9954
S-VEN	4	4	S-VEN	14	4	No	ns	>0.9999
S-VEN	4	4	S-VEN	14	14	No	ns	>0.9999
S-VEN	4	4	R-VEN	1	0	No	ns	0.9991
S-VEN	4	4	R-VEN	1	1	No	ns	0.7758
S-VEN	4	4	R-VEN	4	0	No	ns	0.9994
S-VEN	4	4	R-VEN	4	1	No	ns	0.9873
S-VEN	4	4	R-VEN	4	4	No	ns	>0.9999
S-VEN	4	4	R-VEN	14	0	No	ns	0.9819
S-VEN	4	4	R-VEN	14	1	No	ns	0.8613
S-VEN	4	4	R-VEN	14	4	No	ns	>0.9999
S-VEN	4	4	R-VEN	14	14	No	ns	0.9994
S-VEN	14	0	S-VEN	14	1	No	ns	>0.9999
S-VEN	14	0	S-VEN	14	4	No	ns	>0.9999
S-VEN	14	0	S-VEN	14	14	No	ns	0.9997
S-VEN	14	0	R-VEN	1	0	No	ns	>0.9999
S-VEN	14	0	R-VEN	1	1	No	ns	0.9952
S-VEN	14	0	R-VEN	4	0	No	ns	0.8851
S-VEN	14	0	R-VEN	4	1	No	ns	>0.9999
S-VEN	14	0	R-VEN	4	4	No	ns	>0.9999

Table B 1 (continued).

S-VEN	14	0	R-VEN	14	0	No	ns	>0.9999
S-VEN	14	0	R-VEN	14	1	No	ns	0.9989
S-VEN	14	0	R-VEN	14	4	No	ns	>0.9999
S-VEN	14	0	R-VEN	14	14	No	ns	>0.9999
S-VEN	14	1	S-VEN	14	4	No	ns	0.9963
S-VEN	14	1	S-VEN	14	14	No	ns	0.9729
S-VEN	14	1	R-VEN	1	0	No	ns	>0.9999
S-VEN	14	1	R-VEN	1	1	No	ns	>0.9999
S-VEN	14	1	R-VEN	4	0	No	ns	0.5793
S-VEN	14	1	R-VEN	4	1	No	ns	>0.9999
S-VEN	14	1	R-VEN	4	4	No	ns	0.9994
S-VEN	14	1	R-VEN	14	0	No	ns	>0.9999
S-VEN	14	1	R-VEN	144	1	No	ns	>0.9999
S-VEN	14	1	R-VEN	14	4	No	ns	>0.9999
S-VEN	14	1	R-VEN	14	14	No	ns	>0.9999
S-VEN	14	4	S-VEN	14	14	No	ns	>0.9999
S-VEN	14	4	R-VEN	1	0	No	ns	0.9993
S-VEN	14	4	R-VEN	1	1	No	ns	0.7914
S-VEN	14	4	R-VEN	4	0	No	ns	0.9992
S-VEN	14	4	R-VEN	4	1	No	ns	0.9895
S-VEN	14	4	R-VEN	4	4	No	ns	>0.9999
S-VEN	14	4	R-VEN	14	0	No	ns	0.9848
S-VEN	14	4	R-VEN	14	1	No	ns	0.8735
S-VEN	14	4	R-VEN	14	4	No	ns	>0.9999
S-VEN	14	4	R-VEN	14	14	No	ns	0.9996
S-VEN	14	14	R-VEN	1	0	No	ns	0.991
S-VEN	14	14	R-VEN	1	1	No	ns	0.61
S-VEN	14	14	R-VEN	4	0	No	ns	>0.9999
S-VEN	14	14	R-VEN	4	1	No	ns	0.9454
S-VEN	14	14	R-VEN	4	4	No	ns	>0.9999
S-VEN	14	14	R-VEN	14	0	No	ns	0.9303
S-VEN	14	14	R-VEN	14	1	No	ns	0.7178
S-VEN	14	14	R-VEN	14	4	No	ns	>0.9999
S-VEN	14	14	R-VEN	14	14	No	ns	0.9934
R-VEN	1	0	R-VEN	1	1	No	ns	0.9999
R-VEN	1	0	R-VEN	4	0	No	ns	0.6945
R-VEN	1	0	R-VEN	4	1	No	ns	>0.9999
R-VEN	1	0	R-VEN	4	4	No	ns	>0.9999
R-VEN	1	0	R-VEN	14	0	No	ns	>0.9999
R-VEN	1	0	R-VEN	14	1	No	ns	>0.9999
R-VEN	1	0	R-VEN	14	4	No	ns	>0.9999
R-VEN	1	0	R-VEN	14	14	No	ns	>0.9999

Table B 1 (continued).

R-VEN	1	1	R-VEN	4	0	No	ns	0.1556
R-VEN	1	1	R-VEN	4	1	No	ns	>0.9999
R-VEN	1	1	R-VEN	4	4	No	ns	0.8832
R-VEN	1	1	R-VEN	14	0	No	ns	>0.9999
R-VEN	1	1	R-VEN	14	1	No	ns	>0.9999
R-VEN	1	1	R-VEN	14	4	No	ns	0.9865
R-VEN	1	1	R-VEN	14	14	No	ns	0.9998
R-VEN	4	0	R-VEN	4	1	No	ns	0.4877
R-VEN	4	0	R-VEN	4	4	No	ns	0.9954
R-VEN	4	0	R-VEN	14	0	No	ns	0.4522
R-VEN	4	0	R-VEN	14	1	No	ns	0.2157
R-VEN	4	0	R-VEN	14	4	No	ns	0.9343
R-VEN	4	0	R-VEN	14	14	No	ns	0.7207
R-VEN	4	1	R-VEN	4	4	No	ns	0.9976
R-VEN	4	1	R-VEN	14	0	No	ns	>0.9999
R-VEN	4	1	R-VEN	14	1	No	ns	>0.9999
R-VEN	4	1	R-VEN	14	4	No	ns	>0.9999
R-VEN	4	1	R-VEN	14	14	No	ns	>0.9999
R-VEN	4	4	R-VEN	14	0	No	ns	0.9962
R-VEN	4	4	R-VEN	14	1	No	ns	0.9394
R-VEN	4	4	R-VEN	14	4	No	ns	>0.9999
R-VEN	4	4	R-VEN	14	14	No	ns	>0.9999
R-VEN	14	0	R-VEN	14	1	No	ns	>0.9999
R-VEN	14	0	R-VEN	14	4	No	ns	>0.9999
R-VEN	14	0	R-VEN	14	14	No	ns	>0.9999
R-VEN	14	1	R-VEN	14	4	No	ns	0.996
R-VEN	14	1	R-VEN	14	14	No	ns	>0.9999
R-VEN	14	4	R-VEN	14	14	No	ns	>0.9999

*Note: ns = non-significant.

Table B 2 Tukey's multiple comparison test for R-VEN and S-VEN concentrations (µg/L) found in fish replicates (n = 4-6) in each tank replicate (A, B, and C) on each sampling date (1, 4, and 14 d of exposure).

Treatment 1			Treatment 2			Below threshold?	Summary	Adjusted P Value
Compound	Exposure length (d)	Tank replicate	Compound	Exposure length (d)	Tank replicate			
R-VEN	1	A	R-VEN	1	B	No	ns	0.5512
R-VEN	1	A	R-VEN	1	C	No	ns	0.9856
R-VEN	1	B	R-VEN	1	C	No	ns	0.6489
R-VEN	4	A	R-VEN	4	B	No	ns	0.1579
R-VEN	4	A	R-VEN	4	C	No	ns	0.9783
R-VEN	4	B	R-VEN	4	C	No	ns	0.1146
R-VEN	14	A	R-VEN	14	B	No	ns	0.9951
R-VEN	14	A	R-VEN	14	C	No	ns	0.6961
R-VEN	14	B	R-VEN	14	C	No	ns	0.6629
S-VEN	1	A	S-VEN	1	B	No	ns	0.9876
S-VEN	1	A	S-VEN	1	C	No	ns	0.5528
S-VEN	1	B	S-VEN	1	C	No	ns	0.633
S-VEN	4	A	S-VEN	4	B	No	ns	0.2565
S-VEN	4	A	S-VEN	4	C	No	ns	0.1301
S-VEN	4	B	S-VEN	4	C	No	ns	0.8968
S-VEN	14	A	S-VEN	14	B	No	ns	0.7973
S-VEN	14	A	S-VEN	14	C	No	ns	0.1664
S-VEN	14	B	S-VEN	14	C	No	ns	0.4377

*Note: ns = non-significant.

Table B 3 Two-way ANOVA Tukey comparison test to compare the concentrations (µg/L) for the enantiomers of VEN (R-VEN vs. S-VEN) in fish exposed to 1 µg/L of individual enantiomers for up to 14 d.

Treatment 1		Treatment 2		Below threshold?	Summary	Adjusted P Value
Compound	Exposure length (d)	Compound	Exposure length (d)			
R-VEN	0	S-VEN	0	No	ns	>0.9999
R-VEN	1	S-VEN	1	No	ns	0.5987
R-VEN	4	S-VEN	4	**Yes**	****	**<0.0001**
R-VEN	14	S-VEN	14	**Yes**	****	**<0.0001**
R-VEN	1	R-VEN	4	No	ns	0.1555
R-VEN	1	R-VEN	14	No	ns	0.3323
R-VEN	4	R-VEN	14	No	ns	>0.9999
S-VEN	1	S-VEN	4	**Yes**	****	**<0.0001**
S-VEN	1	S-VEN	14	**Yes**	****	**<0.0001**
S-VEN	4	S-VEN	14	**Yes**	****	**<0.0001**
R-VEN	0	R-VEN	1	**Yes**	****	**<0.0001**
R-VEN	0	R-VEN	4	**Yes**	****	**<0.0001**
R-VEN	0	R-VEN	14	**Yes**	****	**<0.0001**
S-VEN	0	S-VEN	1	**Yes**	****	**<0.0001**
S-VEN	0	S-VEN	4	**Yes**	****	**<0.0001**
S-VEN	0	S-VEN	14	**Yes**	****	**<0.0001**

*Note: Significant results are shown in bold, where **** = $p < 0.0001$, and ns = non-significant.